Amino Acids (1999) 17: 1–129

Amino
Acids

6th International Congress on Amino Acids

Bonn, Federal Republic of Germany
August 3–7, 1999

Abstracts

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This congress was sponsored
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Contents

Neurobiology I

Antidepressant treatment influences responses induced by stimulation of metabotropic glutamate receptors

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Excitatory amino acids (EAA) are abundant in brain and may play a major role in both the physiology and pathophysiology of the central nervous system. EAA act by stimulation of ionotropic and metabotropic glutamate receptors (mGluR). mGluR are divided into three major groups: mGluR I, mGluR II and mGluR III. Our preliminary data indicate that mGluR are modified by prolonged antidepressant treatment [Pilc A., Legutko B. (1995) Neuroreport 7: 85]. The aim of the present study was to investigate further if prolonged antidepressant treatment modifies the biochemical, electrophysiological and behavioural effects of mGluR stimulation.

Male Wistar rats were given 7 electroshocks (ECS) delivered 7 times through ear clip electrodes or 21 daily injections of imipramine (10 mg/kg i.p.). The cyclic AMP or inositol trisphosphate (IP3) formation was measured in the slices from rat cerebral cortex and/or hippocampus. Electrophysiological studies were conducted in hippocampal slices where the response of CA1 neurons to electrical stimulation of Schaffer collaterals was recorded extracellularly. The mGluR1a receptors were visualised using specific monoclonal antibody and avidine-biotine-peroxydase complex method. Behavioural despair test (Porsolt's test) was used to measure potential antidepressant effects of agents acting on group I mGluR.

The ibotenate-mediated stimulation of cyclic AMP accumulation which is due to stimulation of not yet identified subtype of mGluR, was inhibited significantly after both imipramine and ECS treatments. (1S,3R)-1-carboxycyclopentene-3-acetic acid (1S,3R)-ACPD caused a concentration dependent increase in inositol phosphate accumulation, and effect that was not modified by antidepressant treatment. (R,S)-3.5-dihydroxypgenylglycine (DHPG) the selective agonist for group I of mGluR increased by 25% the amplitude of the population spikes evoked by stimulation of the Schaffer collateral pathway in the CA1 cell layer of hippocampus. Repeated but not single ECS produced a significant attenuation of the excitatory effects of DHPG on the population spike. It was found that imipramine and ECS increase the number of MgluR1a immunoreactive neurons in the hippocampus of rats by about 120% of the control value. The increase was the highest in a CA3 piramidal layer. Haloperidol, a neuroleptic drug did not cause similar effects. Thus the changes observed might be specific for antidepressant drugs treatment. (R,S)-1-aminoindan-1,5-dicarbocylic acid (AIDA), an antagonist of group I mGluR after intraventricular injection in rats significantly in a dose dependent manner decreased the immobility time indicating that substances which stimulate group I mGluR express antidepressant-like properties. These results suggest that the antidepressive therapy may alter the responsiveness of group I mGluR, as well as that the blockade of that type of mGluR may induce antidepressant effects.

The role of glutamate receptors in antipsychotic drug action

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It has recently been postulated that diminished glutamatergic neurotransmission may contribute to the pathophysiology of psychotic symptoms.

The aim of the present study was to examine the role of glutamate receptors in the antipsychotic drug action. Haloperidol (1 mg/kg/day) and clozapine (30 mg/kg/day) were administered to rats in drinking water for 3 months. On day 5 of withdrawal, the binding of [^{3}H]MK-801 and [^{3}H]CGP 39653 to NMDA receptors in different cortical areas was analyzed in slices by a quantitative autoradiography, or in parieto-insular cortical homogenates. Haloperidol increased the binding of [^{3}H]CGP 39653 in frontal, insular and parietal cortices. Clozapine increased the binding of that ligand in insular and parietal cortices. The above-mentioned changes were due to increased Bmax values, but not to alternations in the K_D. None of those neuroleptics influenced the binding of [^{3}H]MK-801 to cortical NMDA receptors.

The effect of haloperidol, clozapine and LY 354740 (a group II metabotropic glutamate receptor agonist) on prepulse inhibition was additionally tested. Prepulse inhibition is a model in which a weak subthreshold stimulus (prepulse), applied to an individual before a strong stimulus (pulse), inhibits a startle response to the latter. The deficit of prepulse inhibition induced by phencyclidine in animals has been suggested as a model of a sensorimotor gating deficit in schizophrenia. The deficit induced by phencyclidine (5 mg/kg sc) was reversed by long- (6 weeks or 3 months), but not by short-term (4 days), treatment with haloperidol. Short and long-term treatment with clozapine, as well as acute treatment with LY 354740 (10 mg/kg ip) were ineffective.

The present study suggests that (1) long-term neuroleptic administration increases the density of cortical NMDA receptors labelled with [^{3}H]CGP 39653, (2) the deficit of sensorimotor gating induced by phencyclidine in rats can be reversed by long-term treatment with haloperidol, but not with clozapine, or by acute stimulation of group II metabotropic glutamate receptors. It is proposed that cortical NMDA receptors may play a role in the antipsychotic action of neuroleptics.

The role of metabotropic glutamate receptor (mGluR) ligands in parkinsonian muscle rigidity

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It has been shown that a primary striatal dopaminergic hypofunction, which is at the origin of Parkinson's disease, results in a secondary hyperactivity of glutamatergic neurotransmission. Unfortunately, in the past decade it has been shown that ionotropic glutamate receptor antagonists, in particular antagonists of different binding sites at the NMDA receptor, show a number of unpleasant and even dangerous side-effects. Therefore, in the search for a safer therapy of Parkinson's disease, present study was aimed at determining whether any metabotropic glutamate receptor (mGluR) ligands could evoke an antiparkinsonian effect in the haloperidol-induced muscle rigidity. To this end four mGluR ligands were used: the potent and selective mGluR1 antagonist (RS)-1-aminoindan-1,5-dicarbocylic acid (AIDA), the mixed group II agonist/group I antagonist (S)-4-carboxy-3-hydroxyphenylglycine ((S)-4-C3H-PG), the potent group II agonist (+)-2-aminobicyclo[3.1.0]hexane-2,6,-dicarbocylic acid (LY354740) and the selective group II agonist 3-amino-3,5-pyrrolidinedicarboxylic acid (2R,4R)-APDC. Only the LY354740 penetrated the brain from the periphery, therefore all the other drugs were injected bilaterally into the rostral striatum. Muscle tone was recorded by a mechanomyographic/electromyographic method which mea-

sured the resistance of a rat's hind foot and the reflex response of its muscles to passive movements.

(S)-4C3H-PG (5 and 15 μg/0.5μl) and LY354740 (5 and 10 mg/kp i.p.) diminished the muscle rigidity induced by haloperidol (1 mg/kg i.p.), whereas no such effect was produced by (2R,4R)-APDC (7.5 and 15 μg/0.5 μl) or AIDA (0.5 and 2 μg/0.5 μl).

Our results suggest that stimulation of group II mGluRs may be important to diminution of parkinsonian-like muscle rigidity. However, since (2R,4R)-APDC is without effect on the haloperidol-induced muscle rigidity, it seems that not only striatal group II mGluRs participate in the antiparkinsonian effect but also group II mGluRs located in other brain regions.

On the role of group I metabotropic glutamate receptor subtypes (mGluRs) for motor behaviour of rats

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Glutamate in the basal ganglia has a crucial role in the regulation of motor behaviour. Until now studies addressing the function of glutamate acting via ionotropic receptors predominate. Glutamate however also activates second messenger coupled receptors, the metabotropic glutamate receptors (mGluRs) of which eight subtypes are cloned and classified in three groups so far. Recently we had shown that the mGluRs have important roles for motor behaviour as well, as i.c.v. infusions of the broad-band group I/II mGluR agonist 1S,3R-ACPD dose-dependently induced catalepsy and depressed locomotor activity. Group II receptors at least partially mediate this effect, as the group II selective agonist L-CCG I had qualitatively similar effects. The question which is the contribution of group I mGluRs was not yet directly addressed by agonists, because activation of group I, opposite to group II mGluRs, induces neurodegeneration. As first step we address the contribution of group I receptors, we decided to use (S)-4C3H-PG a ligand, which activates group II mGluRs while blocking group I mGluRs. As second step we address the effects of group I subtypes by subtype specific antagonists.

Effects of (S)-4C3H-PG and respective subtpye specific antagonists on motor behaviour are tested alone and combined with administrations of dopamine D1 and D2 receptor antagonists. We infuse (S)-4C3H-PG locally into the striatum of rats, using chronically implanted guide cannulas. Subtype specific antagonists are administered systematically. Locomotor and sniffing behaviour is analyzed in an open field with holeboard and an experimental chamber, respectively. Cannula placement is verified with standard histological techniques. the detailed data presented are discussed in respect to their possible implications on human basal ganglia disorders, as Parkinson's Disease.

(Supported by the DFG SFB 307/A4.)

Cell-type specificity of mGluRs activation in striatal neuronal subtypes

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The effects of metabotropic glutamate receptor (mGluR) activation were studied in a striatal slice preparation by means of electrophysiological and optical recordings. In pathological conditions involving the striatum, such as Huntington's disease, striatal medium spiny neurons representing the large majority of projecting cells are lost, whereas large cholinergic interneurons (LA) cells accounting for a 3–5% of the entire cell population are spared. The reasons behind this cell-type vulnerability are not known, but a differential sensitivity to glutamate receptor activation has been proposed as a major cause. DCG-IV and L-SOP, agonists for group II and III mGluRs, respectively, produced a presynaptic inhibitory effect of corticostriatal glutamatergic excitatory postsynaptic potentials (EPSPs) in both spiny and LA cells. Activation of group I mGluRs by the selective agonist 3,5-DHPG produced no effect on membrane properties and glutamatergic transmission in spiny neurons, whereas it did cause a membrane depolarization in LA interneurons coupled to increased input resistance. In combined optical and electrophysiological experiments, in spiny neurons 3,5-DHPG enhanced membrane depolarization and intracellular calcium levels induced by NMDA applications, but not in LA interneurons. Activation of protein kinase C (PKC) by phorbol-12,13-diacetate (PDAc) mimicked the enhancement of NMDA-responses by 3,5-DHPG. The PKC inhibitor calphostin C prevented the positive modulatory effect of 3,5-DHPG. In conclusion, these data suggest that activation of group II and III in both neuronal subtypes exerts similar effects. Indeed, the existence of a positive interaction between NMDA and group I mGluR receptors only in medium spiny cells might, at least partially account for the differential vulnerability to excitotoxic damage observed between different striatal neuronal subtypes.

Localization and density of glutamate transporters

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Uptake of glutamate plays important roles in brain physiology and pathology. The extracellular concentration of glutamate must be tightly controlled as both too high and too low concentrations are harmful. Glutamate is removed from the extracellular fluid by cellular uptake mediated by the glutamate transporters. Thus, the importance of the transporters for the long term maintenance of low extracellular glutamate concentrations is well documented. Their roles during the first millisecond after synaptic release of glutamate, however, is currently being debated. We have addressed this question by measuring the transporter densities in young adult rat brains. The total concentration of glial glutamate transporters in the cerebellar molecular layer and in the stratum radiatum of hippocampus CA1 corresponds to 3–5 times the estimated number of glutamate molecules in one synaptic vesicle from each of all glutamatergic synapses. This implies that glutamate transporters are present at sufficiently high average densities to support the notion that they can contribute to glutamate inactivation on the short time-scale by binding rather than by transport. However, their importance in the control of extrasynaptic and intersynaptic glutamate diffusion is likely to vary considerably between different synapses because the transporters are predominantly associated with astrocytes and thereby not evenly distributed in the extracellular space. Mathematical models of the spatiotemporal transmitter profile after synaptic release should therefore take into account the localizations of astrocytic processes in relation to the transmitter release sites.

Excitatory amino acid transporters: Regulation of expression and activity

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High-affinity glutamate transporters so far cloned (GLAST, GLT1, EAAC1, EAAT4, and EAAT5) are believed to maintain low extracellular levels of neurotransmitter glutamate. However, their aberrant functioning leads to excitotoxic neurodegeneration or epileptic seizures. Thus, GLT1, an astroglial transporter that is the most abundant glutamate carrier in the brain, seems to be se-

lectively down-regulated in amyotrophic lateral sclerosis (ALS). The transporter was hardly detectable in pure astroglial cultures, but was induced in the presence of neurons. The induction of GLT1 protein and mRNA was reproduced in pure astroglial cultures supplemented with conditioned media from cortical neuronal cultures. Several growth factors of neuronal origin exhibited moderate effects on the GLT1 expression in astroglial cultures. The regulation of GLT1 by these factors involved overlapping signal transduction pathways. Another transporter, GLAST, being robustly expressed in pure astroglial cultures, was slightly up-regulated in the presence of neurons, but not in the presence of neuron-conditioned media. However, long-term treatment of astrocytes with L-glutamate, kainate or dBcAMP resulted in a selective up-regulation of GLAST, parallelled with significant increase in D[3H]aspartate uptake. The glutamate receptor-sensitive regulation of GLAST seemed to involve both translational and intracellular trafficking mechanisms, whereas the cAMP-dependent pathway utilized primarily transcriptional mechanisms. In conclusion, the expression of GLAST and GLT1 in astroglia may be differentially regulated by neuronal factors via different signalling pathways and expression mechanisms.

(This work was supported by the Danish Medical Research Council, the Norvegian Research Council, and EU BIOMED 2.)

Transient expression of glial glutamate transporters in hippocampal neuronal cultures

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Extracellular glutamate concentration in the brain is mainly regulated by astrocytic transporters despite the presence of neuronal transporters. Among the five identified glutamate transporters, two are primarily astrocytic, GLAST and GLT-1, the three others neuronal EAAC-1, EAAT4 and EAAT5. However, recently, GLT-1 has also been found in neurons and EAAC-1 in astrocytes. Consequently, environmental factors could strongly influence the cell specific expression of both glial and neuronal glutamate transporters. During *in vitro* development of embryonic hippocampal neurons cultured in a defined (serum free) medium, double and triple immunolabelling demonstrated the restricted presence of EAAC-1 on neurons. However, GLAST and GLT-1, present on glial cells, were also detected on a subpopulation of neurons at the first stages of the culture. This expression disappeared with the maturation of the culture. Immunoblots demonstrated the validity of GLT-1 and GLAST antibodies used in this study. Moreover [^{3}H]-glutamate uptake was maximal at 2–3 days of culture, at concentrations (from 2 to 5 μm) favouring GLT-1 activity. These results provide the first evidence of GLAST of neurons and indicate that the two known "glial" glutamate transporters GLT-1 and GLAST could also be transiently expressed on neurons. These transporters appear to be functional. These data indicate that specific culture condition not only influence the level of GLT-1 and GLAST expression but also their cellular localization.

Drug treatments to reduce excitotoxicity: A potential for α_2-adrenoceptor antagonists?

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It is hypothesized that the locus coeruleus-noradrenergic system (LC-NA) controls neuronal compensatory and repair mechanisms in the CNS, and that a deficit in this system is a critical factor in the progression of central neurodegenerative diseases. Pharmacological activation of the LC-NA can be achieved via blockade of α_2-adrenergic autoreceptors. In vivo, α_2-adrenoceptor antagonists increase LC neuronal firing and noradrenaline synthesis and release in rat brain, and are protective against neuronal loss and accelerate spontaneous recovery in some models of brain injury (e.g. trauma, ischemia). Overstimulation of glutamate receptors may also be involved in the pathophysiology of brain injury and progressive neurodegenerative disorders. In order to test the neuroprotective potential of α_2-adrenoceptor antagonists in an in vivo model of excitatory amino acid induced neurotoxicity, the effects of the α_2-adrenoceptor antagonists, (+)-efaroxan (0.63 mg/kg i.p.) or (±)-idazoxan (2.5 mg/kg i.p.), injected i.p. thrice daily for 7 days, were evaluated in rats which received a unilateral lesion of the striatum, produced by intrastriatal injection of the NMDA agonist, quinolinic acid. Both α_2-antagonist treatments resulted in a reduced ipsiversive circling response to apomorphine (measured 10 days post-lesion) and a reduced choline acetyltransferase deficit in the lesioned striatum (14 days post lesion). To investigate the possible mechanisms underlying this neuroprotective effect, striatal extracellular amino acid levels were measured by intracerebral microdialysis in conscious freely moving animals following a single i.p. injection of (+)-efaroxan (0.63 mg/kg). (+)-efaroxan reversibly increased dialysate levels of alanine (+20%) and glutamate (+100%) within the first 3 h post-injection, while a delayed reduction (~50%) in glycine, arginine, asparagine and glutamine was observed after 3 h; aspartate, serine, taurine and tyrosine levels were not significantly affected. These results indicate a neuroprotective effect of (+)-efaroxan and idazoxan in an in vivo model of excitotoxicity. While the mechanisms underlying this drug effect remain to be elucidated, a modulation of the extracellular disposition of amino acids may be involved. These findings suggest a neuroprotective potential for α_2-adrenoceptor antagonists such as (+)-efaroxan and idazoxan in central neurodegenerative disorders where excitotoxicity is implicated.

Implication of poly (ADP-ribose) polymerase (PARP) in excitotoxicity

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Poly (ADP-ribose) polymerase (PARP) is a DNA binding protein which uses NAD^+ as a substrate. When fully activated, notably by free radical-induced DNA single-strand break damage, PARP can deplete NAD^+ and consequently ATP energy stores within a matter of minutes, to an extent which could lead to cell death. Free radicals are known to play an important causative role in excitotoxicity. It has been shown that PARP is activated in the early stages of glutamate-induced toxicity in cerebellar granule neurons [Cosi et al (1994) J Neurosci Res 39: 38–46] and in NMDA-induced toxicity in cortical cells [Zhang et al (1994) Science 263: 687–689] in primary culture, and contributes to the subsequent delayed cell death. However, it has been reported that the *in vivo* intrastriatal injection of non-NMDA glutamate receptor agonists (e.g. kainic acid), and not NMDA agonists (e.g. quinolinic acid), can cause a neurotoxicity that appears to be correlated with free radical formation [Miyamoto and Coyle (1990) Exp Neurol 108: 38–45]. It was therefore of interest to investigate if the intrastriatal injection of a neurotoxic concentration of kainic acid (KA) involved an activation of PARP that might be caused by free radical-induced DNA damage.

PARP activity was measured in striatal extracts both in the absence ("endogenous" activity) and presence ("total" activity) of exogenously-added fragmented DNA. At 6 h after intrastriatal

injection of KA (5 nmols), no significant changes were detected in either endogenous or total PARP activities in comparison to saline-injected (control) striata. At 12 h after KA, both the endogenous and total PARP activities were decreased by 68% and 48%, compared to the 6 h KA group. At 24 h, endogenous PARP activity was increased by 197% compared to saline-injected controls, while total activity did not change. One week after KA, endogenous and total PARP activities were increased by 382% and 60% respectively, compared to the 1-week saline-injected controls. Neither the endogenous nor the total activities of PARP in saline-injected control striata were significantly altered at the time points examined in this study. Cleavage of PARP was not detected at 12 h or at any of the time points following KA treatment. The results indicate that an excitotoxic concentration of KA can produce significant and time-dependent effects on both endogenous and total striatal PARP activity *in vivo*, and suggest a participation of PARP in KA-induced toxicity in the brain *in vivo*.

The nuclear component of excitotoxicity

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In recent years, we have focussed our interest on the role of proteins known to be involved both in cell cycle regulation and in promoting degeneration and apoptosis of neuronal cells. Most of our studies were carried out in primary cerebellar neurons. This in vitro model offers a morphologically defined system for studying transsynaptic regulation of neuronal gene expression and analysing the precise temporal sequence of molecular events following stimulation of specific glutamate receptor subtypes. Exposure of these cells to glutamate results in cell death of both necrotic and apoptotic types. Among the intracellular events triggered by neurotoxic concentrations of glutamate, we identified two transcriptional factors: NF-κB/Rel and the tumour suppressor phosphoprotein p53. Immunocytochemistry and Western analysis demonstrated that glutamate upregulates p50 member of the NF-κB family and p53. Under the same experimental conditions producing cell death, pretreatment of the cultures with aspirin, which inhibits NF-κB/rel activation, or with specific p53 antisense oligonucleotide, which blocks p53 gene transcription, resulted in a complete prevention of glutamate-induced apoptosis. These findings suggest the existence of a transcriptional program activated by glutamate receptor stimulation in which p53 and specific members of the NF-κB family are active contributors.

Then, we analysed the expression of two p53 downstream target genes: p21, which codes for an inhibitor of cdk complexes, and MSH2, which codes for a protein involved in the recognition and repair of a specific type of DNA damage. We found that primary cerebellar neurons expressed both MSH2 and p21 at very low levels in basal conditions. However, very soon after a brief exposure of the cells to glutamate, both MSH2 and p21 expression was dramatically enhanced.

In conclusion, we suggest that glutamate, possibly by increasing intracellular calcium concentration and/or oxygen free radicals production, may activate a restricted number of transcription factors which in turn amplify the signal by recruiting other genes to dictate specific transcriptional programs. A hierarchy of intervention is likely to occur. We propose that NF-κB proteins are among the initial orchestrators of the glutamate-induced apoptotic program. Downstream NF-κB-activation is the transcription factor p53. One of the consequences of the increased transcriptional activity of p53 is the up-regulation of p21 and MSH2. On these bases, we propose that NF-κB, p53, p-21 and MSH2 could be relevant contributors to the glutamate-induced neuronal apoptosis. The fact that these proteins are also involved in cell cycle regulation supports the hypothesis that aberrant expression of mitotic proteins participates in neuronal cell death program.

Can dopamine cytotoxicity be explained by oxidation to aminochrome and ulterior formation of *o*-semiquinone radicals, or by a direct excitotoxic mechanism involving excitatory amino acids?

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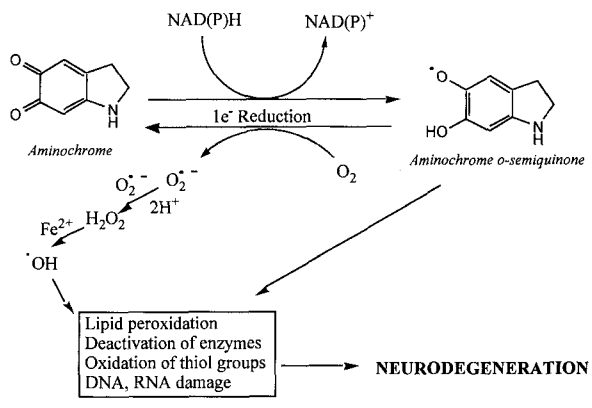
Dopamine have been reported to be toxic in different cell lines and to induce apoptosis, although the molecular mechanism explaining these effects is still not known. One possible explanation to these toxic effects can be associated to a direct effect on glutamate and ulterior excitotoxicity. However, dopamine toxicity can also be due to further metabolism and formation of reactive oxygen species. Dopamine metabolism proceeds along two main pathways, which involve oxidative deamination and O-methylation catalyzed by monoamino oxidase and catechol O-methyl transferase (COMT), respectively. However, under pathological conditions dopamine metabolism can be diverted towards aberrant oxidative pathway. Dopamine, like other catecholamines, can be oxidized in the presence of either oxygen at high pH, or other oxidants, to the corresponding orthoquinone (aminochrome). We postulate that one-electron reduction of aminochrome to *o*-semiquinone radical is the reaction where the reactive oxygen species responsible for the neurodegenerative process in the dopaminergic system is formed. Aminochrome *o*-semiquinone is extremely reactive and autoxidizes in the presence of oxygen giving rise to a redox cycling process, which is accelerated by the antioxidants superoxide dismutase and catalase. In this redox cycling process reactive oxygen species are formed by reducing oxygen to superoxide radical. Hydrogen peroxide can be formed, in the presence of iron or copper, by dismutation of superoxide radicals and hydroxyl radical, a more potent and cytotoxic agent.

Thus, reactive oxygen species, including aminochrome *o*-semiquinone, can induce cytotoxicity by (a) damage of membranes as a consequence of lipid peroxidation, (b) degradation of proteins as a consequence of oxidation of amino acids in their polypeptide chains; (c) deactivation of enzymes, transport pumps and receptors by oxidation of essential thiol groups; and/or (d) damage of DNA or RNA.



Behavioural toxicity of the excitotoxins, domoic acid and kainic acid, at different stages of rat development

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Domoic acid (DOM) and kainic acid (KA) are both naturally occurring excitotoxins known to exert their main effects by acting as agonists at AMPA/kainate subtypes of glutamate receptors. While the pharmacological profiles of these two compounds are very similar (differing mainly in potency), numerous studies have shown that there are also differences between these ligands with respect to receptor binding [Johansen et al (1993) Eur J Pharmacol 246: 195], *in vitro* excitotoxicity [Verdoorn et al (1994) Eur J Pharmacol 269: 43] and *in vivo* behavioural toxicity [Tasker et al (1996) Can J Physiol Pharmacol 74: 1047] in adult rodents. Our objective was to compare the behavioural toxicity profiles of DOM and KA in newborn rats at various stages of postnatal development. Adult Sprague-Dawley rats (200–300 g) were housed in a colony room with food and water available *ad libitum*. Females were harem-mated with males and the time of birth for each litter was recorded. For each litter the first 24 hours following birth was defined as post-natal day 0 (P0). Pups were weaned on P21 where appropriate. Data from male and female pups were analyzed separately. Groups of rat pupy (N = 6 for each) were administered saline or varying doses of DOM on P0, 5, 9, 14, 22 and 30 (females) or 50 (males). Dose response curves for KA were constructed on P8 and P14 for both sexes. Toxicity was recorded as both cumulative behavioural toxicity according to a 4 point scale and as latency to the onset of persistent motor seizures. Analysis of the data revealed that toxicity profiles for DOM were similar in animals up to P14 but that a significant decrease in potency occurred between P14 and P22 (approximately 8-fold). In contrast, the potency of KA decreased by approximately 10-fold between P8 and P14. Prior to P14 KA was about one fourth as potent as DOM whereas this difference increased to almost 30-fold at P14. Behavioural differences between animals receiving DOM or KA were also noted. For example, young rats receiving DOM show both scratching behaviour and whole body tremors whereas KA toxicity was characterized by straub tail and prolonged clonus. No differences were observed between male and female animals at any dose or age. Several possible explanations for these differences, including changes in the properties of different glutamate receptor subunit proteins and differential maturation of inhibitory systems, are currently under investigation and will be discussed.

Modulation of excitatory neurotransmission by terfenadine

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Terfenadine (TEF) is widely used as a non-sedating H1 receptor antagonist, although it presents a broad pharmacological profile. Many studies in non-neuronal cells indicate that TEF may block voltage dependent ion channels and may revert cellular multidrug resistance by binding to glycoprotein P. TEF has also been shown to bind to neuronal calcium channels, although its effects on neurotransmission have been poorly studied. Thus, the aim of present work was to investigate whether TEF could modulate neurotransmission by excitatory amino acids.

Endogenous excitatory amino acid release from cultured cerebellar neurons was stimulated either directly by activating L-type voltage sensitive calcium channels (VSCC) with Bay-K 8644, or indirectly through the activation of voltage sensitive sodium channels (VSSC) with veratridine. In both cases, the release of endogenous excitatory amino acids led to the quick appearance (15–30 min) of excitotoxicity, that could be fully prevented by the NMDA receptor antagonist MK-801. Surprisingly, TEF as low as 10 nM antagonized approx. 50% of excitotoxicity by Bay-K 8466 (2 μM). Excitotoxicity by direct exposure to either NMDA (100 μM) or glutamate (40 μM) was unaffected by TEF. TEF decreased both calcium influx induced by Bay-K 8644 in the presence of MK-801, as measured by confocal laser microscopy, and calcium-dependent cGMP synthesis. The kinetics of TEF inhibition of intracellular calcium increase by Bay-K 8644 indicated that TEF may act as an activity-dependent channel blocker.

TEF also prevented both excitotoxicity following activation of VSSC as well as non-excitotoxic neurodegeneration by persistent activation of VSSC. 50% neuroprotection was achieved at approx. 50 nM terfenadine. TEF produced a significant but not complete reduction of cGMP increase following veratridine exposure, indicating that calcium-dependent second messenger formation following neuronal depolarization was only partially blocked.

Our data suggest that TEF can fully reduce the occurrence of excitotoxicity by endogenous excitatory amino acid release following neuronal depolarization, while allowing partial formation of second messengers, and therefore should be considered as a prototype for therapeutic drugs in the treatment of diseases that involve excitatory amino acid neurotransmission.

Regulation of the nigrostriatal pathway by metabotropic glutamate receptors during development

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Dopamine neurons in the substantia nigra heavily innervate the striatum making it the nucleus with the highest levels of dopamine in the adult brain. The present study analyzed the time course and the density of striatal innervation by nigral dopamine neurons and characterized the role of the neurotransmitter glutamate during the development of the nigrostriatal pathway.

For this purpose, organotypic cultures containing the cortex, the striatum, and the substantia nigra (triple cultures) were prepared. Coronal sections (350–400 μm) from rat brains at postnatal day 0–2 were cut on a microslicer. Selected tissue regions were arranged in serial order on a small rectangular piece of a Millicell-CM membrane (Millipore, Marlborough, MA). Plasma and thrombin were added and cultures were grown using the standard "rollertube" technique for up to 60 days in vitro (DIV). After 8 DIV, cultures were divided into 4–5 groups containing 6–8 triple cultures and drugs were added directly to the culture medium. The pharmacological treatment was repeated at 12 DIV. Cultures were fixed at 16 DIV and were processed for immunohistochemistry. Dopamine fibers and neurons were labeled using tyrosine hydroxylase (TH) immunohistochemistry. Striatal TH-ir fiber density was quantitatively analyzed using confocal laser scanning microscopy (CLSM).

In long-term triple cultures (44 ± 3 DIV), the striatal dopamine fiber density was high and was weakly correlated with the number of nigral dopamine neurons. The high striatal dopamine fiber density mainly resulted from an enhanced ingrowth and ramification of dopamine fibers from nigral neurons during 8–17 DIV. The metabotropic glutamate receptor (mGluR) antagonist L(+)-2-amino-3-phosphonopriopionic acid (L-AP3; 100 μM) selectively inhibited this dopaminergic inner-

vation of the striatum, whereas ionotropic GluR antagonists had no effect. The L-AP3 mediated inhibition was prevented by the mGluR agonist 1S,3R-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD; 1000 μm). (2S,3S,4S)-2-Methyl-2-(carboxycyclopropyl)glycine (MCCG; 200 μm), a compound that is active at group II mGluR, had no effect. The inhibition of the striatal dopaminergic innervation by L-AP3 was further confirmed by anterograde tracing of the nigrostriatal projection with *Phaseolus vulgaris leucoagglutinin.*

These results indicate that glutamate, by acting on group I mGluRs, plays an important "trophic" role for the development of the nigrostriatal dopamine pathway.

Organotypic slices of striatum and meso-striatal organotypic co-slices: models for analysis of biochemistry and function of signalling molecules

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Organotypic slices of brain tissue have recently been used for investigation of biochemistry, function and electrophysiology of signalling molecules. Here we describe the properties of single striatal slices and mesencephalic-striatal co-slices in relation to input, output and intrinsic neurons, as well as their regulation by growth factors and drugs.

Culturing striatal slices significantly reduced the density of dopaminergic nerve fibers and substance P, a peptidergic marker for striato-nigral GABA neurons. Decreased expression of substance P was counteracted by glial cell line-derived neurotrophic factor (GDNF). The single slice model was also used to determine c-fos expression by ecstasy (3,4 methylene-dioxy metamphetamine) via NMDA and dopamine receptors. A further study was addressed to the question whether GDNF has trophic effects on dopamine neurons in an organotypic meso-striatal slice model. Striatal dopamine and acetylcholine tissue levels significantly decreased during culturing. When co-slices were incubated with 10 ng/ml GDNF for 2 weeks dopamine tissue levels were increased. Single ventral mesencephalon slices incubated for 4 weeks with GDNF showed enhanced nerve fiber density. Meso-striatal co-slices incubated without GDNF revealed markedly increased nerve fiber density in the mesencephalic tissue, but no nerve fiber ingrowth into the striatal part. However, meso-striatal co-slices incubated with GDNF for 2–6 weeks showed an increased nerve fiber ingrowth into the striatum. The single slice model is, therefore, useful to discover direct molecular effects of drugs and growth factors whereas in the co-slice model we found that GDNF has potent dopaminotrophic effects on neuronal survival of dopamine neurons but more predominantly on nerve fiber re-growth into the striatum in an organotypic slice model.

Synaptic corelease of glutamate and dopamine from midbrain neurons

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The contemporary version of the "Dale Principle" states that a neuron releases a single classical neurotransmitter from all of its synapses. However, several lines of evidence suggest that CNS monoamine neurons may also be glutamatergic [1, 2]. To examine this question at the synaptic level, we used postnatally-derived neuronal cultures of midbrain dopamine neurons. Amperometric recordings at varicosities on the axons demonstrated quantal dopamine release. We found quantal events were elicited by depolarization, and consisted of ~3,000–20,000 molecules of dopamine over approximately 200 μsec, depending on conditions [3]. To determine if the neurons also released glutamate synaptically we used single neuron microcultures. These formed extensive autaptic synapses *(autapses)*. In the majority of dopamine neurons, as identified by immunoreactivity for the dopamine synthetic enzyme tyrosine hydroxylase, action potentials triggered during whole cell patch recordings elicited subsequent excitatory postsynaptic currents (EPSCs). The EPSCs were blocked by both NMDA and AMPA-type glutamate antagonists, indicating that these neurons exhibit stimulation-dependent glutamate release [4]. While no dopamine-evoked currents were observed directly following action potentials, there was D2 dopamine autoreceptor-mediated presynaptic inhibition of glutamate release, as observed by sulpiride potentiation of excitatory postsynaptic potentials (EPSPs) in a paired pulse protocol.

In the single cell microcultures, dopamine neurons gave rise to some varicosities immunoreactive for both tyrosine hydroxylase and glutamate, and others immunoreactive mainly for glutamate, which were found near the cell body. At the ultrastructural level, dopamine neurons formed occasional dopaminergic varicosities with symmetric synaptic spezializations, but more commonly non-dopaminergic varicosities with asymmetric synaptic specializations. Most dopamine neurons immunostained for glutamate; they were also immunoreactive for phosphate-activated glutaminase, the major source of neurotransmitter glutamate. This colocalization of glutamate and dopamine synapses may also occur *in situ* as ventral midbrain sections from rat and monkey were immunoreactive for both tyrosine hydroxylase and glutamate.

In contrast to Dale's principle, stimulation of individual dopamine neurons evoked quantal dopamine release from a subset of axonal varicosities and glutamate from a partly overlapping subset of axonal varicosities. The glutamate EPSCs showed presynaptic inhibition due to concomitant dopamine release. Thus, dopamine neurons can exert rapid synaptic actions by releasing glutamate and slower modulatory actions by releasing dopamine. Dopamine modulates release of both glutamate and dopamine release via presynaptic D2 autoreceptors. Together with evidence for glutamate cotransmission in serotonergic raphe neurons [5] and noradrenergic locus coeruleus neurons [6], the results suggest that glutamatergic cotransmission may be the rule for central monoaminergic neurons.

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Monoamine, excitatory amino acid and NOS systems in triple organotypic cultures: effects of perinatal asphyxia

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At the Karolinska Institutet, a novel non-invasive model for studying the short- and long-term consequences of perinatal asphyxia has been developed, consisting in placing foetus containing uterus horns, taken by hysterectomy from ready to deliver rats, in a water bath at 37° C for various periods of time. Following asphyxia, the uterus horns are opened, the pups are removed, stimulated to breathe and studied immediately after birth, or long after development at the care of surrogate mothers. Using this model, we have demonstrated that anoxia produces region specific impairment of metabolism, and a delayed apoptosis-like neuronal death, mainly affecting the striatum, frontal cortex and cerebellum, in absence of gross neuroanatomical changes. Furthermore, when reaching adulthood, these animals present marked alterations in the nigrostriatal dopamine system, which are accompanied by motor deficits revealed during the exposition of the animals to a new environment, suggesting an impairment of mechanisms of habituation and learning. Thus, we have proposed that severe perinatal asphyxia can be a concurrent factor for the development of neurodegenerative diseases with a clinical onset at late stages.

The effect of perinatal asphyxia is now studied with the Organotypic Culture Model developed by Gähwiler in Basel, Switzerland, and by Plenz and colaborators in Memphis, TN, USA. At postnatal day 3 the brain of control and asphyctic pups is dissected to sample tissue from the nigrostriato-cortical or mesolimbic cingulate systems. Organotypic triple cultures are prepared and grown for a 4 weeks period. The general survival and growth of the cultures is monitored directly with inverted microscopy and, after biochemical sampling from the culture medium, by assaying catecholamines, amino acids, hypoxanthine and xanthine at 4, 8, 16 and 28 days in vitro (DIV). At 28 DIV the cultures are fixed with a 4% paraformaldehyde solution, and studied with histochemistry.

The goal of the project is to find out what are the critical parameters by which perinatal asphyxia primes the development of the central nervous system, aiming to describe and to test new therapeutic strategies to prevent and/or reverse the long-term consequences induced by metabolic/anoxic insults occurring at time of birth.

Excitatory amino acid neurotoxicity and modulation of glutamate receptor expression in organotypic brain slice cultures

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Slices of developing postnatal brain tissue can be grown in tissue culture for weeks with preservation of the normal, basic cellular and connective organization of the donor brain regions, thereby providing an easily accessible experimental model for studies of toxic, degenerative and plastic developmental changes in the brain.

By growing organotypic slice cultures of *hippocampus* from newborn to 7 day old rats on semiporous membranes in chemically defined, serum-free medium, we have studied the excitotoxic effects of acute doses kainic acid (KA) and AMPA and the neurotoxin trimethyltin. The resulting neurodegeneration and cell damage, monitored quantitatively by densitometric measurements of the cellular uptake of the fluorescent dye propidium iodide (PI), cellular staining by Flouro-Jade, lactate dehydrogenase (LDH) release to the medium, and immunostaining for microtubulin-associated protein 2 (MAP-2), corresponded to the regional pattern of neurotoxicity observed *in vivo*. By long-term exposure of developing hippocampal cultures to low, non-toxic doses of KA, we have moreover shown that the susceptibility of the cultures to a subsequent higher and normally toxic dose of KA is reduced, while long-term treatment of the cultures with the AMPA/KA antagonist NBQX results in increased susceptibility to KA. Following long-term exposure to KA there was a reduced expression of glutamate receptor subunit GluR6 and KA2 mRNA, as demonstrated by quantitative in situ hybridization. When the expression of GluR1 protein was examined by Western blot and GluR1 mRNA, by in situ hybridization only the protein, but not the mRNA expression was found to be reduced. In cultures long-term treated with NBQX the hybridization signals for KA1 and KA2 mRNA were increased.

After application of KA and AMPA to *corticostriatal slice cultures,* with striatum and the overlying neocortex present *en bloc* in the slices, we found a dose-response related correlation between cellular PI uptake, Flouro-Jade staining for degenerating neurons and release of cytosolic LDH to the culture medium. Acute exposure of the corticostriatal cultures to 24 μM KA or 6 μM AMPA mixed with 0–9 μM NBQX showed that NBQX had neuroprotective effects in both striatum and cortex, but with better (lower dose) protection against AMPA in striatum, and better protection against KA in cortex.

From the results obtained, we conclude that organotypic brain slice cultures, combined with standardized procedures for demonstration and quantitation of cell damage and receptor subunit changes is of great potential use for studies of excitotoxic, glutamate receptor-induced neuronal cell death, receptor modulation and related neuroprotection.

(Supported by the Danish MRC, the Danish Biotechnolgy Program, and the EU Biotech Program.)

Long-term exposure to the AMPA/kainic acid antagonist NBQX increases the susceptibility of hippocampal slice cultures to acute doses of kainic acid

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We have previously shown that long-term exposure of hippocampal slice cultures to a low, non-toxic dose of kainic acid (KA) makes the cultures more resistant to a subsequent high and normally toxic dose of KA, and that the long-term treated cultures displayed a downregulation of GluR6 mRNA and KA2 mRNA by quantitative *in situ* hybridization.

In this study hippocampal slice cultures were long-term treated with the AMPA/KA antagonist, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX) and then examined for changes in their susceptibility to KA.

Organotypic slice cultures were prepared of the hippocampus from 7 days old rats and grown on semiporous membranes. Initially the slices were grown in serum containing medium, but after 4 days this was replaced by a chemical defined medium (Neurobasal with B27 supplement; Gibco) containing 0.3 μM NBQX, with renewal of the medium NBQX twice a week. After 3 weeks of NBQX exposure the cultures were exposed to a medium containing 3 or 5 μM KA for 48 h followed by another 48 h in normal medium. The excitotoxic effects of KA was monitored by Propidium Iodide uptake into damaged cells, immunohistochemical staining for neurons by the NeuN antibody and general cell staining.

In cultures long-term treated with 0.3 μM NBQX and then acutely exposed to 3 or 5 μM KA the uptake of PI was significantly increased in all subfields of the CA3 pyramidal cell layer compared to corresponding sets of control cultures without NBQX long-term treatment. Corresponding to the PI uptake there was ex-

tensive loss of Nissl staining in the CA3 pyramidal cell layer. In NeuN immunocytochemical staining the CA3 pyramidal cells were darkly stained with a condensed morphology. The CA1 region which normally is less sensitive to KA than CA3 displayed no changes in susceptibility due to the NBQX treatment.

To what extent the NBQX-induced change in KA susceptibility is accompanied by glutamate receptor modulations and subunit expression is currently being investigated.

Differential protective effects of NBQX against KA and AMPA excitotoxicity in striatum and cortex of corticostriatal slice cultures

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This study aimed to determine excitotoxic effects of kainic acid (KA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and neuroprotective effect of the glutamate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)-quinoxaline (NBQX) on striatum and the overlying neocortex in corticostriatal slice cultures. Trimmed, coronal brain slices containing the two structures were prepared from neonatal rats and grown in serum-free medium on semiporous membranes for 3–4 weeks before exposure to KA or AMPA for 48 h. With PI added to the medium, digitized fluorescent microscope images showed a dose (0–24 μM KA; 0–6 μM AMPA) and time (0–48 h) dependent PI uptake in both striatum and cortex. There was no general differences in PI uptake related to the doses of KA and AMPA, but when exposed to 24 μM of KA and increasing doses of NBQX (0–9 μM), cortical neurons were protected by NBQX (IC50 <0.1 μM NBQX) at significantly lower doses of NBQX than striatal neurons (IC50 = 2.5 μM NBQX). The opposite was the case when cultures were exposed to AMPA (IC50 = 0.3 μM NBQX for cortex; and IC50 ~0.07 μM NBQX striatum). For the evaluation and comparison of markers for degenerating neurons we compared the densitometric measurements of PI uptake with measurements on sections of the same KA exposed corticostriatal cultures stained with the fluorescent dye Fluoro-Jade, which is a newly introduced marker for degenerating neurons. For both striatum and cortex we found a significant correlation between the PI uptake and Fluoro-Jade staining, just as the total PI uptake was significantly correlated with the amounts of cytosolic lactate dehydrogenase released to the culture medium after the excitotoxic lesions. We conclude that the protective effects of NBQX against AMPA and KA is different in striatum and neocortex and probably reflects different glutamate receptor subunit expressions in the two regions, and that PI uptake under standardized conditions is a feasible marker for excitotoxic neurodegeneration in corticostriatal slice cultures.

Modeling of amyloid pathology and neuroinflammation in hippocampal organotypic cultures

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The neuropathological hallmarks of Alzheimer's disease (AD) are deposition of extracellular amyloid plaques, the occurrence of intracellular neurofibrillary tangels and degeneration of vulnerable neurons, including pyramidal cells of the hippocampus. A principal component of amyloid plaques is the β-amyloid peptide (Aβ), a metabolic product of β-amyloid precursor protein (APP). Inflammatory changes are seen proximal to amyloid deposits in AD brains, with gliosis and the presence of cytotoxic factors, such as cytokines, free radicals and excitotoxic species. We have developed an *in vitro* model of AD by the use of organotypic cultures, where slices of rat neonatal hippocampus are grown in a roller-drum for several weeks. In the cultures a cellular monolayer of different neuronal populations, such as hippocampal pyramidal neurons and GABA containing interneurons, as well as macroglial and microglial cells can be found. Following culturing in the presence of Aβ (25–35) a concentration dependent loss of pyramidal neurons, identified by their expression of the NMDAR1 receptor subunit, is observed concomitant with plaquelike deposits in the tissue. Antioxidants, such as the spin trapping agent a-phenyl-N-tert-butyl nitrone (PBN) and trolox, and the microglial 3-hydroxyanthranilic acid 3,4-dioxygenase (3-HAO) enzyme inhibitor NCR-631 exert potent and dose-dependent neuroprotective effect. Also exposure to Salmonella abortus equi lipopolysaccaride (LPS 1–100 ng/ml), a general pro-inflammatory bacterial endotoxin, lead to a pronounced pyramidal neuronal loss, which can be counteracted by the GABA agonist chlometiazole and NCR-631 [Luthman et al (1998) Amino Acids 14: 263–269], but not with trolox. While the cytokine IL-1β (3–25 IU/ml) also induces concentration-dependent pyramidal cell loss no neurodegeneration could be detected following exposure to TNFα (10–30 IU/ml), IL-2 (0.1 ng/ml), IL-6 (70 ng/ml) or INF-γ (200 IU/ml). Hence, two aspects of AD neuropathology, i.e. amyloid pathology and neuroinflammation can be mimicked in the highly differentialted organotypic hippocampal cultures. The relative involvement of different cytotoxic factors implicated in AD, such as Aβ, cytokines, free radicals and excitotoxins, can be explored in this model in order to identify possible therapeutic agents for AD.

Differences in rates of trapping between AR-R15896 and other uncompetitive NMDA receptor antagonists

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NMDA receptor antagonists have suffered numerous difficulties in moving from *in vitro* and whole animal studies to clinical trials. At least some of the problems are due to the inability of antagonists to selectively block pathological activity while leaving basal activity relatively normal. In both epilepsy and stroke, pathological activity is very likely to be associated with hyperactivity of the NMDA receptor. Therefore, uncompetitive, or use-dependent, antagonists should be better at selecting between basal and pathological activity than competitive or non-competitive antagonists. However, use-dependent antagonists of the NMDA receptor can become trapped at the receptor when agonist leaves. When this occurs, activity is effectively blocked prior to subsequent agonist presentation and the block is no longer use-dependent. However, recent reports from Blanpied et al. and Mealing et al. demonstrate that some use-dependent NMDA receptor antagonists can partially escape from the closed channel and retain at least some degree of use-dependence. This property, closed-channel egress resulting in partial trapping, can explain the better safety profile of compounds such as memantine and AR-R15896. We will present data to discuss the concept of closed-channel egress as important for developing clinically useful NMDA receptor antagonists.

Low affinity uncompetitive NMDA receptor antagonists: A success story

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Previous attempts to introduce high affinity competitive or uncompetitive NMDA receptor antagonists into human patients

met with failure due to the untoward effects encountered at therapeutic levels of the compounds. On the other hand, several uncompetitive low affinity NMDA receptor antagonists have advanced into clinical trials for dementia (memantine, remacemide); epilepsy (remacemide [dextromethorphan, ADCI, both discontinued]), head trauma (HU-211, NPS 1506), acute stroke (remacemide, HU-211, NPS 1506, AR-R 15896 AR [dextromethorphan, discontinued]), Parkinson's disease (amantadine, budipine, remacemide, memantine for associated spasticity), and Huntington's disease (remacemide). These compounds differ from their high affinity counterparts in that there is wider separation between doses providing efficacy and the following side effects: 1) Abuse liability; 2) lack of motor in-coordination; 3) no effects on learning and memory; 4) μM vs. nM affinity at the ion channel site of the receptor; 5) more rapid on, especially off, receptor kinetic rates; 6) limitation, but not inhibition, of $[Ca^{2+}_{in}]$ entrance through the NMDA receptor-operated ion channel; 7) less membrane trapping of the compound between NMDA pulses; 8) differential affinity between forebrain- and cerebellum-type receptors – greater affinity for the latter; 9) NMDA subunit specificity – tendency for more affinity at NMR1/NMR2b,c,d subunits than for NMR1/NMR2a receptor subunits; and 10) lack of neurotoxicity and lower incidence of transient vacuoles.

Why are moderate affinity NMDA receptor channel blockers well tolerated?

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Fast open channel blocking/unblocking kinetics and strong voltage-dependency are important characteristics of all moderate affinity NMDA receptor channel blockers. MRZ 2/579 and memantine bind to the same channel site (d = 0.8) and have similar fast blocking kinetics (K_{on} 10.7 and 7.5 * 10^4 M^{-1} sec^{-1}, K_{off} 0.20 and 0.21 sec^{-1} at –70 mV respectively). In the continuous presence of MRZ 2/579 or memantine (10 μM) voltage-steps from –70 mV to +70 mV caused near complete relief of blockade with similar rapid, double-exponential kinetics [$t_{off}1 = 121 \pm 33$ (39%), $t_{off}2 = 953 \pm 69$ ms; $t_{off}1 = 99 \pm 38$ (44%), $t_{off}2 = 725 \pm 122$ ms respectively]. Recent data confirm that partial untrapping following agonist removal in the continuous presence of memantine and MRZ 2/579 is around 15–20%. This may release a proportion of previously blocked channels for subsequent physiological activation (Blanpied et al., 1997). NR2C receptors subtype selectivity was also proposed to be important. Memantine, amantadine and MRZ 2/579 had the following IC_{50}s at NR2A, 2B, 2C or 2D receptors expressed with NR1a in *Xenopus* oocytes at –70 mV: Memantine 0.9, 0.4, 0.3 and 0.3 μM; amantadine 26, 18, 10 and 10 μM; MRZ 2/579 0.5, 0.6, 0.4, 0.5 μM. This weak subtype selectivity of memantine was increased slightly under more "physiological" conditions (–30 mV, with 1 mM Mg^{2+}): IC_{50}s of 10.3, 2.0 and 1.9 μM at NR2A, 2C and 2D respectively. MRZ 2/579 showed no subtype selectivity, but has a similar promising profile to memantine in animal models. As such, the moderate potency and associated biophysical properties of all three antagonists – fast, voltage-dependent blockade and associated partial untrapping – are probably more important for good therapeutic profiles.

NMDA channel blockers: memantine and amino-alkylcyclohexanes – *in vivo* characterization

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Out of NMDA receptor antagonist only channel blockers like memantine, dextromethorphan, remacemide, or amantadine have been used clinically. All of these agents are rather low to moderate than high affinity antagonist indicating that this feature is crucial for therapeutic tolerability. In line with this trend, we describe NMDA antagonistic properties of over 40 new agents showing *in vitro* certain similarities to memantine (see Parsons et al., this meeting for *in vitro* and primary *in vivo* characterization). Although they all produced stereotyped behaviour in mice and rats, this effect only appeared at relatively high doses. Selected agents (e.g. MRZ 2/579) were characterized in a number of animal models which indicate that they have therapeutic potential in opioid abuse, alcohol abuse, inhibition of tolerance to the analgesic effects of morphine and strong neuroprotective activity. Moreover, MRZ 2/579 inhibited haloperidol-induced catalpsy, reserpine-induced sedation and produced rotation in rats with unilateral lesion of the nigro-striatal system (10–30 mg/kg) suggesting antiparkinsonian-like activity. MRZ 2/579 failed to show anxiolytic activity in the plus-maze test, did not change prepulse inhibition and produced partial generalization to ethanol cue in drug discrimination test. Hence, MRZ 2/579 and related agents represent a new chemical class of NMDA channel blockers with favourable behavioural profile that could possibly be used in drug dependence, inhibition of drug tolerance, Parkinson's disease and as neuroprotective agents.

NPS 1506, a moderate affinity uncompetitive NMDA receptor antagonist: Preclinical summary and clinical experience

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NPS 1506 is a novel NMDA receptor antagonist with an IC_{50} of 470 nM. NPS 1506-induced block increases as the concentration of NMDA is increased, consistent with uncompetitive block. NPS 1506-induced block is use- and voltage-dependent, consistent with open-channel block. NPS 1506 is equipotent at blocking all NR2 subunits.

NPS 1506 is neuroprotective *in vivo* in models of focal ischemic stroke, hemorrhagic stroke, and head trauma. In models of temporary ischemic stroke, neuroprotection was present when treatment was delayed 2 hours after the onset of ischemia. Doses of NPS 1506 providing neuroprotection ranged from approximately 0.1 to 1.0 mg/kg. Doses were more effective when administered twice, four hours apart. A loading dose plus constant-rate infusion regimen provided neuroprotection in a model of temporary focal ischemic stroke. Peak plasma concentrations following neuroprotectant doses were between 8 and 80 ng/ml.

NPS 1506 does not elicit in rodents the side effect profile that is typical of potent open-channel blockers. MK-801-like behaviours were not noted. NPS 1506 did not generalize to PCP, did not elicit neuronal vacuolization, did not impair pre-pulse inhibition of startle, did not produce a lasting impairment of spatial learning, and did not impair learning in a simple passive avoidance task.

An initial Phase I study of NPS in healthy volunteers has been completed. Intravenous doses of NPS 1506 from 5–100 mg were well tolerated and provided plasma concentrations in excess of those required for neuroprotection in rodents. A Phase Ib study in stroke patients is in progress.

The role of NMDA receptors in behavioural sensitization

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If repeated intermittent administration of a drug causes a progressive augmentation of a behavioural response, this phe-

nomenon is called development of behavioural sensitization. Sensitization is a very robust phenomenon and outlasts the period of drug administration. Experimentally, this can be shown by administration of the drug under which sensitization developed after long drug free periods (challenge). Since most drugs of abuse have been shown to induce behavioural sensitization, it is considered to play a role in the development of addiction [Wolf M E (1998) Prog Neurobiol 54: 679–720]. The environment (context) in which sensitization develops can become conditioned to the behavioural response and can influence the development and expression of sensitization (in animals mostly sensitization of locomotion has been studied). Thus sensitization encompasses context-independent and context-dependent components. But there are also cases in which locomotor sensitization can be completely context dependent.

Sensitization reflects a form of neural plasticity. Since several forms of neural plasticity require activation of glutamatergic NMDA receptors, it has been studied whether or not NMDA receptor antagonists are able to block behavioural sensitization. In fact numerous studies have shown that sensitization was blocked by competitive and uncompetitive NMDA receptor antagonists and in turn, the use of NMDA receptor antagonists has been proposed as a possible treatment for addiction. However, recently the situation became much more complicated: In most of the published studies, locomotion was studied after the first administration of the sensitizing drug, then, the animals were treated for several days in their home cage. To test for sensitization, the animals received a challenge injection of the sensitizing drug and locomotion was measured. An increased response shows that sensitization has taken place. If the NMDA receptor antagonist MK-801 had been coinjected during the treatment phase, the animals displayed locomotion at the same level as animals that have received the sensitizing drug for the first time. This led to the conclusion that NMDA receptor antagonists block sensitization. However, recent experiments in which animals were tested during the phase of sensitization showed that during the sensitization period, a day to day increase occurred also in the group of animals receiving the sensitizing drug plus MK-801.

If after a period of daily treatments with a drug plus MK-801 the animals are challenged with the drug alone, the animals react as if they were receiving the drug for the first time [Tzschentke TM, Schmidt WJ (1998) Trends Pharmacol Sci 19: 447–451]. To conclude that MK-801 has blocked sensitization would mean to ignore the day to day increase seen under MK-801. The view will be discussed, that NMDA receptor antagonists do not block the development of sensitization but make the expression of sensitization strongly state dependent.

[Supported by the BMBF (FSP Suchtforschung, Universität Tübingen 01EB9420).]

Susceptibility of neostriatal dopamine nerve terminals to oxidative injury by L-DOPA and related chemical species

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L-dihydroxyphenylalanine (L-DOPA) generates reactive O_2 species (ROS) and is suspect as being cytotoxic to dopamine (DA) nerves and accelerating progression of parkinsonism. To model L-DOPA effects in parkinsonism, the neostriatum was DA-denervated (99% reduction in endogenous DA) by 6-hydroxydopamine (6-OHDA) treatment (134 µg, bilateral icv) of neonatal rats. At 10 weeks IP carbidopa (12.5 mg/kg) was given 30 min before vehicle, L-DOPA (60 mg/kg) or a known ROS generator, 6-hydroxydopa (6-OHDOPA). Salicylic acid (10 µl, saturated soln), given 30 min later and 15 min before sacrifice, was the trapping agent for hydroxyl radical ($\bullet OH^-$). Both L-DOPA and 6-OHDOPA reduced neostriatal content of the spin trap product 2,3-dihydroxybenzoic acid (2,3-DHBA) in fully innervated neostriatum but did not markedly alter higher levels of 2,3-DHBA in DA-denervated neostriatum. Findings suggest that L-DOPA is not damaging to DA nerves in treated parkinsonians and may be neuroprotective.

Functional aspects of the ventral pallidum – Neurochemical and behavioural studies

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The ventral corticoaccumbo-thalamocortical loop of the basal ganglia is critically involved in the control of limbic-motor integration, e.g. locomotor behaviour and reinforcement. The ventral pallidum (VP) is part of this ventral loop and is innervated by glutamatergic projections from cortical, subcortical and limbic areas, by GABAergic projections from the nucleus accumbens and by a distinct dopaminergic projection from the ventral tegmental area (VTA). Despite these comprehensive innervations and interactions, only limited information is available about the functional implication of VP in the integrative process of the corticoaccumbo-thalamocortical loop.

We investigated the effects of glutamate receptor stimulation in the VTA and VP on dopamine and glutamate release in the two structures in awake rats implanted with one microdialysis probe in the VTA and another in the VP. In parallel, effects on motor behaviour (i.e. locomotion) were analyzed.

The microdialysis studies show that dopamine is released in the VP and that this release is under control of NMDA and AMPA receptors located in the VTA and also the VP. Moreover, glutamate release in the VTA and VP is controlled by effects mediated by NMDA and AMPA receptors located in the respective structure. Correlation of the neurochemical findings to motor outcome suggests that NMDA receptor-mediated effects but less those of AMPA receptors are important for the processing of limbic-motor integration in the ventral loop.

[Supported by the DFG (SFB 307 A4).]

The role of the rat medial prefrontal cortex in the mediation of brain reward

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The medial prefrontal cortex (mPFC) is thought to be part of the brain reward system. Evidence for this comes from self-administration, self-stimulation, and place preference conditioning (CPP) studies. Stimulation of the mPFC is presumed to be rewarding because rats will learn to press a lever in order to self-administer the stimulation. Experimenter-applied stimulation of the mPFC causes dopamine (DA) release in nucleus accumbens septi (NAS), presumably by activating glutamatergic fibers that project to the ventral tegmental area (VTA), origin of the mesolimbic DA system. However, a similar increase in subcortical DA in self-stimulating rats has not been shown yet. Therefore, we have sought to determine whether glutamate and DA release in VTA and NAS are seen at parameters of stimulation that are rewarding to the animal, using microdialysis in freely-moving, self-stimulating rats. It was found that all frequencies and only frequencies that reliably sustained self-stimulation behaviour produced an increase in extracellular glutamate in the VTA and a corresponding increase in extracellular DA in the NAS. Both lever-press re-

sponding and the increase in NSA DA could be prevented by intra-VTA infusion of the glutamate antagonist kynurenic acid.

In a second set of experiments the effects of mPFC subarea-specific quinolinic acid lesions on drug-induced CPP were examined. It was found that lesions of the infralimbic mPFC disrupted the development of morphine- and CGP37849-induced CPP, and lesions of the prelimbic mPFC disrupted cocaine- and CGP37849-induced CPP. Lesions of the anterior cingulate mPFC disrupted only CGP37849-induced CPP. None of the lesions (incl. lesions of the whole mPFC) had effects on the development of amphetamine-induced CPP. These findings suggest that a double dissociation exists for the role of the mPFC in reward. While its function is important for the rewarding effects of several drugs, it is not important for the mediation of amphetamine-induced reward. In addition, different subareas of the mPFC seem to be important for the mediation of the rewarding actions of different drugs.

Taken together, these findings, along with the demonstrations that cocaine and NMDA receptor antagonists are self-administered into the mPFC, clearly suggest that the mPFC has an important function in the brain reward system. Reward can be initiated directly within the mPFC, and even in cases where reward is not initiated there the mPFC appears to the important, possibly by conveying reward-relevant information to the VTA and/or NAS.

(Supported by the BMBF and NIDA.)

Suppression of conditioned avoidance response by AMPA receptor blockade

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Several lines of evidence indicate that major behavioral effects of schizophrenomimetic, non-competitive NMDA receptor antagonists, especially at low doses, are mediated largely via the mesocorticolimbic dopamine (DA) system. Systemic administration of MK-801 causes, indeed, an increase in release and metabolism of DA in e.g. the nucleus accumbens (NAC). Moreover, the concomitant locomotor stimulation seems to be specifically related to activation of AMPA and/or kainate receptors in the ventral tegmental area (VTA), probably due to enhanced glutamatergic input. Thus, both systemic and intra-VTA administration of AMPA receptor antagonists effectively suppress the behaviorally activating effect of MK-801 as well as the increased DA output in the NCA (see Mathé et al., 1998). Subsequently, we observed that systemic administration of GYKI52466, a non-competitive AMPA receptor antagonist, causes a significant and selective suppression of the conditioned avoidance response (CAR) in the rat, an effect shared by essentially all effective antipsychotic drugs (Svensson et al., 1998). In addition, we have now investigated the effect on the CAR of LY326325, a novel AMPA receptor antagonist, with a different chemical structure. Administration of LY326325, 18 mg/kg subcutaneously, also caused a selective suppression of the CAR, without effect on escape behavior or intertrial crosses. These results underline the notion that the suppression of CAR by LY326325 is not related to general sedation or impaired motor performance *per se*. In addition, no catalepsy was observed. Our results taken together indicate an antipsychotic effect of AMPA receptor antagonists with a low liability for extrapyramidal side effects and hence, in principle an atypical antipsychotic profile.

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Antioxidant compounds EGB-761 and BN-520 21 attenuate heat shock protein (HSP 72 kD) response, edema and cell changes following hyperthermic brain injury. An experimental study using immunohistochemistry in the rat

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Heat stress and associated hyperthermia induces profound cell injury and edema in may parts of the brain [1]. The probable mechanisms of brain pathology following hyperthermia are not well known. Our experimental observation in the rat model show that many neurochemicals such as serotonin, prostaglandin, histamine and opioid peptides are involved in the breakdown of the blood-brain barrier (BBB) permeability, vasogenic edema and cell injury following heat stress [1]. Since heat stress is associated with oxidative stress and free radical formation, in present investigation we examined the influence of a new antioxidant compound EGB-761 and BN-520 21 (Institute Henri Beaufor-IPSEN, Paris, France) on edema formation and cell changes in the brain in our rat model [1–3]. Furthermore, to find out the molecular basis of cellular injury in heat stress and its possible modification with the antioxidant compound EGB-761 and BN-520 21 we also examined immunostaining of heat shock protein (HSP 72 kD), a universal response of cells following noxious heat stimulus [3]. Rats subjected to 4 h heat stress at 38° C in a biological oxygen demand (BOD) incubator (relative humidity 50–55%, wind velocity 20–25 cm/sec) resulted in profound edema and cell injury in many parts of the cerebral cortex, hippocampus, cerebellums, thalamus, hypothalamus and brain stem. Immunostaining of HSP 72 kD showed marked upregulation in the damaged and distorted neurons located within the edematous area. Pretreatment with EGB-761 (50 mg/kg/day, p.o.) and BN-520 21 (3 mg/kg, i.p.) per day for 5 days and 30 min before stress [1] significantly reduced the edematous swelling and cell injury and resulted in a marked attenuation of HSP response. Our results show that EGB-761 and its component gingkolides, BN-520 21 has the capacity to reduce edema and cell changes following hyperthermic brain injury and this effect of the compound is somehow associated with a reduction in cellular HSP response, a subject which require further investigation.

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Neurotrophic factors influence upregulation of constitutive isoform of heme oxygenase in the spinal cord following trauma. An experimental study using immunohistochemistry in the rat

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Carbon monoxide (CO) is considered as a gas responsible for signal transduction in the CNS and is involved in cell injury caused by brain trauma and hyperthermia [1]. In the CNS, CO is produced by an enzyme known as heme oxygenase (HO) [1, 2]. HO is present in two isoforms, i.e. HO-1 and HO-2, which corresponds to the inducible and constitutive isoforms respectively. HO-2 is constitutively expressed in normal animals and few pos-

itive neurons in the spinal cord gray matter can be seen using immunohistochemistry. Recent reports suggest that traumatic, ischemic or haemorrhagic insult to the CNS induces upregulation of HO in the nervous system [1]. The probable mechanisms and significance of such upregulation are still unknown.

The present investigation was undertaken to find out whether a focal trauma to the rats spinal cord induces an upregulation of HO-2 like nNOS upregulation seen previously [3]. Secondly, whether the upregulation of HO is involved in the pathophysiology of cell injury. In order to examine the potential contribution of HO-2 in cell injury we used pretreatment with BDNF and IGF-1 which is known to induce neuroprotection in this model [3, 4].

Our results show that subjection of rats to 5 h spinal trauma induced by an incision of the right dorsal horn at T10–11 segment significantly upregulated the HO-2 in the adjacent spinal cord segments (T9 and T12) compared to normal rats. Pretreatment with BDNF or IGF-1 significantly attenuated the upregulation of trauma induced HO-2 expression in the cord. These results probably for the first time show that spinal cord injury is associated with upregulation of HO-2 expression and this increase in HO-2 expression is significantly attenuated with BDNF and IGF-1 pretreatment. Our observations, thus opened a new line of investigation in the field of spinal cord trauma which suggest that CO is involved in the pathophysiology of spinal cord injury and growth factors somehow influence the CO metabolism, not reported earlier.

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Growth factors attenuate alterations in spinal cord evoked potentials and cell injury following trauma to the rat spinal cord

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Experiments carried out in our laboratory suggest a strong correlation between decrease in SCEP amplitude following trauma with later development of spinal cord edema and cell injury [1, 2]. This investigation is undertaken to examine the role of traumatic stress in causing early disturbances in SCEP and edema formation following trauma. We used two exogenous growth factors, brain derived neurotrophic factor (BDNF) and insulin-like growth factor-1 (IGF-1) to attenuate the consequences of cell injury following trauma and to see whether this neuroprotection is related with cellular stress as examined with expression of heat shock protein (HSP 72 kD) response.

A focal trauma to the rat spinal cord produced by incision of the right dorsal horn at T10–11 segment resulted in an immediate depression of SCEP amplitude (mean depression 60%) which lasted for about 1 h [1, 2]. At the end of 5 h there was some recovery in SCEP amplitude [4]. Morphological examination showed profound edema and cell injury 5 h after injury and the expression of HSP was pronounced in the perifocal T9 and T12 segments.

Repeated topical application of BDNF or IGF-1 (20 µg/kg for 30 sec) applied 30 min before followed by 0 min (at the time of injury), 10 min, 30 min, 60 min after and thereafter every 1 h, i.e. 120 min, 180 min, 240 min and 300 min [3], resulted in a marked protection of SCEP amplitude seen after injury. In addition, the traumatized cord which received neurotrophins showed significantly less edema and cell injury. Interestingly, the occurrence of HSP 72 kD upregulation was not much pronounced in these growth factors treated rats. These observations for the first time provide evidence that (i) cellular stress is involved in SCEP disturbances, edema and cell injury and (ii) growth factors somehow attenuate the stress response following trauma and improve spinal cord conduction. It would be interesting to see whether similar application of BDNF or IGF-1 as post-trauma treatment at various intervals after the primary injury is still neuroprotective and can restore spinal cord conduction, a feature which is currently under investigation in our laboratory.

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Spinal nerve lesion induces upregulation of constitutive isoform of heme oxygenase in the spinal cord. An immunohistochemical investigation in the rat

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The neurochemical basis of the pathophysiology of neuropathic pain and associated neurodegeneration is not well understood. Recently involvement of carbon monoxide (CO) in many physiological and pathophysiological functions of the CNS is suggested [1, 2, 4, 5]. However, its role in neurodegeneration and cell injury is still unclear [1, 2]. The synthesis of CO in the nervous system is mediated by the enzyme heme oxygenase (HO) which is normally present in two isoforms, HO-1 and HO-2 in some nerve and glial cells in the CNS [2]. There are experimental evidences which suggest that a focal trauma to the rat spinal cord is associated with an upregulation of HO-1 and HO-2, activity in neurons of the perifocal segments [1]. This upregulation of HO is closely related with the pathological reaction of nerve cells indicating a putative role of CO in cell injury. The present investigation was undertaken to find out whether a chronic spinal nerve lesion is associated with alteration in HO-2, the constitutive isoform of the enzyme activity, and if so, whether this alteration in HO expression is related to cell injury. Spinal nerve lesion at L-5 and L-6 level was produced according to the Chung model of neuropathic pain and rats were allowed to survive for 8 weeks [3]. Sham operated rats, in which the spinal nerve was exposed but not ligated, served as controls. HO-2 upregulation was examined on Vibratome section (60 µm thick) obtained from L-5 segment of the lesioned control and sham operated rats according to the standard protocol [3, 4]. Selected tissue pieces of the cord from each group were embedded in epon for routine light and electron microscope [5]. Ligation of spinal nerves in rats resulted in an upregulation of HO-2 expression which was most pronounced in the ipsilateral gray matter of the spinal cord compared to the contralateral side. In these rats, ultrastructural investigations showed distorted neurons, membrane disruption, synaptic damage and myelin vesiculation. Sham operated rats did not show either HO-2 upregulation or structural

changes in the spinal cord. These observations for the first time suggest an important role of CO in the pathophysiology of chronic nerve lesion. Further studies using selective HO inhibitors, neuroactive drugs and growth factors in this model may provide a new strategy to treat chronic neuropathic pain or to minimize neurodegeneration in the patients suffering from such diseases of the nervous system.

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A new antioxidant compound H-290/51 attenuates nitric oxide synthase and heme oxygenase expression following hyperthermic brain injury. An experimental study using immunohistochemistry in the rat

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Previous investigations from our laboratory suggest that heat stress and associated hyperthermia induces profound cell injury and edema in many parts of the brain [1]. The probable mechanisms of brain pathology following hyperthermia are not well known. Recently, nitric oxide (NO) and carbon monoxide (CO) has been implicated in the pathophysiology of ischemic or traumatic brain injuries [2]. However, the role of these gaseous molecules in hyperthermia induced brain pathology is still unclear. Our observations in heat stress suggest that upregulation of constitutive isoform of nitric oxide synthase (nNOS) and heme oxygenase (HO-2) occurs in several parts of the brain following heat stress [1, 2]. However, the functional significance of such finding is not well understood. It seems likely that upregulation of NOS and HO represent production of NO and CO respectively which contributes to cell injury in the CNS.

Available drugs influencing NOs and HO synthesis are not very specific [2]. Thus, the role of NO and CO in cell injury is very difficult to ascertain. There are evidences that heat stress is associated with oxidative stress which plays an important role in generation of NO and CO. Thus, it seems quite likely that pretreatment with antioxidants may shed some light on the mechanisms of NO and CO function in the pathophysiology of cell injury in the heat stress. The present investigation was undertaken to find out whether pretreatment with a new antioxidant compound H-290/51 influences expression of NOS and HO in the CNS following heat stress in relation to the cell injury.

Our observations show that rats subjected to 4 h heat stress at 38° C in a biological oxygen demand (BOD) incubator (relative humidity 50–55%, wind velocity 20–25 cm/sec) resulted in profound edema and cell injury in many parts of the cerebral cortex, hippocampus, cerebellum, thalamus, hypothalamus and brain stem. Immunostaining of nNOS and HO-2 showed marked upregulation in the damaged and distorted neurons located within the edematous area [2, 3]. Pretreatment with H-290/51 (50 mg/kg, p. o., 30 min before heat stress) [1, 3] significantly reduced the edematous swelling and cell injury and resulted in a marked attenuation of nNOS and HO-2 expression. These observations strongly suggest that upregulation of NOS and HO are somehow contributing to the cell injury and pretreatment with antioxidant compound H-290/51 is neuroprotective in heat stress.

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Regulation of quinolinic acid-induced damage in the hippocampus

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Quinolinic acid may cause neuronal damage in disorders such as the AIDS-dementia complex, in which brain quinolinic acid levels are elevated to neurotoxic levels. Approaches to reducing either the levels or the actions of quinolinic acid could be a valuable therapeutic strategy in such cases. Quinolinic acid is known to produce damage following localised administration into several regions of the CNS. Its activity at receptors for N-methyl-D-aspartate (NMDA) [Stone TW, Perkins MN (1981) Europ J Phamacol 72: 411–412] is thought to account for this damage. However, there is evidence to suggest that quinolinic acid can promote lipid peroxidation in brain tissue, and the possibility arises that this effect may contribute to the neuronal damage. In this study we examined the effect of melatonin, a potent free radical scavenger, and deprenyl on the excitotoxic effects of quinolinic acid. Quinolinic acid was injected directly into the hippocampus of anaesthetised rats at doses ranging from 50–250 nmols in 1 *1. Melatonin was co-injected in some animals at doses of 1 and 5 nmols, simultaneously with quinolinic acid. An intraperitoneal injection of melatonin 20 mg/kg was also made. After 7 days the animals were killed and the hippocampus removed for histology and Nissl staining. Cell counts showed that the higher dose of melatonin protected significantly against quinolinic acid damage. Comparable experiments with NMDA 2.5 nmols showed no such protection. Inclusion of the melatonin receptor antagonist luzindole did not prevent the protection by melatonin. Deprenyl was administered at 10 or 50 nmols and again the higher of these doses afforded significant protection. An examination of lipid peroxidation products using fragments of hippocampus in the presence of quinolinic acid, with or without the addition of iron, indicated a significant increase of 4-hydroxynonenal and malondialdehyde which could be reduced by the presence of melatonin but not deprenyl. The results suggest that quinolinic acid induced-neuronal damage is partly mediated by the generation of free radicals. Approaches to reducing the neuronal damage due to quinolinic acid in the AIDS-dementia complex could include the use of free radical scavengers or inhibitors.

Potent *in vivo* and *in vitro* physiological effects of kynurenines

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The kynurenine pathway of tryptophan metabolism produces kynurenic acid (KYNA), which is an antagonist of the glycine co-agonist site of the NMDA receptor at relatively low concentrations (K_D approximately 10 M), and both AMPA and NMDA receptors at higher concentrations. The low concentration of endogenous KYNA has cast doubt on its potential physiological significance. Nonetheless, there are several aspects of kynurenine metabolism and its modulation which indicate that endoge-

nous KYNA might be physiologically active. This hypothesis was addressed by parallel *in vivo* and *in vitro* approaches.

Hippocampal electrophysiological recordings were made using brain slices and conventional extracellular and intracellular recording techniques. Samples of the buffer which perfused the slices were made to quantify KYNA production, under various conditions. *In vivo* recordings were made in urethane-anesthetized rats with stereotaxic methods, using a jugular catheter to infuse drugs while recording in the hippocampus.

Initial experiments examined the efficacy of kynurenic acid (KYNA) to antagonize evoked responses in hippocampus. *In vitro* and *in vivo,* KYNA potently inhibited evoked responses. Population spike amplitude and paired pulse facilitation were reduced in area CA1 with as little as 5 M KYNA. Surprisingly, KYNA was more potent in area CA1 than the dentate gyrus. KYNA infusion by a jugular catheter had similar effects to KYNA exposure *in vivo,* suggesting that endogenously-synthesized KYNA, which is produced in highest concentration in the periphery, would not necessarily be limited in its potential central effects by the blood brain barrier.

The effects of the immediate precursor to KYNA, L-kynurenine (L-KYN) were also tested *in vivo*. Although evoked responses were not affected by L-KYN immediately, consistent with previous reports that L-KYN is not directly neuroactive, prolonged L-KYN incubation in slices had anticonvulsant effects. Thus, L-KYN incubation decreased spontaneous epileptiform activity that was induced by exposure to buffer containing nominal 0 mM magnesium. The anticonvulsant action could be due to the conversion of L-KYN to KYNA, given that slices can convert L-KYN to KYNA via glia, and that KYNA was measured in the superfusate after L-KYN exposure. Consistent with this possibility, increased KYNA concentrations occurred after exposure to higher concentrations of L-KYN, and this correlated with increased anticonvulsant action.

Surprisingly, even 1 M L-KYN had anticonvulsant activity. The amount of KYNA produced in these experiments was in the range of endogenous KYNA levels (20–50 nM), indicating that endogenous KYNA might have anticonvulsant potential in certain situations. Pyruvate (10 mM) enhanced KYNA production and anticonvulsant effects. Because the antagonism of spontaneous epileptiform activity occurred in the absence of antagonism of evoked responses, the results support the potential therapeutic value of kynurenines as anticonvulsants in the future.

Other experiments addressed potential differences between endogenously-synthesized KYNA and synthetic KYNA *in vitro*. A comparison was made between the anticonvulsant effects of 200 M L-KYN and the effects of 2 M KYNA. These concentrations were chosen because 2 M KYNA was measured in our slices after exposure to 200 M L-KYN, suggesting that L-KYN produces approximately 100-fold less concentrated KYNA, consistent with previous reports. The results demonstrated that 200 M L-KYN had greater anticonvulsant effects than 2 M synthetic KYNA, suggesting that endogenously-synthesized KYNA is more effective than synthetic KYNA in this model of epileptiform activity. If the mechanism underlying this difference could be exploited, it might lead to an improved design of anticonvulsants based on the kynurenine pathway.

In vivo assessment of kynurenate neuroprotective potency and quinolinate excitotoxicity

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Quinolinate (QUIN) and kynurenate (KYNA) are kynurenine metabolites that interact with NMDA-receptors. As QUIN is an agonist, its accumulation in the brain may be hazardous to neurones [1]. On the contrary, KYNA is an antagonist of all ionotropic glutamate-receptors with preferential affinity for the NMDA-receptor glycine site and, therefore, favouring KYNA synthesis may protect against excitotoxicity [2]. The purpose of our *in vivo* studies was to address the following questions: (1) Which extracellular levels of QUIN may be neurotoxic? (2) Which extracellular levels of KYNA may control excessive NMDA-receptor function? (3) Can these KYNA levels be reached by inhibition of kynurenine-3-hydroxylase (i.e. decrease of QUIN formation and shunt of kynurenine metabolism toward KYNA)?

Microdialysis probes incorporating an electrode [3] were implanted in the brain of halothane-anaesthetised rats and used for the following procedures: (i) perfusion of QUIN (1, 2 or 3 mM) or NMDA (150–200 μM) at 1 μl/min for 2 min; (ii) recording of the resulting local depolarisations as shifts of the extracellular direct current (DC)-potential; (iii) co-perfusion of KYNA (0.1 to 1 mM); and (iv) for question (3), measurement of changes in dialysate KYNA after administration of the kynurenine-3-hydroxylase inhibitor, 3,4-dimethoxy-*N*-[4-(3-nitrophenyl)thiazol-2-yl]benzene-sulfonamide, Ro-61-8048 [2*].

In this preparation, QUIN produced a concentration-dependent depolarisation with an estimated EC_{50} of 1.22 ± 0.03 mM ($n = 7$). If we assume that around 1/10 of this concentration was delivered to the surrounding tissue, then the extracellular level of QUIN for excessive excitation is still >15 times higher than those measured in the immune-activated brain (7.3 μM) [1]. With regard to KYNA, at least 100 μM had to be perfused through the probe to produce a detectable attenuation of responses to NMDA (150 μM) or QUIN (2 mM). Ro-61-8048 (100 mg/kg i.p.) increased the dialysate levels of KYNA from 3.0 ± 0.9 to 33.8 ± 8.0 nM ($n = 6$) 4 h post-injection, but did not reduce responses to 200 μM NMDA. These data challenge the notion that extracellular accumulation of endogenous QUIN may contribute to neuronal death in some neurological disorders [1], and the suitability of kynurenine-3-hydroxylase inhibition as an anti-excitotoxic strategy [2].

(Kindly supplied by A. M. Cesura, Hoffmann-La Roche Ltd, Basel, Switzerland.)

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Pharmacological manipulations of brain kynurenate levels affect striatal vulnerability to excitotoxic insults

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Kynurenine pathway metabolites such as 3-hydroxykynurenine, quinolinic acid and kynurenic acid (KYNA) have been speculatively linked to the pathophysiology of a spectrum of human brain diseases. In particular, it has been proposed that fluctuations in brain KYNA content preferentially affect the function of NMDA receptors and, hence, susceptibility to excitotoxic injury. The development of compounds which selectively inhibit kynurenergic enzymes, and the discovery of mechanisms that influence the dynamics of cerebral kynurenine pathway metabolism, have recently made it possible to experimentally address pertinent cause-effect relationships.

Systemic administration of the dopamine D1 or D2 receptor agonists SKF 38393 (5 mg/kg) and quinpirole (2 mg/kg) results in a brain-specific, acute decrease in the brain content of KYNA in immature (post-natal day 14) rats. This effect can be prevented by co-treatment with the appropriate dopamine receptor antagonists (1 mg/kg SCH 23390 and 2 mg/kg raclopride, respectively) or by kynurenine 3-hydroxylase inhibitors, i.e. compounds that shift kynurenine pathway metabolism towards an increased formation of KYNA.

Treatments with dopamine receptor agonists, alone or in combination with their respective receptor antagonist, or with kynurenine 3-hydroxylase inhibitors [m-nitrobenzoylalanine (100 mg/kg), FCE 156561 (50 mg/kg) or Ro 61-6048 (20 mg/kg)] were used as experimental vehicles to examine possible changes in the excitotoxic potency of NMDA *in vivo*. In all cases, reductions of KYNA levels were associated with a heightened vulnerability of striatal neurons to a focal injection of 2 μmol NMDA, i.e. a dose that normally causes only very moderate excitotoxic damage. This pro-excitotoxic effect of dopamine receptor agonists was not observed in animals in which the KYNA decrease was attenuated by the additional administration of either a dopamine receptor antagonist or kynurenine 3-hydroxylase inhibitors. Qualitatively identical biochemical and pro-excitotoxic effects were obtained using a d-amphetamine (5 mg/kg, i.p.) to stimulate dopaminergic activity.

Taken together, these studies demonstrate that a down-regulation of endogenous KYNA levels in the brain is functionally significant, rendering the brain more susceptible to NMDA receptor activation. Since abnormal cerebral kynurenine pathway metabolism, often including altered KYNA levels, has been observed in several human brain diseases and in relevant animal models, targeted intervention to affect the function of brain KYNA constitutes a promising strategy for the treatment of neurological and psychiatric diseases.

(This work is supported by USPHS grants NS 16102, NS 28236 and HD 16596.)

In vitro neuroprotective and *in vivo* anticonvulsant effects of 3-HAO inhibition

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The putative neuropathogen quinolinic acid (QUIN) is formed in the kynurerine pathway of tryptophan metabolism through the conversion of 3-hydroxyanthranilic acid (3-HANA) by the enzyme 3-HANA 3,4-dioxygenase (3-HAO). In order to investigate the possible role of this metabolic step, a novel 3-HAO inhibitor, 4,6-dibromo-3-hydroxyanthranilic acid (NCR-631), has been developed [Linderberg et al. (1999) Eur J Med Chem (in press)]. Studies on NCR-631 in liver homogenate showed irreversible, noncompetitive, inhibition of 3-HAO, as determined by spectrophotometric measurement of the immediate product α-amino-β-carboxymuconic acid ω-semialdehyde. However, analysis of the initial rate exhibited reversible competitive characteristics with a Ki of 82 nM, suggesting that NCR-631 acts as a tight-binding inhibitor. NCR-631 exerted no effects on monoamine, muscarinic or NMDA receptor binding, or on NMDA-sensitive depolarization of spinal cord neurons. NCR-631 was found to provide neuroprotective actions *in vitro* against anoxia-, LPS-, IL-1β- and β-amyloid peptide-mediated loss of hippocampal pyramidal neurons in organotypic cultures of rat hippocampus [Luthman et al. (1998) Amino Acids 14: 263–269, and this issue]. The *in vivo* characteristics of NCR-631 to inhibit 3-HAO were determined in Sprague-Dawley rats by studies on *de novo* production of QUIN after administration of the substrate 3-HANA, given either into the brain, through intracerebroventricular (i.c.v.) injections, or systemically. In Sprague-Dawley rats NCR-631 given i.c.v. (300 nmol) was found to reduce the time until, but not the severity of, pentylenetetrazole (PTZ)-induced seizures. Also when given systemically, at a dose of 250 mg/kg s.c., NCR-651 was shown to reduce the time until PTZ-induced seizures in NMRI mice, while again no significant effect was observed on the severity of the seizures. The effect of NCR-631 in the NMRI mice was found to be short lasting, with a loss of its action when given more than 15 min before PTZ. A dose-dependent anticonvulsant effect of NCR-631 was moreover found in the audiogenic-induced seizure model in young DBA mice. On the other hand no neuroprotective effects of NCR-631 could be detected in the gerbil model of global forebrain ischemia, or in trimethyltin-induced hippocampal lesions in rats. The findings demonstrate that the 3-HAO inhibitor NCR-631 has neuroprotective properties *in vitro* and anticonvulsant actions *in vivo*, while its neuroprotective potential *in vivo* remains to be established. NCR-631 constitute a useful tool to further determine the role of QUIN formation in neuropathological processes.

Neurobiology II

Modulation of the high-affinity excitatory amino acid transporter (EAAC1) by benzodiazepines

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The high affinity excitatory amino acid transporter, EAAC1, concentrates glutamate and aspartate across plasma membranes of brain neurons providing a presynaptic uptake mechanism to terminate the excitatory action of these released neurotransmitters and keeping their concentration below toxic levels. In the present work the effect of benzodiazepines on a putative presynaptic target, EAAC1, was investigated. EAAC1-mediated transport was measured in stably-transfected Chinese Hamster Ovary (CHO) cells and in microinjected *Xenopus laevis* oocytes by trancer flux measurements and voltage-clamp techniques. Substrate uptake was activated (up to 200% of control velocity) in EAAC1-transfected CHO cells at low micromolar concentrations (10 μM), on the contrary, concentrations at the millimolar range (1 mM) inhibited (up to 50%). From all 1,4-benzodiazepines tested, the strongest effects were exerted by derivatives with lactam and hydroxylactam structures. Such compounds behaved similarly in EAAC1-expressing *Xenopus laevis* oocytes. Another sodium co-transporter, the sodium D-glucose co-transporter SGLT-1, stably transfected in CHO cells was not affected by benzodiazepines at the same conditions as described above. Electrophysiologically, benzodiazepines induced no inward currents in cRNA microinjected *Xenopus laevis* oocytes, indicating that these compounds are not transported by EAAC1. Potential measurements gave evidence that benzodiazepines did not alter the membrane potential significantly.

Thus, in vivo low concentrations of benzodiazepines may reduce presynaptic excitatory amino acid concentration by increased transport activity, providing an additional mechanism to regulate neuronal excitability.

Differential protein expression in the human brain

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We investigated protein expression levels in the human brain, which are unrelated or related to neurological disorders. Human brain extracts from the frontal, parietal, temporal, occipital lobes of the cortex and the cerebellum, thalamus and striatum from 10 patients with Down's syndrome, 10 with Alzheimer's disease and 10 controls were analyzed by two-dimensional electrophoresis. A two-dimensional database, comprising approximately 200 human brain proteins was constructed. The identified proteins were mainly structural and heat shock proteins and enzymes with various catalytic activities and were localized in the cytoplasma and in mitochondria. Quantification of the proteins in the control samples revealed the presence of several, probably allelic, disease-unrelated differences. These differences were mainly of quantitative nature and concerned structural proteins and enzyme isoforms. In the Down's syndrome brain, we identified variable expression levels for certain enzymes involved in glucose metabolism. The expression levels of synaptosomal proteins were reduced, whereas the levels of glial fibrillary acidic protein and 14-3-3 proteins were increased in the brain of patients with Down's syndrome or Alzheimer's disease.

Differential regulationship between the expression of arginase I and II, and of iNOS in rat alveolar macrophages

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L-arginine is the substrate of nitric oxide synthase (NOS) and arginase. In macrophages (MΦ), including alveolar MΦ (AMΦ) both enzymes can be coexpressed and compete for the common substrate, eventually resulting in a limitation of L-arginine utilization by either pathway [1, 2]. As little is known about the regulation of the expression of the two arginase isoforms in MΦ, the effects of lipopolysaccharides (LPS) and interferon-γ (IFN-γ), two potent inductors of iNOS in AMΦ, were studied.

Rat AMΦ, freshly prepared or cultured for various times in the absence or presence of 1 μg/ml LPS or 500 U/ml IFN-γ were used to isolate total RNA and to extract cellular proteins. The mRNA for arginase I and II, iNOS and β-actin were determined by semi-quantitative RT-PCR and protein levels of arginase I and II, iNOS and α-tubulin were measured by immunoblotting. In freshly prepared AMΦ mRNA for both isoforms of arginase as well as the corresponding proteins were detected. After a 20 h culture period mRNA for arginase II was markedly reduced. Presence of LPS during the culture period prevented the decline in arginase II mRNA and enhanced arginase I mRNA above initial levels. This was accompanied by an increase in protein levels of arginase I and II after 20 h culture in the presence of LPS. IFN-γ, which like LPS caused a marked induction of iNOS mRNA and protein, had no effect on the expression of arginase I, but enhanced both the mRNA and protein levels of arginase II. Time course experiments showed that an inductive effect of LPS on arginase I mRNA was present after 5 h, whereas an inductive effect of LPS on iNOS mRNA was already seen after 2 h. Dexamethasone (10 μM), known to induce the expression of arginase I in the liver [3], had no such stimulatory effect in AMΦ, on the contrary, it opposed the LPS induced upregulation of both arginase isoforms as well as the LPS-mediated induction of iNOS.

In conclusion, rat AMΦ coexpress arginase I and II, but their expression is differentially regulated. The levels of both arginase isoforms are enhanced by LPS, but via different mechanisms. IFN-γ, like LPS an inductor of iNOS, only enhanced arginase II, excluding a simple link between the expression of iNOS and the arginases.

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Parallel regulation of L-arginine transport and NO synthesis, and the expression of cationic amino acid transporters and inducible NO synthase in rat alveolar macrophages (AMΦ)

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The cellular availability of L-arginine (L-Arg) can limit NO synthesis, particularly in AMΦ in which iNOS is responsible for NO synthesis [1]. As NFκB is an essential transcription factor for the induction of iNOS [2], a possible role of NFκB in the regulation of L-Arg transport in rat AMΦ was studied.

Rat AMΦ ($0.5*10^6$ cells/well) were cultured for 5 or 20 h, in the absence or presence of LPS (1 μg/ml) and/or other test substances. L-Arg uptake was studied by measuring cellular radioactivity after 2 min of incubation with ^{3}H-L-Arg (37 kBq, 0.1 μM). Nitrite accumulation and mRNA levels for iNOS and different cationic amino acid transporters (CATs) was determined.

^{3}H-L-Arg uptake in control AMΦ amounted to 4.7 ± 0.3 pmol/mg protein. After exposure to LPS, ^{3}H-L-Arg uptake was enhanced by 173 ± 15%. Two different NFκB inhibitors (Pyrrolidine dithiocarbamate, PDTC, 60 μM and N^{α}-p-tosyl-L-lysine chloromethyl ketone, TLCK, 100 μM) caused a reduction of ^{3}H-L-Arg uptake by 30 ± 8% and 63 ± 5%, resp., and each of them inhibited the stimulatory effect of LPS. Likewise dexamethasone (10 μM) caused a reduction of L-Arg uptake by 41 ± 6 and largely attenuated the respective stimulatory effect of LPS. Culture in the presence of IFN-γ caused an increase in ^{3}H-L-Arg uptake by 157 ± 10%, an effect inhibited also by either TLCK or PDTC. Nitrite accumulation showed always changes in parallel to the observations on ^{3}H-L-Arg uptake. LPS caused a marked increase in the mRNA for iNOS and CAT-2B, but had no clear effects on mRNA for CAT-1. Time course experiments showed that the stimulatory effect of LPS on mRNA for both iNOS and CAT-2B was present after 2 h, and maximally after 5 h, and PDTC and TLCK prevented these LPS effects on mRNA. On the other hand dexamethasone did not affect the initial (5 h) LPS induced increase in mRNA for iNOS and CAT-2B, but downregulated mRNA for iNOS and CAT-2B during a 20 h incubation period.

In conclusion, NFκB, known to be an essential transcription factor for the induction of iNOS, appears also to play an important role in the upregulation of L-Arg transport in rat AMΦ.

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Developmental regulation of glutamate receptor expression differs in neurons vs. glial cells of the hippocampus

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To compare ontogenetic changes in glutamate receptor properties of pyramidal neurons and astrocytes of the CA1 area of the

hippocampus, cells were acutely isolated and analyzed with the patch clamp technique. Rats and mice were pooled in three age groups, ranging between postnatal day 5 and 45.

The complete block of receptor currents by GYKI 53655 and the absence of modulation by Concanavalin A proved that the cells exclusively expressed non-NMDA receptors of the AMPA subtype. Accordingly, dose-response curves of kainate-induced currents demonstrated a low affinity for kainate at the glutamate receptors.

Analysis of reversal potentials of currents evoked in high $CaCl_2$ solution revealed a low Ca^{2+} permeability of receptor channels in the pyramidal neurons and an intermediate Ca^{2+} permeability in astocytes which remained constant during development.

To investigate flip/flop splice variant expression, receptor modulation by cyclothiazide (CTZ) was compared during development. In pyramidal neurons, potentiation of kainate-induced currents by CTZ decreased with continuing maturation. In contrast in the adjacent astrocytes, an increase in CTZ potentiation was observed. These functional data suggested an opposite regulation of AMPA receptor splice variants in pyramidal neurons and astrocytes early in postnatal life.

6-Hydroxykynurenic acid antagonizes AMPA and NMDA receptors in hippocampal neurons: A Patch clamp study

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A large number of different derivatives of the nonselective excitatory amino acid antagonist kynurenic acid (KYNA) has been pharmacologically analysed. In this study, we used excised patches and acutely isolated neurons from the pyramidal layer of the CA1 region of the hippocampus to characterize the effects of 6-hydroxykynurenic acid (6-HKA) on NMDA and AMPA receptors and compared it to the actions of KYNA.

6-HKA proved to be a full antagonist at both glutamate receptor subtypes. However, when compared to KYNA, 6-HKA turned out to be less potent at the NMDA (IC_{50} = 122 and 52 µM, for 6-HKA and KYNA, respectively) but much more potent at the AMPA subtype. Thus, AMPA receptor currents evoked by 1 mM glutamate were reduced by 50% (0.2 mM 6-HKA) and by 35% (0.2 mM KYNA).

Recently, KYNA has been reported to be a rapidly dissociating antagonist at AMPA receptors. By means of a rapid perfusion technique we compared the unbinding kinetics of these substances. Both antagonists (0.2 mM, continuously present) were displaced by 1 mM glutamate. Although KYNA possessed a faster displacement (π_{KYNA} < 1.3 ms), the time constant for 6-HKA still was on a millisecond time scale ($\pi_{6\text{-}HKA}$ < 1.7 ms).

Taken together, 6-HKA turned out to be a broad-spectrum antagonist, similar to KYNA. Nevertheless, the hydroxylation at the C6 atom results in a considerable change of the pharmacological profile.

Effect of MK-801 (NMDA receptor antagonist) applied during pregnancy concomitantly with cadmium on the central dopamine (DA) receptors reactivity in adult offspring rats

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Cadmium (Cd), a heavy metal, is an element present in environment that can cause serious damages of the central nervous system (CNS), especially in young and developing animals. It is known that prenatal exposure to Cd alters the neurotransmitters levels in offspring increased dopamine (DA) and their metabolites DOPAC levels in the metencephalon but decreased in mesencephalon, can change also serotonine (5-HT) and 5-HIAA contents in some areas of the brain [1]. Previously we have shown that ethanol (EtOH) applied together with Cd to pregnant rats prevent some toxic effect of the metal and its deposition in the brain of the offspring [2]. Molecular mechanisms of this phenomena are still unknown. Some hopes have set with diazepam and MK-801, because of their similarities with EtOH in molecular action on the CNS. It appeared from our previous study, that diazepam does not prevent the toxic effects on the CNS in offsprings caused by Cd [3].

In the present study female Wistar rats were exposed during pregnancy to Cd in concentration of 50 ppm (water solution, ad libitum), or with daily injected MK-801 0.5 mg/kg SC, concomitantly or separately. Control rats received tap water only. Behaviour of 2 months old male offsprings was investigated by several psychopharmacological methods. Oral activity, yawning, catalepsy, stereotypy, locomotor activity and other were recorded following respective central DA receptors agonists and antagonists administration. Beside the level of the Cd in the brain and some peripheral organs was estimated.

The results indicate that Cd and MK-801 applied during pregnancy separately or jointly produce moderate functional changes in the brain of offsprings. MK-801 does not prevent the Cd central effects and its deposit in examined tissues of offspring rats.

(Supported by grant from Silesian Medical University.)

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Effect of the amino acid L-arginine and its analog nitro-L-arginine methyl ester (L-NAME) on amphetamine and apomorphine induced stereotyped behavior and DOPAC release in the striatum of rats

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Nitric oxide (NO), a novel intracellular messenger of mammalian brain, affects a variety of physiological and pathological functions. Previously, we showed that NO modulates central dopamine (DA) D_1 and D_2 receptor reactivity to agonists (SKF 38393 and quinpirole, respectively) in rats. In the current study we examined the possible role of NO in amphetamine- (AMPH-) and apomorphine- (APO-) induced stereotyped behavior and release of dihydroxyphenylacetic acid (DOPAC) from neostriatum of adult Wistar rats. Using the rating scale of Costall and Naylor, L-NAME (25 mg/kg IP) but not L-arginine (L-ARG; 300 mg/kg IP) was found to attenuate AMPH (4 or 10 mg/kg IP) induced stereotyped behavior of male and female rats. Neither L-NAME nor L-ARG modified the APO stereotypy score or when given alone. Using differential pulse voltammetry in the neostriatum, L-NAME but not L-ARG prevented the reduction in DOPAC in chloral hydrate anesthetized male rats acutely treated with AMPH (4 mg/kg IP). Findings indicate that NO is essential in mediating effects of AMPH on DA release and in mediating AMPH-induced behaviors.

[Supported by the Silesian Academy of Medicine, grant NN-4-30/98 and Forgarty International Science Exchange Program with Poland (RMK).]

Increased extracellular glutamate in the ventral tegmental area in response to electrical stimulation of the prefrontal cortex: Functional and neuroanatomical correlates

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VTA neurons receive a major excitatory projection from the PFC and there is evidence that excitatory amino acids modulate DA cell activity in the VTA. In this study we confirmed by microdialysis in vivo that electrical stimulation of the PFC causes glutamate release in the VTA. Secondly, we determined whether PFC stimulation is associated with functional activation of VTA neurons, as assessed by Fos immunohistochemistry. Thirdly, by using a retrograde tracer, we determined whether VTA Fos-positive neurons project to nucleus accumbens. Monopolar electrodes were implanted monolaterally in the prefrontal cortex (anterior cingulate or prelimbic subregions) and dialysis probe ipsilaterally in the VTA. Rats were stimulated with trains of 60 Hz pulses of 0.1 msec width and 30 μA intensity, delivered for 1 sec every 10 sec for the duration of 10 min. Glutamate was measured in 10 min-perfusate samples by HPLC coupled to fluorescence detection following pre-column derivatization with OPA/β-mercaptoethanol. Basal perfusate glutamate concentration was 0.02"0.2 μM at 0.6 μl/min. PFC stimulation increased extracellular glutamate to 280% of baseline values. This response was dependent on the frequency of stimulation (8–120 Hz) and was prevented by the perfusion through the probe of the sodium channel blocker tetrodotoxin (10 μM), suggesting that the increase in extracellular glutamate is of neuronal origin. Stimulation of the PFC increased the expression of Fos-like immunoreactivity in VTA neurons, suggesting that glutamate release mediates transynaptic activation of VTA neurons. Fos-positive cells were observed bilaterally with a significantly higher number ipsilateral to the stimulated side (60%). Combined application of fluorescent retrograde tracers to PFC and VTA indicate that the activation of controlateral VTA neurons by PFC stimulation can occur by bilateral activation of PFC neurons through transcallosal connections between homotopic PFCs. Approximately 10% of Fos-positive cells were double-labeled by the fluorescent retrograde tracer FluoroGold, applied to the nucleus accumbens one week before PFC stimulation, evidence that activated VTA neurons project to nucleus accumbens. This study provides direct evidence for a functional glutamatergic control of VTA neurons by the PFC and supports the hypothesis that PFC excitatory efferents to midbrain DA neurons constitute a primary neurobiological substrate for the cortical regulation of subcortical DA function.

Glutamate as the metabolic coupling signal between neurons and astrocytes

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Camillo Golgi and its followers have proposed that astrocytes could regulate the passage of metabolic substrates from blood to neurons. We recently provided evidence to support such an important function. Thus, it was shown that glutamate, not acting at specific receptors but rather via its uptake, stimulates glucose utilization and lactate release by astrocytes. We have demonstrated that the mechanism involves a direct stimulation of glial Na^+/K^+ ATPase activity by glutamate uptake as a consequence of increased Na^+ entry. Pharmacological analysis with the inhibitor ouabain has revealed that the increase in Na^+/K^+ ATPase activity is due to the mobilization of a distinct isoform highly sensitive to ouabain, most likely the α_2 isoform. Since lactate does not cross membranes easily, it requires the presence of a transport system to become an adequate energy substrate. Using in situ hybridization and immunocytochemistry, we have observed an abundant expression of two monocarboxylate transporters, MCT1 and MCT2, in the cortex, the hippocampus and the cerebellum of the mouse brain. Lactate is converted to pyruvate by lactate dehydrogenase which exists as five tetrameric forms. The muscle form (LDH_5) is abundant in glycolytic tissues while the heart from (LDH_1) is enriched in oxidative tissues. Using specific antibodies, we have shown that neurons in human hippocampus were only labeled by anti-LDH_1 antibodies while other antibodies against the LDH_5 form only revealed a population of astrocytes. Finally, we have determined the capacity of lactate to support TCA cycle activity in cultured cortical neurons as compared to glucose. We have found that lactate can fully substitute for glucose as a metabolic substrate for oxidative metabolism and that monocarboxylate transporters are essential to ensure efficient utilization. In conclusion, these data provide evidence to support the existence of a transfer of lactate from astrocytes to neurons which would be regulated by the level of synaptic activity, via the release of glutamate, its reuptake into astrocytes, and activation of aerobic glycolysis in this cell type.

The use of microdialysis for the evaluation of amino acid neurotransmission in conscious animals

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Microdialysis has become a frequently used method to study extracellular levels of GABA and glutamate in the central nervous system. Various authors have reported that basal levels of GABA and glutamate in microdialysates are virtually tetrodotoxin- and calcium-independent. This implicates that amino acids in dialysates do not fulfil the classical criteria for exocytotic release, which questions the neuronal origin of amino acids in dialysates. Given the fact that evidence for non-exocytotic release mediated by reversal of the uptake sites as a release mechanism relevant for normal neurotransmission is so far limited to conditions of "excessive stimulation", basal levels of amino acids in dialysates most likely reflect a non-neuronal pool of amino acids. Extracellular GABA and glutamate can be stimulated by a wide variety of pharmacological and physiological manipulations. However, it is presently impossible to ascertain that the stimulated GABA and glutamate in dialysates are of neuronal origin. On the other hand, under certain stimulatory conditions, increases in amino acid transmitters can be obtained in the presence of tetrodotoxin, again suggesting that aspecific factors not directly related to neurotransmission underly these changes in extracellular levels. It is concluded that synaptic transmission of GABA and glutamate is strictly compartimentalized and as a result, these amino acids can hardly leak out of the synaptic cleft and reach the extracellular space where the dialysis probe samples.

Two populations of $GABA_A$ receptors in rat cerebellar granule cells in culture

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Cerebellar granule cells from neonatal rats have been studied in cell cultures, at 5–12 DIV, by the whole cell patch clamp technique to study GABA activated chloride channels.

Several characteristics of the GABA activated chloride current point to the presence of two different populations of $GABA_A$ receptors in different cellular compartments.

In the first place, upon continued 10 μM GABA perfusion the chloride current activated by the neurotransmitter decays according to two kinetics components, a fast and a slow one. Treatment of the cells with activators of either PKA or PKG decreases selectively the fast component. The same result is found upon

pretreatment of the granule cells with NMDA: the fast component is selectively decreased.

In addition, the fast component is the only one which is positively modulated by a benzodiazepine agonist, flunitrazepam. At relatively basic external pH the fast component is comparatively enhanced so to make the overall positive modulation by flunitrazepam of the chloride current statistically significant.

The two components are equally affected by the so called "run-down"-phenomenon, occurring when Mg^{++}/ATP are omitted in the recording pipette and due to tyrosine dephosphorylation. On the basis of the overall data, we suggest that in rat cerebellum granule cells there are two $GABA_A$ receptors populations. One, synaptic and present in dendrites, desensitises rapidly and it is benzodiazepine sensitive. The other one is extrasynaptic and present in the cell body; it contains the δ subunit and desensitizes slowly. This benzodiazepine insensitive receptor population is most probably at the basis of the tonic GABA mediated inhibition of cerebellar granule cells.

Effect of gabapentin (GBP) on memory process

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The antiepileptic drug GBP was designed as a GABA analogue which crosses the blood-brain barrier. preliminary clinical results demonstrated better performance on tests of intelligence, memory and attention with GBP alone than with GBP plus carbamazepine.

We investigated the action of a single i.p. injection of a range of GBP doses on the retention of memory, its possible interaction with endogenous levels of amino acids in cerebral cortex (CC) and hippocampus (HIC) and the ability of *in vitro* GBP to modulate [^{3}H]-glutamate binding.

We used male Wistar rats (150–200 g) and compared with other species such as Swiss male mice (25–30 g) (only in behavioral assay). As behavioral test we used an inhibitory avoidance task. Quantification of the amino acids was performed by HPLC. The [^{3}H]-glutamate binding assay to NMDA receptors was performed on synaptic membranes isolated from CC of the rat.

Posttraining i.p. administration of 10 mg/kg GBP facilitated 48 h retention in mice ($p < 0.05$). In contrast in rats, we found an impaired retention after 100 mg/kg GBP ($p < 0.05$).

The administration of 100 mg/kg i.p. GBP increased the endogenous levels of glutamate ($p < 0.05$) while decremented the contents of glycine ($p < 0.05$) on HIC.

The [^{3}H]-glutamate binding in the presence of 10^{-5} M GBP increased B_{max} and K_D in the CC without reaching statistical significance.

In conclusion GBP produces facilitation of memory in mice, in an inhibitory avoidance task, while in rats we observe the opposite effects. In rats these changes may correlate with the increase on the endogenous content of glutamate and decrease on glycine levels. We also note a tendency to an increment the density and affinity of [^{3}H]-glutamate for NMDA receptors from CC in the presence of GBP; this might be indicating a possible modulation of NMDA receptors by GBP in the memory process.

Are GABA release and GABA synthesis modulated by $GABA_B$ autoreceptors?

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Gamma aminobutyric acid (GABA) is synthesized within GABA terminals through a highly compartmentalized process in which glial-derived glutamine (gln) is a major precursor, and its release is modulated by $GABA_B$ autoreceptors. The aim of this work was to ascertain whether or not GABA synthesis and release are coupled in the rat brain through a $GABA_B$ autoreceptor mediated comodulation. It was found that (-)baclofen (30 μM) reduces the K^+-stimulated release of [^{3}H]GABA in synaptosomes but not (10 μM) in cerebral cortical prisms while at the same concentrations (-)baclofen failed to modify the incorporation of radioactivity from [^{3}H]glutamine into [^{3}H]GABA in slices and prisms from hypothalamus and cerebral cortex, and cortical synaptosomes. In this latter preparation, identical results were observed when (-)baclofen was added to Krebs-Tris media, containing either 5 or 15 mM K^+ concentration. In agreement with these later results glutamic acid decarboxylase (GAD) activity measured within hypothalamic prisms was not affected by 1–100 μM (-)baclofen. Similar results on GABA synthesis were also observed when 1–100 μM 3-aminopropil(methyl)phosphonic acid or GABA was used instead of (-)baclofen to stimulate $GABA_B$ autoreceptors in cortical prisms. [^{3}H]GABA release, [^{3}H]GABA synthesis from [^{3}H]glutamine and GAD activity were also insensitive to the action of the $GABA_B$ antagonist CGP52432 (10–100 μM). Likewise, Muscimol (0.3–100 μM), used as a control, did not have any effect of GABA synthesis. Our results indicate that unlike GABA release GABA synthesis is not modulated by $GABA_B$ autoreceptors.

(Supported in part from grants: 26370N Conacyt, México; and IN230198 DGAPA, UNAM, México.)

Effect of polyamines on NMDA receptors from the retina

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Endogenous polyamines (PA), as spermine and spermidine, are synthesized from omithine via omithine decarboxylase (ODC). Inhibition of PA synthesis abolishes calcium entry through various receptor-linked or voltage-gated calcium channels; this property would place PA in a powerful position regarding the control of neurotransmission in the CNS. Recently, spermidine and spermine have been postulated as neuromodulators or neurotransmitters. On this line, the presence of specific binding sites for [^{3}H]spermidine and [^{3}H]spermine in rat brain has been documented, although the nature and affinity of such sites is still controversial.

PA interact with the *N*-methyl-D-aspartate (NMDA) type of glutamate receptor evoking distinct effects, which indicates the presence of more than one polyamine binding site on this receptor channel. Two of these effects are related to the coagonist glycine. In the presence of saturating concentrations of glycine, spermine and spermidine stimulate whole-cell current and also [^{3}H]MK-801 binding, possibly due to an increase in the frequency of NMDA receptor-channel opening. On the other hand, at subsaturating concentrations of glycine, spermine and spermidine increase the affinity of NMDA receptors for this amino acid.

Results from our group demonstrate that the effects of spermine on NMDA receptors from plexiform layers of chick retina, differ from those in other regions of the central nervous system (CNS). Spermine inhibits specific [^{3}H]glycine binding to retinal membranes through a reduction in B_{max} without affecting K_d. In addition, specific [^{3}H]spermine binding to both plexiform layers of the retina has been characterized, and found to concentrate in the inner plexiform layer compared to the outer plexiform layer, as also seen for [^{3}H]glycine binding. These results suggest that the heteromeric assembly of NMDA receptors in the retina might differ from that in the CNS, probably due to the expression of NR2C subunit which is restricted to the retina and the cerebellum.

Little is known about the mechnisms controlling PA activity in

the brain. In rat striatum, spermine and spermidine are selectively released by NMDA-receptor activation and sodium-pump inhibition by ouabain. Calcium-dependent release of [^{3}H]spermidine upon depolarizing stimuli has also been observed in brain slices.

Our results demonstrate that in the retina [^{3}H]spermine is released by calcium-independent K^+-depolarization, but not by the activation of ionotropic glutamate receptors. These data suggest that in the retina spermine is released by the reverse activity of the transporter, and further supports a distinct role of PA in this tissue.

(Partially supported by Grant IN-210998 from DGAPA, UNAM to AMLC.)

Methyl bromide: Is the glutathione transferase pathway the key to its neurotoxicity?

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Methyl bromide (MB) is used worldwide as a disinfectant mostly in soils and transports of perishables. This gas has pronounced mutagenic and neurotoxic effects. Its methylating action accounts directly for its genotoxicity while a more complicated mechanism seems to underline its neurotoxicity.

As we were able to show in neurons of the rat hippocampus in vitro, MB is not acutely neurotoxic up to a concentration of 1.4 mM within one hour. Neither membrane input resistance, membrane resting potential, discharge activity or synaptic transmission was changed significantly. We only observed a slight diminution of excitatory postsynaptic potentials reducing excitability by about 13% (at 1.4 mM). This reduction could be fully explained by the action of bromide that inevitably is set free when MB methylates proteins and other cellular compounds.

The delayed neurotoxic action of MB, known also from clinical cases of intoxication, has been related to a metabolic pathway whose first step is a conjugation with glutathione by means of a glutathionetransferase. Further metabolism leads to toxic compounds like methanethiol and formaldehyde. The human glutathionetransferase displays a polymorphism. If products of the glutathionetransferase pathway really account for neurotoxicity, the latter should be more pronounced in conjugators than in non-conjugators. In an ongoing field study performed in workers in Chile we determine exposure levels and conjugator status. Neurobehavioral tests will reveal the level of damage to be correlated with the enzyme status of the persons involved in the study.

Aspartate as a neurotransmitter in the vertebrate rod photoreceptors; 3H-aspartate uptake and release mechanisms in the isolated frog retina

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Currently there is little doubt that aspartate and glutamate may be neurotransmitters for photoreceptor cells in vertebrate retinae. However, many details of aspartat/glutamatergic transmission still remain to be clarified. But because of its highly ordered and well defined layered structure, and the possibility to use light as an instrument for functional studies, the retina offers considerable advantages for investigation of fine details of chemical transmission. In this respect it is of interest that in darkness photoreceptors are partially depolarized [1], which may cause a continuous release of depolarizing neurotransmitter from photoreceptors [2]. Light stimulation causes hyperpolarization of photoreceptors and hence under light a depression of transmitter efflux from the photoreceptor cell would be expected. In this study attempts are made: 1) To estimate kinetic characteristics of accumulation of 14D-L-aspartate and 3H-kainate by the retina. 2) To reveal a picture of fine distribution of 14C-L-aspartate. 3H-D-aspartate and 3H-kainate in the retina after their accumulation by high affinity uptake mechanism at different time exposures (5, 15, 30 and 60 min.) in the frozen retina. 3) To clarify the effect of changes in illumination levels on the efflux of 14C-L-asparate from the perfused retina. Throughout this study isolated retinae of the frog *Rana ridibunda* were transport systems for aspartate with Km – 10.8 mkM, VM – 3.7 nM/g/min and KM – 704.7 mkM, Vm – 177.8 nM/g/min correspondingly and only a high affinity transport system for kainate with Km – 10.8 mkM, Vm – 1.8 nM/g/min. This study establishes that because of a high affinity transport system both L and D forms of aspartate accumulate mainly in the photoreceptor layer, with the highest peak on inner segments in the ellipsoid region and a slightly lower second peak in the region of synaptic endings. It is of interest that kainate mainly accumulates in photoreceptor cells only in the ellipsoid region of inner segments, which indicates that kainate is binding with the aspartate/glutamate transporter rather than with their receptor. An efflux experiment showed a decrease of C14-aspartate release from the photoreceptor cells at illumination and its sharp increase under de-illumination of the retina previously under weak 0.05 lx or strong 40 lx illumination. On the basis of data obtained, the conclusion can be made that there exists a circular flow of aspartate in the rod photoreceptor cell, the regularity of which depends on changes in levels of illumination. The release and reuptake mechanisms are more than likely equally important for aspartate base chemical transmission. Hence the aspartate transporter, which is localized in the ellipsoid region, probably plays a key role along with the aspartate receptor in chemical transmission between the photoreceptor cells and second order neurons of the retina.

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Inheritance of migraine and its progressive worsening: What a role for NMDA activation?

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We showed that subjects healthy except for migraine (M) differ from controls having a negative family and personal history for primary pain since M are characterized by a generalized hyperalgesia distinguishing them from controls who never complain it. Recently, we performed a statistical study suitable for determining the inheritance of hyperalgesia, in 2 matched groups of adolescents-preadolescents. Group 1 (447 healthy control, 221 malges, age 8.1 + 1.2) was compared to group 2 (497 healthy subjects having ascendants suffering from M, 235 males, age 8.4 + 1.3). Notwithstanding the absence of any primary pain or secondary pain so severe and iterative to promote hyperalgesia, group 2 showed a significantly higher number of subjects complaining hyperalgesia and evident index of genetic aggregation for hyperalgesia. That hyperalgesia occurs in subjects without any primary or secondary iterative, severe pain lead us to label this pain independent hyperalgesia "third inheritable hyperalgesia" to distinguish it from historical first and secondary hyperalgesia, both dependent on pain. What mentioned above evidenced that a large part of the M sufferers' descendants can inherit an abnormal set-up of their brain stem. Since it is known how hyperalgesia/allodynia depends on hyperactivity of excitatory amino acids neurons, especially of NMDA receptor sites, data suggest that descendants of M sufferers can be born with the feature of an abnormally functioning of NMDA ionotropic receptor. In case these subjects meet the condition of M, a vicious circle arose where primary and secondary hyperalgesia states act on the ground of "third hyperalgesia" so evoking a redundancy of hyper-

algesia/allodynia leading subjects to suffer from severer headache till to chronic headache. The role of hyperalgesia seems evidenced in a large clinical pharmacology study comparing placebo, lidocaine and the antagonist at the NMDA ionotropic receptor ketamine in chronic M ($p > 0.0001$ versus baseline, placebo and lidocaine). Finally subjects characterized by "third hyperalgesia" showed a highest level ($p > 0.0001$) of NO, measured as L-citrulline (nmol/ml 31.2 + 9.4) than controls (nmol/ml 11.9 + 2.7). The difference between controls and matched M sufferers (nmol/ml 29.4 + 3.5) was even more evident ($p > 0.0001$). These values regarded blood sampled during hyperalgesia-inducing maneuvers. Even if NO/NOS elicited by several stimuli it remains the simultaneity between the cropping out of hyperalgesia symptoms and the increase of NO plasma level.

Upregulation of a calcium binding protein, calbinidin-D28k, in cerebellar Purkinje cells is dependent on excitatory amino acids, but not on protein synthesis

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A protective role from excitatory amino acids (EAA) toxicity has been postulated for the calcium buffer protein Calbindin-D28k (CaBP). Using a CaBP specific antibody, changes in immunoreactivity (IR) in cerebellar Purkinje cells (PC) exposed to EAA were studied. IR was very rapidly and dose dependently increased in PC superfused with excitatory and excitotoxic doses of glutamate or its ionotropic analogs, kainic acid and AMPA. The CaBP up-regulation was independent of calcium influx but was blocked by CNQX, the AMPA receptor antagonist. With trans-ACPD, the metabotropic EAA agonist, a much smaller increase of the IR was obtained and LAP3, the specific metabotropic antagonist, instead of blocking, significantly increased the IR. Moreover, LAP3 by itself also significantly increased the IR therefore behaving as an agonist. The protein synthesis inhibitors, cycloheximide and emetine as well as the transcription inhibitors, actinomycine-D and α-ammanitine did not prevent the IR increase, not even after prolonged pre-incubations of 12 hours. These results indicate that i) CaBP-containing neurons increase their calcium buffer capacity through the activation of specific EAA receptors; ii) the IR increases with EAA are independent on calcium entry; iii) the increase of the IR is not from a *de novo* synthesis of CaBP and may therefore be the result of a conformational change of the protein.

In vivo and *in vitro* γ-aminobutiric acid inhibition of Purkinje cells of the cerebellar cortex

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Type A and type B γ-aminobutiric acid (GABA) receptors are found in the Purkinje cell (PC) of the cerebellar cortex. They are mediated by PC recurrent collaterals and by stellate cells in the dendrites and basket cells in the soma. GABA-induced inhibitory activity of the PCs has been studied *in vivo* and *in vitro* (sagittal cerebellar cortex slices) using specific agonists and antagonists. The $GABA_A$ receptor agonists, Muscimol and THIP, dose dependently inhibited PCs activity *in vivo* and *in vitro* by opening Cl^- channels, their effects were reduced by the specific antagonist, bicuculline. Baclofen, the $GABA_B$ receptor agonist, dose dependently inhibited PC activity *in vivo* and *in vitro,* by opening K^+ channels, the effect was reduced by the specific antagonists, 2-hydroxysaclofen and CGP55845A. With CGP27492, a drug considered to be a $GABA_B$ receptor agonist more potent than baclofen, applied to PCs in slices, the dose response inhibition was complex but similar to the response induced by GABA, the endogenous neurotransmitter, it opened Cl^- channels and was antagonized by bicuculline and partially by CGP55845A. These results concern postsynaptic receptors since they persisted when presynpatic activity was blocked by TTX. They demonstrate that PC inhibition is mediated by both types of receptors. The possibility of the existence in the cerebellar cortex of a particular subtype of $GABA_B$ receptor will be discussed.

Bilobalide inhibits GABA-induced depolarization in mouse cortical wedges

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Bilobalide, the major non-flavonic neuroprotective constituent of *Ginkgo biloba* leaves, is an unique non-nitrogenous molecule containing three condensed γ-butyrolactone rings on a cyclopentane skeleton. This study investigated the effects of bilobalide on NO-711 (a GABA-uptake inhibitor), magnesium-free medium and NMDA-induced depolarizations in mouse cortical wedges using grease-gap recording with Ag/AgCl electrodes.

Perfusion with NO-711 (25 μM) for 15 minutes onto the cortex induced regular depolarizations for up to 6 hours. Bilobalide, concentration-dependently, significantly reduced the frequency of these recurrent depolarizations with an IC_{50} of 31.4 μM. At lower concentrations (10–50 μM) the effects of bilobalide were reversible whereas 100 μM abolished the depolarizations for more than 1 hour following removal. Bicuculline (10 μM), tetrodotoxin (0.5 μM) and removal of calcium or bicarbonate from the perfusing fluid also blocked NO-711-induced depolarizations. In concentrations up to 2 mM no inhibitory effect of γ-butyrolactone or of γ-hydroxybutyrate was observed on these NO-711-induced depolarizations.

Spontaneous, fast depolarizations, induced by perfusing the slices with magnesium-free medium, were not blocked by bilobalide at concentrations up to 200 μM and neither were NMDA-induced depolarizations at concentrations of bilobalide up to 400 μM.

These observations, together with our current understanding of the neuropharmacological profile of bilobalide (and that on the mechanisms involved in the GABA uptake-induced depolarizations) suggest that a reduction in glutamate release could be involved in the effect of bilobalide. These effects were not mimicked by γ-butyrolactone or γ-hydroxybutyrate.

Excitatory amino acid stimulation of the survival of rat cerebellar granule cells in culture is associated with an increase in SMN, the spinal muscular atrophy disease gene product

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The survival of cerebellar granule cells in culture is stimulated by activation of excitatory amino acid (EAA) receptors, the N-methyl-D-aspartate (NMDA)-preferring subtype being especially effective. Although a rise in intracellular calcium appears to be essential for cell survival, little is known of other components of the survival signalling pathway triggered by EAA. Spinal muscular atrophy is a motor neuron degenerative disease that results from deletions or mutations in the Survival of Motor Neurons (SMN) gene. SMN is an ubiquitous protein found in the cytoplasm and in the nuclear dot-like structures called gems. Its function is linked to mRNA biogenesis. The aim of this study was to determine the relationship between the expression of SMN and the stimulation of granule cell survival elicited by EAA.

At the key period for cell survival *in vitro* (4 days; 4 DIV) the vast majority of cerebellar granule cells grown under the standard conditions, i.e., FCS-KCl 25 mM (K25), expressed SMN both in the nucleus (one/two gems) and in the cytoplasm (diffuse pattern), as revealed by immunofluorescent analysis. Culturing the cells under "suboptimal" conditions, i.e. FCS-KCl 10 mM (K10) or serum-free chemically defined medium (CDM), drastically reduced the percent of cells expressing nuclear gems. NMDA or glutamate treatment which promoted the survival of K10 cultures also promoted the expression of nulcear gems in the cells. These findings suggest that SMN may be a component of the survival signalling pathway triggered by EAA.

(Supported by MURST 60% to M.L.E. and Telethon-grant 828 to C.B.)

Effect of GABA receptor antagonist picrotoxin on ethanol intake and cardiovascular system in hypertensive rats

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Recently, particular attention has been directed to study the relationship between hypertension and ethanol (ETOH) consumption. It has been known that GABAergic system involved in cardiovascular regulation may be also implicated in behavioral effects of ETOH. To clarify if the GABA neurotransmission plays an important role in ETOH action, influence of picrotoxin on ETOH self-administration and cardiovascular system of hypertensive rats was examined. Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR) were exposed to a procedure in which increasing contents of ETOH (from 2% to 10%) were presented in a free choice with water on an alternative day schedule. Before and after ETOH injection systolic blood pressure (SBP) and heart rate (HR) were recorded. Chronic injection of picrotoxin at a dose of 2 mg/kg i.p. was more effective in SHR than in WKY rats. Fall in alcohol intake was longer in SHR for 2% ETOH and was absolutely inhibited for more concentrated (Table 1).

Table 1

Strain	Group	Intake ETOH (g/kg)	
		2%	4%
WKY	ETOH	1.75±0.1	1.28±0.3
	ETOH + PIC	0.84±0.1	0.64±0.1
SHR	ETOH	1.12±0.2	0.82±0.3
	ETOH + PIC	0.38±0.2	0

Strain	Group	Intake ETOH (g/kg)		
		6%	8%	10%
WKY	ETOH	0.94±0.21	0.58±0.2	0
	ETOH + PIC	0.82±0.4	0.54±0.1	0.76±0.2
SHR	ETOH	0.78±0.2	0	0
	ETOH + PIC	0	0	0

In both strains, chronic ETOH treatment had no effect on SBP but HR was suppressed. Co-dosage ETOH with picrotoxin decreased HR only in SHR rats.

Table 2

Strain	HR (beats/min)				
	control	ETOH	control	ETOH + PIC	P
WKY	363 ± 7	336 ± 6*	357 ± 7	355 ± 15	* < 0.05
SHR	377 ± 4	328 ± 2**	375 ± 10	341 ± 2*	** < 0.01

Conclusion: GABAergic activity is associated with ethanol self-administration. Besides, these results confirm the efficacy of ETOH to inhibit the tachycardia.

Extrasynaptic action of neurotransmitters in the brain

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Experimental evidence has recently emerged to suggest that basal transmission at conventional "fast" CNS synapses exerts actions beyond the synaptic cleft: synaptically released neurotransmitter molecules (in particular, glutamate) appear to bind to receptors and transporters at a considerable distance from the activated synapse [1, 2]. Whether these mechanisms can mediate significant cross talk between neighbouring synapses remains unclear.

To address this issue, we employed the well-defined hippocampal synaptic circuitry and, firstly, assessed inter-synaptic distances from stereological analyses of electron micrographs. In hippocampal areas CA1 and dentate gyrus, the estimated mean nearest-neighbour distance between synapses was ~0.5 μm [3, 4].

Secondly, quantitative data on perisynaptic ultrastructure allowed us to build the "typical" geometry of the synaptic microenvironment. Based on this geometry, we investigated mechanisms that control extrasynaptic action of glutamate by simulating its diffusion, uptake and receptor activation at distances on the scale of 0.5 μm [4, 5].

Thirdly, we tested our theoretical predictions in electrophysiological experiments where pharmacologically-isolated synaptic currents (NMDA-, AMPA- and kainate receptor-mediated) were recorded in individual hippocampal neurons. We also investigated the activation of extrasynaptic metabotropic receptors. The link between extracellular diffusion and the spatio-temporal profile of synaptically released neurotransmitter was probed by altering viscous properties of the extracellular medium [6].

These data allowed us to reveal critical parameters that regulate cross talk between neighbouring synapses through the extracellular action of excitatory neurotransmitters like glutamate [7]. In cases where such cross talk is significant, the conventional paradigm of synaptic transmission may need revisiting.

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Toxicological estimation of biologically active GABA-derivatives

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Parameters of substances' toxicity are basic for their safety evaluation. In the present work toxicity screening and evaluation of phiperone (phenotropile), karphedone (cyclic derivatives of GABA), gammoxin and mephebute (linear derivatives of GABA) after acute treatment in rats were investigated. On the basis of the received data it was made the forecast of the prospec-

tive mechanism of neuromediator action. Probability of the forecast of karphedone, phiperone, gammoxin and mephebute actions on catecholaminergic and cholinergic structure was testified by methods of the pharmacological analysis.

The experiments were carried out on 300 mature rats of both sexes weighing 180–250 g. Dose-dependent profile of nootropic drugs' toxicological action (karphedone, phiperone, gammoxin and mephebute) was studied by the method of S. Irvin [1] in arithmetically progressing doses (per os). Observation of animals was done continuously within the first day, and the next two weeks periodically. Possible action of drugs on catecholaminergic and cholinergic structures was considered by influence of karphedone, phiperone, gammoxin and mephebute on effects of D-amphetamine sulfate *Sigma* (10 mg/kg, i.p.) and arecoline *Sigma* (15 mg/kg, i.p.). The studied drugs were given orally in effective and toxic doses either 40 min before D-amphetamine and arecoline injections.

It was shown that drugs belong to a low toxic drugs group. The LD_{50} for karphedone 2700 mg/kg, phiperone – 700 mg/kg, mephebute – 1800 mg/kg, gammoxine – 3500 mg/kg. The death of animals from toxic doses of karphedone and phiperone was observed at the end of first – beginning of the second day. The death of animals after toxic dose of gammoxin and mephebute treatments was observed within the first three hours.

The changes of movement activity character of animals were common for all tested drugs, and karphedone, phiperone and mephebute treatment in effective and 3–5 times exceeding doses led to decrease of movement activity, and gammoxin to an increase. Rats treated with tested drugs in doses 5 times more than effective one (up to lethal dose) showed increase of motor activity at first and stereotyped locomotion further on. The revealed uniformity in character of dose-dependent influence of karphedone, phiperone, gammoxin and mephebute on movement activity of animals apparently can testify to possible presence of catecholaminergic component in the mechanism of their actions. The results of the carried out pharmacological analyses are submitted in the Tables 1 and 2.

All data are presented as the means (± SEM). Boldfaced means are statistical significant from control, $P \leq 0.05$.

Table 1

Variants of tests	Doses *mg/kg*	n	Latency period onset, *min*	Hyper-kinesis, *min*
control	-	12	2.8±0.2	22.9±0.7
mephebute	10	6	**2.2±0.2**	**10.8±0.6**
	100	6	3.6±0.6	**13.0±1.1**
karphedone	50	6	2.3±0.4	**19.1±1.0**
	250	6	2.8±0.1	22.2±0.2
phiperone	30	6	2.9±0.3	**12.7±1.3**
	150	6	3.2±0.4	21.1±0.7

Table 2

Variants of testes	Doses *mg/kg*	n	Latency period onset, *min*	Loco-motion, *min*	Stereo typy, *min*
control-1	-	8	11.3±0.3	13.6± 2.0	198.0±12.7
karphedone	50	7	**9.0±0.0**	**33.2± 2.0**	**260.0± 4.2**
	250	6	**7.3±0.3**	**34.4± 2.0**	**259.2± 4.1**
phiperone	30	6	12.4±4.0	24.2± 9.0	200.0± 4.5
	150	6	**8.6±2.7**	32.6± 7.0	242.0±37.0
control-2	–	6	18.3±4.0	28.3±11.0	142.0± 7.2
gammoxin	30	6	22.6±8.0	21.4± 0.4	132.4± 5.0
	200	6	15.3±0.3	42.6± 0.5	160.0±22.0
mephebute	10	6	16.1±0.5	21.2± 6.0	145.6± 7.5
	100	6	13.2±1.0	32.3± 8.0	160.4±21.6

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Developmental plasticity in inhibitory transmission in the central auditory system

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In the central auditory system, lateral superior olivary nucleus (LSO) integrates binaural cues for sound localization in adults by integrating excitation from the ipsilateral ear and inhibition from contralateral ear. In gerbils, inhibitory synapses innervating the LSO that arise from the medial nucleus of the trapezoid body (MNTB) are modified during early development as demonstrated by: a) The refinement of single arbors of the MNTB neurons; b) intracellular measures of convergence, and c) the distribution of glycine receptors in the LSO (Sanes et al 1987, Sanes, 1993, Sanes and Takács, 1993). We have used whole cell recording technique in brain slice preparation to explore developmental plasticity of synaptic inhibition. Our recent studies suggest that inhibitory transmission itself changes dramatically during development. Furthermore, inhibitory transmission affects the development of excitatory amino acid receptors.

We found a profound transition from GABAergic to glycinergic transmission in the LSO during the first two postnatal weeks. Inhibitory postsynaptic currents (IPSCs) evoked by electrical stimulation of the MNTB were characterized as GABAergic and glycinergic components by blocking transmission with either bicuculline (BIC) or strychnine (SN), respectively. There was a dramatic change in the GABAergic IPSC component, decreasing from 78% at postnatal days (P) 3–5 to 12% at P12–16. There was an equal and opposite increase in the glycinergic component during this same period. Consistent with the electrophysiological observations, there was a reduction in staining for the β2,3 $GABA_A$ receptor subunit from P4 to P14, while staining for the glycine receptor associated protein, gephyrin, increased. Brief exposure to baclofen depressed transmission at excitatory and inhibitory synapses for ‰ 15 mins, suggesting a $GABA_B$-mediated metabotropic signal. These data demonstrate corelease of two inhibitory amino acid transmitters, and a striking switch from GABAergic to glycinergic transmission.

How does inhibitory transmission influence the development of excitatory synapses? Two in vivo manipulations were designed to decrease glycinergic transmission in the developing LSO. First, contralateral cochlear ablation functionally denervated the glycinergic pathway from the MNTB to the LSO. Second, continuous release of a glycine receptor antagonist, strychnine, was used to decrease transmission. The strength of excitatory synapses was examined with whole-cell current clamp recordings from LSO neurons in a brain slice preparation. Ipsilaterally evoked EPSPs were of unusually long duration following either manipulation. Hyperpolarizing the neuron to –90 mV, or exposing it to AP-5, an NMDA receptor antagonist, significantly shortened these long EPSPs. Since both cochlear ablation and strychnine rearing were initiated prior to the onset of sound-evoked activity, the results suggest that *spontaneous* glycinergic transmission influences the development of NMDA receptor function. Collectively, our data show a profound degree of plasticity of inhibitory amino acid transmission in the developing auditory system.

(Supported by NIH DC00540.)

NMDA receptors: Targets for anticraving and antirelapse compounds

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The role of the glutamatergic/NMDA receptor system in modulating alcohol self-administration has only recently been examined. The functional NMDA receptor antagonist acamprosate was effective in a series of animal studies in reducing alcohol consumption and relapse behaviour. We suggest that acamprosate decreases glutamatergic neurotransmission and selectively alters the transcription of NMDA receptor subunits thereby counterbalances chronic alcohol-induced changes within the glutamatergic/NMDA receptor system. Altough competitive as well as uncompetitive NMDA receptor antagonists attenuate operant responding for ethanol the selectivity of such an effect is questionable. However, uncompetitive NMDA receptor antagonists with relatively low affinity, fast blocking/unblocking kinetics and strong voltage dependency may allow therapeutically relevant concentrations to block chronic, low level pathological activation of NMDA receptors whilst leaving their synaptic activation intact. Memantine and MRZ 2/579 fullfil those biophysical characteristics and indeed proofed to be very effective in preventing the alcohol deprivation effect in an animal model of long-term alcohol self-administration with repeated deprivation phases. Our results suggest that these uncompetitive NMDA receptor antagonist can be classified as potential new anticraving/antirelapse compounds. Moreover our drug discrimination studies indicate that memantine and MRZ 2/579 dose-dependently generalized the ethanol cue. It is therefore concluded, that both compounds might exert their anticraving/antirelapse properties by substituting for some of the stimulus properties of ethanol.

Excitatory amino acid transporters (EAAT) in the mammalian retina

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Glutamate is the major excitatory neurotransmitter of the mammalian retina and glutamate uptake is essential for normal transmission at glutamatergic synapses.

At the first synapse in the retina, between photoreceptors and second order neurons, increases in light intensity are signaled by decreases in the concentration of glutamate within the synaptic cleft. In such a system, it is critical to precisely control the glutamate concentration in the synaptic cleft, not only because of the neurotoxic effects of glutamate at high concentration, but also because the glutamate concentration represents the light signal. The precise control of glutamate in the synaptic cleft is thus essential. High-affinity glutamate transporters are thought to contribute to this process.

So far, we have cloned five subtypes of mammalian high-affinity glutamate transporters from the mammalian retina: GLAST1, GLT1, GLT1C, EAAC1 and EAAT5. EAAT4, another member of the EAAT-gene family, is not expressed in the mammalian retina. A comparative immunoblot analysis suggested the following quantitative rank order of transporter expression in retina: GLAST1 $\geq$ EAAT5 > EAAC1 > GLT1. GLAST1 is localized only in glial cells; EAAT5 in glial and neuronal cells; GLT1 and EAAC1 in neuronal cells. Ultrastructural localization confirmed the labeling of Müller cells and some processes in the inner plexiform layer of the retina. EAAT5 localization overlaps that of GLAST1 in Müller cells. Unlike GLAST1 localization, however, which appears to be in regions abutting synaptic terminals, EAAT5 is found in the main process and endfeet of Müller cells.

Heterologous expression of these transporters revealed that they are electrogenic, sodium-dependent and mainly driven by the transmembranous, retrograde sodium/potassium ion gradient. In addition of being cotransporters, they also behave as glutamate-gated chloride channels; this property is particularly prominent in EAAT5. The multifunctional properties of the uptake system suggest that its functions are more complex than simple transmitter removal. The specific subcellular expression pattern of GLAST1 and EAAT5 in one and the same cell, together with their mechanistic differences (e.g. chloride conductance) may reflect the differential roles these glutamate transporters have in Müllers cells:

High capacity glutamate uptake by GLAST1 and glutamate sensoring by EAAT5.

Taurine

Taurine uptake activity in the rat retina: Protein kinase C-independent inhibition by chelerythrine

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Taurine, a free amino acid found in high quantities in mammalian tissues, particularly in the central nervous system, has been linked to many important physiological processes including osmoregulation, calcium homeostasis, and neurotransmission, and in particular, visual transduction in the retina. Taurine is abundant in the retina, comprising 40–50% of the total free amino acids and reaching concentrations as high as 79 mM. The relatively high concentration of taurine appears to be a functional necessity, albeit the exact mechanism of action of taurine is yet unknown. Although some degree of endogenous biosynthesis exists in certain mammalian species, much of the taurine present is exogenous in its source, requiring a very efficient transport system to achieve the high tissue concentrations that are observed. Thus, in these present studies, taurine uptake was characterized in both whole retinal preparations and in isolated rod outer segments (ROS) in terms of uptake kinetics and possible protein kinase C (PKC)-dependent regulation. Two uptake systems, a high and a low-affinity system was found in the isolated ROS. All the uptake systems that were characterized were inhibited by guanidinoethanesulfonate (GES), a well-known competitive inhibitor of taurine uptake. Stimulation and inhibition of PKC activity with phorbol myristate acetate and with staurosporine, respectively, produced no significant effect on taurine uptake. Taurine uptake was also independent of the Ca^{2+} concentration. These data suggest that taurine uptake is not under regulation by PKC activity. However, chelerythrine (CHT), a documented potent PKC inhibitor, was observed to cause significant inhibition of the two taurine uptake systems, presumably through a PKC-independent mechanism. These data demonstrate that CHT may be a useful tool in studying taurine uptake in the retina and specifically in the ROS.

(This research was supported by a grant from the RGK Foundation, Austin, Texas.)

Taurine-depletion enhances cell death due to angiotensin II-induced apoptosis

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We have previously shown that cells exposed to medium containing taurine inhibit several of the actions of angiotensin II. Conversely, taurine deficient cells exhibit enhanced angiotensin II activity. These observations have led to the suggestion that cellular taurine content may influence the development of congestive heart failure by altering the hypertrophic activity of angiotensin II. Similarly, one could argue that taurine might alter the onset of heart failure by altering angiotensin II-induced apoptosis, a process important in the ventricular modelling phase of heart failure development. In accordance with the latter hypothesis, we found that the taurine deficient cardiomyocyte shows increased susceptibility to angiotensin II-induced apoptosis. While the extent of cellular apoptosis was 23.6 ± 2.7% in the control myocyte exposed for 24 hr to 1 nM angiotensin II, the apoptotic rate was 33.4 ± 3.8% in the taurine deficient myocyte treated with 1 nM angiotensin II. The promotion of apoptosis by angiotensin II appears to be caused in part by a 60% increase in the content of the pro-apoptotic factor, Bax. Although taurine deficiency had no effect on Bax content in the absence of angiotensin II, Bax levels increased 120% in the presence of angiotensin II. Angiotensin II also reduced Bad phosphorylation in both the control and taurine deficient cell. Since unphosphorylated Bad is considered pro-apoptotic while the phosphorylated form of the regulator lacks activity, angiotensin II-induced dephosphorylation of Bad will also promote apoptosis. Thus, angiotensin II-induced apoptosis is enhanced in the taurine deficient cell by a stimulation in the activity of the pro-apoptotic factors, Bax and Bad.

Protective effects of taurine in alcohol-induced liver dysfunction: A. Preliminary investigations

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Alcohol was administered chronically to female rats (Sprague-Dawley) in a nutritionally adequate totally liquid diet for 28 days. With some animals taurine was co-administered with alcohol. Control animals were pair fed. Urine samples were collected throughout the treatment and blood was taken at the end of the experiment. After the 28 day treatment period animals were exsanguinated under anaesthesia, the liver was removed for histology and the following biochemical determinations carried out: triglycerides, lipid peroxidation, taurine, homocysteine, glutathione and cytochrome P450 level and CYP2E1 and methionine synthase activity. Plasma was used for clinical chemical analysis and for determination of taurine and homocysteine.

In the animals treated with alcohol alone hepatic steatosis was detected both histologically and biochemically. Lipid peroxidation was increased when measured as malondialdehyde production. When taurine was co-administered with the alcohol there was a significant reduction in steatosis and lipid peroxidation was completely prevented. The cytochrome P450 level was raised and the activity of CYP2E1 was significantly increased after alcohol treatment. However when taurine was co-administered the CYP2E1 activity was significantly decreased to levels which were barely detectable. However, bile acids and alkaline phosphatase were raised in serum of animals treated with alcohol and alcohol plus taurine indicating that a slight cholestasis had resulted from the treatment.

These results suggest that co-administration of taurine with alcohol will significantly protect against the fatty liver and lipid peroxidation but that cholestasis is not prevented by taurine. The significant decrease in CYP2E1 in the taurine plus alcohol treated animals may be due to the effect of taurine conjugated bile acids and may underlie the protective effects of taurine.

Sodium nitroprusside-induced seizures and taurine release from rat hippocampus

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It is now controversial whether nitric oxide (NO) acts as a pro- or anticonvulsant. We have recently reported that sodium nitroprusside (SNP, an NO donor) induces seizures which are associated with an increase in the basal release of aspartate and glu-

tamate from the rat hippocampus [Kaku et al. (1998) Jap J Physiol 48 (Suppl): S121]. In this study, to determine whether taurine release occurs with the SNP-induced seizures, we examined the effects of SNP, diethyldithiocarbamate (DETC, an NO trapper), dithiothreitol (DTT, a superoxide radical scavenger) and oxypurinol (OP, alloxanthine: a xanthine oxidase inhibitor) on EEG activity and basal and high K^+-evoked taurine release from rat hippocampus *in vivo* using microdialysis. Perfusion with 0.5 mM SNP tended to increase high K^+-evoked taurine release and increased significantly taurine release with the occurrence of the seizures during reperfusion with artificial cerebrospinal fluid. Coperfusion with 0.5 mM SNP and 5 mM DETC significantly abolished the SNP-induced seizures and reduced the taurine release during and after perfusion with the drugs. Coperfusion with 0.5 mM SNP and 1 mM DTT reduced both the frequency of SNP-induced seizures and the taurine release during and after perfusion with the drugs. Coperfusion with 0.5 mM SNP and 1 mM OP did not reduce the frequency of SNP-induced seizures, but tended to decrease the taurine release during and after perfusion with the drugs. These results demonstrate that SNP-induced seizures are triggered by an increase in both NO and in part peroxynitrite and related to an increase in taurine release from the rat hippocampus *in vivo*.

Protective effects of taurine in alcohol induced liver dysfunction: B. Further studies and mechanistic implications

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We have previously shown in rats [1] that taurine protects against the alcohol induced accumulation of liver triglycerides and reduces the production of malondialdehyde when co-administered with alcohol. The following study examined the ability of taurine to reverse alcohol induced damage by treating animals with taurine for two days following withdrawal of the alcohol containing diet.

Alcohol was administered chronically to female rats (Sprague-Dawley, 125–150 g). Control rats were pair fed. After 28 days alcohol was removed from the diet and half of the alcohol and control animals were given taurine (3%) in the liquid diet for a further 2 days. Alcohol induced triglyceride accumulation and malondialdehyde production was measured as well as other markers of liver injury including serum liver enzymes and biochemistry. Cytochrome P450 2E1 activity was also assessed as well as histological examination of liver samples.

Table 1. The effect of alcohol and taurine treatment on biochemical parameters

Treatment (0–28 days)	No alcohol	Alcohol
Treatment (28–30 days)	None	None
Liver wt (% body wt)	2.08 ± 0.11	5.05 ± 0.16***
Hepatic TRIG (mg/g)	8.3 ± 0.9	15.2 ± 1.2***
Hepatic MDA (μmol/g)	1.37 ± 0.21	4.28 ± 0.52***
Serum TRIG (mmol/L)	0.33 ± 0.04	0.57 ± 0.90***
Treatment (0–28 days)	No alcohol	Alcohol
Treatment (28–30 days)	+ Taurine	+ Taurine
Liver wt (% body wt)	2.91 ± 0.07	5.13 ± 0.07***
Hepatic TRIG (mg/g)	6.8 ± 0.8	6.7 ± 0.6
Hepatic MDA (μmol/g)	1.41 ± 0.21	2.94 ± 0.73***#
Serum TRIG (mmol/L)	0.35 ± 0.04	0.77 ± 0.12***#

Values are means ± sem, N = 5, *P < 0.05, **P < 0.01, ***P < 0.001 pair fed controls; #P < 0.001 alcohol treatment compared with alcohol followed by taurine treatment. *MDA* malondialdehyde; *TRIG* triglycerides.

Taurine treatment may have reversed the hepatic triglyceride levels by increasing lipid secretion into the blood and may have contributed to the reduction in malondialdehyde. However histology showed that hepatocytes remained enlarged. Taurine treatment did not reduce P450 2E1 activity as shown previously [1], although total P450 levels were higher in animals treated with alcohol followed by taurine.

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Physiological significance of taurine and taurine transporter in intestinal epithelial cells

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The absorption of dietary taurine is carried out via the transport system in the intestinal epithelium. We have observed that the human intestinal epithelial cell line, Caco-2, expresses taurine transporter and the transport activity is regulatable by various factors. The taurine transport is subject to adaptive regulation, being down-regulated on culturing the cells in taurine-containing medium and up-regulated in taurine-free medium. This adaptive regulation was associated with changes in Vmax of taurine transport and also with changes in Km. Changes in the mRNA level of the taurine transporter in this regulation were also observed. The presence of such a regulatory mechanism to maintain the intracellular taurine content at a certain level suggests that taurine plays an important role in the intestinal epithelial cell functions. The increase in the intracellular taurine content was observed when the Caco-2 cells were exposed to a hypertonic stress. The taurine transporter was up-regulated via the increased expression of the transporter gene in the hypertonic cells. On the other hand, hypertonicity did not affect the activity of L-Leu, L-Lys or L-Glu transport nor the intracellular content of these amino acids, suggesting that only taurine serves as an osmolyte and protects the intestinal cells from the osmotic stress. The similar up-regulation of taurine transporter was observed in the small intestine of water-deprived rats. Our recent studies on the osmoregulatory mechanism has demonstrated that calmodulin-dependent protein kinase may play a part in the osmoregulation of taurine transporter.

Renal excretory responses of the taurine-depleted rat to a selective antagonist of vasopressin receptors

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Taurine is implicated in the regulation of vasopressin secretion [J Comp Neurology (1997) 381: 513–523] and the taurine-depleted rat manifests altered renal excretory function [Amino Acids (1998) 15: 109–116]. We tested the hypothesis that increased vasopressin activity contributes to altered renal excretory function in the taurine-depleted rat. Male Wistar-Kyoto rats were given either tap water (n = 5; control) or 3% β-alanine (n = 5; taurine-depleted) for 3 weeks. Thereafter, renal excretory responses were monitored in the conscious animal prior to and after a bolus injection (100 μg/kg; i. v.) of a selective antagonist for renal vasopressin receptor subtype 2 [i.e. V_2; (Adamantaneacetyl, D-Tyr $(Et)^2$, Val^4, Abu^6, $Arg^{8,9}$) vasopressin]. Baseline fluid excretion was lower in the taurine-depleted than control rats. The administration of the V_2 receptor antagonist significantly increased fluid and sodium excretion in both the control and the taurine-depleted rats. However, renal excretions of fluid and sodium were not only

delayed but also significantly lower in the taurine-depleted than control rats following administration of the antagonist. Moreover, baseline sodium to fluid ratio, an index of urine concentrating ability, was higher in the taurine-depleted than control rats. Following drug administration, taurine-depleted rats manifested a delayed but more prominent reduction in sodium to fluid ratio. The reduction in sodium to fluid ratio in response to the V_2 receptor antagonist was restored in the control, but not taurine-depleted, rats 90 minutes after administration of the drug. These findings suggest that taurine depletion augments renal actions of vasopressin. However, it remains to be determined whether the effect of taurine depletion is mediated by alterations in renal V_2 receptor affinity, number, or post-receptor events.

(Supported by MCG School of Dentistry and Taisho Pharmaceutical Company of Japan.)

Taurine release mechanisms as studied *in vivo* under physiologic and pathophysiologic conditions

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The sulfur-containing amino acid taurine is supposed to act as a neurotransmitter, as a neuromodulator, and as a regulator of Ca^{++} fluxes in the brain. In addition, it has been shown to be an osmoregulator under various conditions. Recently, we reported on its release in the zone surrounding a thrombotic infarct [Scheller et al. (1997) Eur J Pharmacol 338: 243–251]. We showed that the neuroprotective drug lubeluzole prevented the rise of extracellular taurine.

To get more insight into the possible mechanism of action of the drug, we implanted a microdialysis membrane into the cortex of anesthetized rats. As possible triggers to stimulate taurine release, we applied NMDA, NO or a hypotonic solution locally via the perfusate with or without the addition of the NMDA antagonists APV or Ketamine, or the NO synthase inhibitor L-NAME. Alternatively, we applied the neuroprotective drug lubeluzole but intravenously.

NMDA, NO or the hypotonic solution stimulated the release of taurine. The effect of NMDA could be inhibited by APV or Ketamine or the NO synthase inhibitor L-NAME. Lubeluzole had not effect under these conditions. Interestingly, the effect of the hypotonic solution could not be influenced by L-NAME, but could be reduced by lubeluzole.

These data suggest that the NMDA-induced release of taurine is mediated via the NO cascade *in vivo* too. However, the release occurring subsequently to the hypotonic stimulus is not related to the NO cascade. Since lubeluzole had been postulated to act through the NO pathway but downstream the NO synthase, its inhibitory effect on the taurine release can not be related to the NO system. Its effect supports the observation that the osmotically induced taurine release occurs via an other regulatory system.

Taurine modulates expression of transporters in rats as revealed by "gene hunting"

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Taurine is known to increase ATP-dependent calcium ion uptake in retina. The involvement of a cation channel activation has been proposed [1]. Moreover the modulation of the intracellular taurine pool has been shown to affect the cellular calcium homeostasis and myocardial contractile function [2]. The molecular mechanism by which taurine concentration modulates cellular ion transport has not been elucidated yet.

We used the gene hunting technique of subtractive hybridization to investigate a possible gene regulating effect of oral taurine administration in Sprague-Dawley rats. Rats were fed with taurine 100 mg/kg body weight per day for a period of three days. Hearts (total ventricular tissue) of experimental animals and control animals were pooled and used for mRNA extraction. Both groups of mRNAs were used for subtractive hybridization.

Two clones of the subtractive library have been identified to be homologous to a bacterial Mg^{2+}-dependent cation-transporting ATPase and to an outer membrane channel protein respectively. These proteins are upregulated by oral taurine administration in myocardial cells. The Mg^{2+}-dependent cation-transporting ATPase is an active regulator of cation homeostasis. The outer membrane channel protein is induced by organic solvents and in turn enables an active efflux of these solvents. These findings provide a genetic basis for the taurine-dependent mechanism of membrane transport regulation.

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Evidence that taurine modulates osmoregulation by modification of osmolarity sensor protein EnvZ-expression

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Taurine is known as a very important osmolyte factor. Some recent studies show that taurine regulates the osmotic balance in the neonatal cardiomyocyte, modulating size and shape of the cells [1]. Moreover taurine depletion in rats effects renal excretory responses to a hypotonic, but not a hypertonic, saline solution [2]. Nevertheless the mechanism for this taurine function is still unclear.

We used the gene hunting technique of substractive hybridization to investigate a possible gene regulating effect of oral taurine administration in Sprague-Dawley rats. Rats were fed with taurine 100 mg/kg body weight per day for a period of three days. Hearts (total ventricular tissue) of experimental animals and control animals were pooled and used for mRNA extraction. Both groups of mRNAs were used for subtractive hybridization.

Two clones were found to be homologous (61 and 65%) to the bacterial osmolarity sensor protein EnvZ. This protein is an inner membrane protein important for osmosensing and required for porin gene regulation. It undergoes autophosphorylation and subsequently phosphorylates OmpR, which in turn binds to the porine (outer membrane protein) promoters in order to regulate the expression of OmpF and OmpC, major outer membrane porines. Down regulation of the EnvZ homologous protein in rat cardiac cells by oral taurine administration points to a mechanism by which the taurine concentration influences the osmotic status of the cells.

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Reactivity of the aorta from rats supplemented with taurine

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Taurine has been reported to produce a hypotensive effect in several animal models of hypertension [Adv Exp Med Biol (1994) 359: 149–157]. Although the exact mechanism for the effect of the amino acid on blood pressure remains elusive, it is plausible to suggest that taurine-induced alterations in vascular reactivity to endogenous substances contributes to its hypotensive effect. This study tests the hypothesis that taurine supplementation attenuates contractility but enhances relaxation of blood vessels to vasoactive agents. Six week old male Wistar-Kyoto rats were given either tap water (control) or 1% taurine (taurine-supplemented) for 6 weeks. Animals were then sacrificed and the thoracic aorta was carefully removed from each rat. Aortic rings were prepared and suspended in organ baths for isometric tension measurement. Contractile responses to the endogenous agonist norepinephrine (NE) and to the depolarizing agent KCl were determined cumulatively in preparations from both groups of animals. Endothelium-dependent and independent relaxant responses to acetylcholine (ACh) and sodium nitroprusside (SNP) were also determined in rings precontracted with 10^{-7} M NE. The taurine-supplemented rats did not exhibit significant changes in blood pressure compared to controls. Contractile responses to NE and KCl were significantly attenuated in aortae from taurine-supplemented animals as compared to controls. On the other hand, ACh and SNP-induced relaxations were markedly augmented in tissues from rats supplemented with taurine compared to the responses obtained in control preparations. The data suggest that taurine supplementation attenuates contractility but enhances relaxation of blood vessels non-specifically. These observations may be of relevance to the hypotensive effect of taurine previously reported in hypertensive animals.

(Supported by Taisho Pharmaceutical Company and MCG School of Dentistry.)

Taurine modulates the activity of the classical hypothalamo-neurohypophyseal system

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The mammalian hypothalamo-neurohypophyseal system (HNS) consists of magnocellular neurons located within hypothalamic supraoptic (SON) and paraventricular nuclei (PVN) which synthesize the nonapeptides arginine-vasoppressin (AVP) and oxytocin (OXT). The axons of these neurons project to the neurohypophysis to release AVP and OXT into the systemic circulation after appropriate stimulation. In addition to this peripheral secretion, HNS neurons are capable of releasing AVP and OXT from their somata and dendrites into the extracellular fluid of both the SON and PVN. Recent investigations demonstrated that this intrahypothalamic release can be dissociated from the peripheral secretion under defined stress conditions. The aim of the present study was to further investigate the mechanisms that control this dissociated release. Therefore, we used microdialysis (i) to monitor intrahypothalamic release patterns of inhibitory amino acids and (ii) to administer specific antagonists for gamma-aminobutyric acid (GABA) and taurine while forcing the animals to swim for 10 min in 20° C warm water. Forced swimming triggered significantly intra-SON release of taurine (up to 350%; $p < 0.05$ versus basal values obtained under unstressed conditions) but not GABA (approx. 100%, n.s.). Blockade of the taurinergic and GABAergic neurotransmission at the level of the hypothalamus by local administration of a specific taurine antagonist (TAG) or a $GABA_A$ antagonist (bicuculline) into the SON via retrodialysis caused only in case of TAG a significantly increased release of AVP within the SON (upto 800%, $p < 0.01$) and into plasma (up to 800%; $p < 0.05$ versus basal values). In contrast neither TAG nor bicuculline affected peripheral OXT release. Taking together out data suggest taurine as a candidate for dimming the activity of the magnocellular vasopressinergic part of the HNS during swim stress.

The effects of β-alanine on the hepatotoxicity of alcohol in rats

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Alcohol administration to rats causes hepatic steatosis and lipid peroxidation. Taurine has been shown to protect against this hepatotoxicity [1]. The aim of this study was to discover if a structural analogue of taurine, β-alanine, which depletes taurine, would alter the susceptibility of animals to the hepatic toxicity of alcohol. Alcohol was administered to animals (Sprague-Dawley rats, female, n = 6, 125–150 g) in a nutritionally adequate totally liquid diet for 28 days [2] and β-alanine also was administered in the liquid diet to some animals. There were four treatment groups; a) controls, no alcohol, b) alcohol, c) 3% β-alanine and d) alcohol plus 3% β-alanine. After 28 days of treatment, livers were removed for histolocigal processing and biochemical assessment of hepatic damage. Blood war removed for the preparation of serum.

Alcohol treatment resulted in the accumulation of hepatic triglycerides and increased malondialdehyde levels in liver samples. Hepatic steatosis in the alcohol plus β-alanine treated animals was slightly but significantly less than in alcohol treated animals. However lipid peroxidation as indicated by malondialdehyde levels was not affected by β-alanine treatment. Alcohol and β-alanine given separately may both have caused mild cholestasis and/or slight bile duct damage as both bile acids and serum alkaline phosphatase were significantly raised. However, these parameters were raised significantly more when alcohol was given with β-alanine which may suggest an additive or synergistic effect. Serum transaminases (ALT, AST) were also raised by alcohol but were slightly but significantly reduced by alcohol plus β-alanine treatment. However the levels were not as high as might be expected following liver necrosis. Serum taurine and liver taurine were depleted in the β-alanine and alcohol plus β-alanine treated animals.

Histology, however, appeared to contradict the biochemial findings as there appeared to be more fat accumulation (stained with Oil REd O) in alcohol plus β-alanine treated animals. The reason for this discrepancy is unknown but may reflect the differences in triglyceride synthesis in different lobes. Therefore histology alone would suggest that alcohol-induced fat accumulation is exacerbated by β-alanine treatment.

These *in vivo* findings demonstrate that a) β-alanine may have some protective properties against alcohol induces fatty liver; b) depletion of taurine did not increase susceptibility to alcohol induced lipid peroxidation; c) β-alanine treatment (or taurine depletion) may increase susceptibility to alcohol induced biliary dysfunction.

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Influence of a taurine containing drink on cardiac parameters at rest and after exercise

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Introduction: Previous double-blind studies have shown an effect of the drink "Red Bull" on various physiologic parameters during and after exercise. Especially the endurance performance increased, the heart rate and the norepinephrine concentrations at a given workload were lower under taurine [Geiß et al. (1994) Amino Acids 7: 45–56]. With the present study we wanted to elucidate if this is due to an improved function of the heart.

Subjects and methods: Thirteen endurance athletes training at least 10 hrs/week since at least 3 years participated in the study. The test design was planned double-blind and cross-over. The drinks had the following compositions:

D1: Glucose 10.5 g, saccharose 43 g (500 ml, "Placebo")

D2: Caffeine 160 mg, glucose 10.5 g, saccharose 43 g (500 ml, "Control")

D3: "Red Bull" original drink containing taurine 2 g, glucuronolacton 1.2 g, caffeine 160 mg, glucose 10.5 g, saccharose 43 g (500 ml, "Verum").

The subjects cycled with 5 increasing intensities in 6 min intervals until near exhaustion and after a pause of 25 min with fast increasing intensity up to a heart rate of 160 within 1 min. The following parameters were determined by echocardiography: left ventricular enddiastolic and endsystolic diameters, systolic and diastolic left ventricular volumes were calculated from the Teichholz formula, also the stroke volume. Echocardiographic examinations were performed before the ingestion of the test drink, 20 min thereafter immediately prior to the exercise, and in the regeneration period.

Results: The stroke volume after exercise was only sign. increased in the verum experiment due to a higher fractional shortening and sign. lower endsystolic volume. Also the inflow velocity at the mitral walve increased only sign. after ingestion of the drink D3 ("Verum").

Conclusion: The present study demonstrates an influence of the original taurine containing drink "Red Bull" on cardiac parameters after exercise. Further evidence for an increased contractility of the left ventricle and atrium might be given by studies which observe left ventricular function during exercise by stress echocardiography. The observed results might be due to metabolic effects to the combination of taurine and caffeine and/or taurine effects on electrical membrane stability and/or on calcium channels.

Regulation of taurine transporter

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The β-amino acid taurine is abundantly present as a free amino acid in a variety of excitable and peripheral tissues. These tissues primarily depend on a high-affinity taurine transporter for their intracellular supply of taurine. We isolated a taurine transporter encoding clone from a mouse retinal cDNA library, functionally expressed the cDNA clone in *Xenopus* oocytes, and localized the sites in the mammalian eye expressing this transporter specific mRNA (Vinnakota et al., 1997). To elucidate mechanism(s) by which various cellular factors might regulate the taurine transporter activity and/or expression, we have examined the effects of changes in the extracellular pH, activation protein kinases, and treatment with redox agents on the mTauT function.

The taurine uptake in oocytes expressing the mTauT protein varied in an S-shaped manner as the pH of the uptake buffer was changed from 5.3 to 8.5. Independent activation of protein kinase A and protein kinase C inhibited the taurine uptake. Pre-incubation with the oxidizing agent diamide or the reducing agent DTT had no significant effect on the mTauT function; however, addition of ascorbic acid directly to the uptake buffer inhibited the taurine uptake in a concentration dependent manner.

Restoration of plasma taurine levels in patients with Gaucher disease during enzyme replacement therapy (ERT)

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Gaucher disease is caused by an autosomal-recessive deficiency of glucocerebrosidase. Cells of monocytic/macrophagic origin accumulate glucosylceramide. This eventually leads to hepatosplenomegaly, bone destruction, thrombocytopenia and anemia. Enzyme replacement therapy (ERT) with macrophage-targeted glucocerebrosidase leads to normalization of these parameters. Osmolytes (betaine, choline, taurine, inositol) are capable of regulating macrophage function. The way of macrophage activation in Gaucher disease is not known and little is known about the role of amino acids in this lysosomal storage disease. Therefore, the role of plasma taurine in Gaucher disease was studied.

Fasting plasma levels were measured from blood samples of healthy control subjects (n = 29, m:f = 11:18, mean age 37 ± 3 years), from untreated Gaucher patients (n = 11, m:f = 6:5, mean age 49 ± 5 years) and those treated for more than a year (n = 53, m:f = 19:34, mean age 47 ± 2 years). Amino acid analysis was carried out in a BioChrom amino acid analyzer.

In the untreated patients, plasma taurine was 45 ± 3 μM, as compared to the controls with a plasma taurine of 63 ± 4 μM ($p < 0.05$). The average increase of plasma taurine during the first year of ERT was 19 ± 8 μM. Patients treated for more than one year (range 1–9 years of ERT) had a plasma taurine of 60 ± 2 μM (n = 53), which was not different from the controls.

It is concluded that therapy of Gaucher disease might correct taurine deficiency. It has to be established, whether decreased taurine availability might influence the function of glucosylceramide-storing monocytes/macrophages in these patients.

Effects of taurine application on visual stress due to VDT works

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Healthy male college students aged 20–25 years participated in a double-blind study. After supplement of either 1 g taurine or placebo three times a day for 12 days, changes in pattern visual evoked potential (PVEP) and critical fusion frequency (CFF) caused by visual display terminal (VDT) works for 2.5 hrs were investigated.

The latency (ms) of negative (N) 75 and positive (P) 100 of PVEP became longer, and the amplitudes of the both became lower after the VDT works. In addition the CFF became lower after VDT works.

The supplement of taurine prevented the P100 amplitude reduction due to VDT works. Furthermore, it was also the case for the CFF reduction.

Taurine as a key substance in the vitamin A affinity channel transport of the retinoids in the retinal rod photoreceptor cells

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We have used different approaches to clarify the nature of connections between disc and plasma membranes in the rod outer segments (ROS). Using osmotic explosion as well as freeze fracture technique we showed that these membranes as well as adjacent discs in the ROS are connected to each other via filamentlike structures [1, 2]. Application of colloidal lanthanum, which is a marker for extracellular compartments, in combination with hyperosmotic sucrose incubation indicate that connections between disk rims and plasma membrane in the ROS are channelized [3]. Rose-like profiles of these channels recently were found on the surface of the ROS plasma membrane [4]. They consist of six subunites and function like a diaphragm to close or open the channel, depending on the surrounding calcium concentration [2]. During these studies we have succeeded in showing that a synthetic conjugate of taurine with vitamin A, retinylidentaurine, which we name TAURET, can selectively penetrate from an incubation medium into intradisc spaces via these channels [2]. It is important to note that because of conjugation with taurine vitamin A becomes highly water soluble. However because tauret is an easily hydrolyzed substance, with about 7 minutes half time of hydrolysis, vitamin A can be released after penetration and then can incorporate into rhodopsin. Our thin layer chromotographical and high pressure liquid chromotographical measurements showed that tauret is an endogenous substance in the retina and pigment epithelium [5]. During microspectrophotometrical investigations we revealed that rhodopsin can regenerate in the bleached ROS because of 11-cis tauret [5]. It is necessary to note that among the well understood functions of taurine one is its conjugation with bile acids. Probably this ability of taurine to promote the solubility of lipids in water has been noted by early natural evolution over hundreds million years since the origin of vision. All of this presented data combined with our recent finding that tauret can be synthesized in the retina after injection of 3H-taurine into the eye indicates that connections between disks and plasma membrane of the ROS are vitamine A selective molecular channels and that taurine seems to play a key role in their function. Also in light of the above, taurine is probably one of the most potent protectors against light induced damage, especially in emergency situations such as when photoreceptors are exposed to too bright light after deep dark adaptation.

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Reflux bile acid, *Helicobacter pylori* and trace mineral in peptic ulcer patients (taurine conjugated deoxycholic acid)

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Purpose: We examined the interrelationship of *Helicobacter pylori* (H.P.), trace mineral concentration (Zn) of the gastic mucosa, reflux bile acid concentration in the stomach (taurine conjugated deoxycholic acid [TCDC]) and the biopsied gastric mucosal change (atrophic change and cystic gland dilatation) – with reference to the pathophysiologic significance.

Objects, methods: We examined 53 patients suffering from gastric ulcers and 8 patients suffering from the atrophic gastritis as control. We proved H.P. infection by culture, microscopy and unrease test. We graduated reflux bile acid concentration with high performance liquid chromatography and trace mineral concentration in the flakes of gastric mucosa by application of thin metal film method. We examined gastric mucosal flakes under the microscope grading by atrophic changes and cystic gland dilatation, which biopsied at 4 points (Sano method).

Results: 1. The concentration of reflux bile acid (TCDC) of H.P.(–)group was higher than that of H.P.(+)group. 2. The gastric atrophy becomes more severe in proportion to the TCDC concentration. 3. Cystic gland dilatation of the gastric mucosa becomes more severe in proportion to the TCDC concentration. 4. Trace mineral concentration (Zn) of the gastric mucosa of H.P.(–)group is higher than that of H.P.(+)group. 5. The gastric mucosal atrophy of H.P.(+)group is more severe than that of H.P.(–)group. 6. Cystic gland dilatation of the gastric mucosa of H.P.(+)group is more severe than that of H.P.(–)group.

Discussion and conclusion: The reflux bile acid concentration in the stomach (taurine conjugated deoxycholic acid [TCDC]) takes part in the optimum gastric mucosal condition that *Helicobacter pylori* inhabits. The trace mineral concentration (Zn) of the gastric mucosa may be changed in relation to the reflux bile acid concentration (TCDC), *Helicobacter pylori* and the mucosal change (atrophic changes and cystic dilatation).

Chemical properties of N-chlorotaurine sodium, a novel disinfectant in human medicine

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N-chlorotaurine *(NCT),* 25 years ago detected in human blood cells, is known to play an important role in the human defence system. Generally prepared *in situ* from equimolar solutions of hypochlorite and taurine, *NCT* is also available as pure sodium salt enabling a simpler approach for a thorough investigation of its chemical properties.

The sodium salt of *NCT* forms colourless crystals which decompose at 135–140 °C. Compared to other N-chloroamino acids known only in solution, the aqueous solution is distinguished by an outstanding stability showing a decomposition rate of only 1–2%/day at room temperature which decreases to ~0.03%/day if kept at 2–5 °C.

The chemistry in aqueous solution reveals *NCT* as a mild oxidizing (R-SH → R-SS-R and R-SO_{1-3}H) and chlorinating (R-NH_2 → R-NHCl) agent. The equilibrium constant of the reaction of *NCT* with NH_3/NH_3^+ forming NH_2Cl and taurine

comes to K_{NHCl2} = $[NH_2Cl]$ [taurine]/[*NCT*] $[NH_4^+]$ = 0.2 ± 0.004 (25 °C) which discloses an appreciable grade of transformation in presence of NH_4^+. Auto-chorination causes a pH-dependent disproportionation forming N,N-dichlorotaurine *(NDCT)* and taurine with $K_{Dispr.}$ = [*NDCT*] [taurine]/[*NCT*]$^2aH^+$ = 4.5 ± 0.8 * 10^6 mol^{-1}. In pure aqueous solution showing a stable pH 7.8 -8.1, however, disproportionation is negligible.

The unique status of *NCT* concerning stability, bactericidal and virucidal properties, its endogenous occurrence (it is produced at the stimulation of human phagocytes), and the low toxicity suggests the sodium salt of *NCT* as a mild disinfectant in human medicine.

N-chlorotaurine, a product of stimulated leukocytes: Evidence for its antimicrobial function in the human defence system

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N-chlorotaurine ($ClHN-CH_2-CH_2-So_3^-$, NCT), an active N-chloro compound produced by stimulated granulocytes and monocytesis known to provied sustained oxidation capacity at inflammatory sites and to be involved in the human immune regulation, while its participation in destruction of pathogens is discussed controversely. Therefore, we investigated NCT on antibacterial effects at physiological concentrations.

In buffer solution, 12.5 – 50 µM NCT demonstrated significant bactericidal activity against gram-positive and gram-negative pathogens with a > 3 log_{10} reduction in viable counts after incubation times of 6–9 h at pH 7.0. In acidic milieu (pH 5.0), this effect was achieved within 1–3 h.

The supernatant of stimulated human granulocytes showed a similar bactericidal activity at pH 5.0 and a bacteriostatic effect at pH 7.2. Inactivation of active N-chloro compounds by addition of thiosulfate removed the antimicrobial effects.

Transmission electron microscopy of staphylococci treated with 50 µM NCT revealed changes of the cell membrane and cytoplasmic disintegration.

Due to its high stability, bactericidal levels of NCT may be assumed to accumulate *in vivo*. Our results suggest that NCT contributes significantly to the antimicrobial activity of human granulocytes, especially at acidic pH present in lysosomes of these cells.

Taurine chloramine affects functions (proliferation, cytokines synthesis) of synoviocytes from rheumatoid arthritis (RA) patients

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Fibroblast-like synoviocytes B play an important role in the pathogenesis of RA. Endogenously produced cytokines (TNFα, IL-1β and IL-17) trigger synoviocytes B to synthesize pro-inflammatory cytokines (IL-6, IL-8), while the excessive growth of these cells results in synovial membrane hyperplasia. The aim of present study was to investigate whether taurine chloramine (TauCl) affects these functions of RA synoviocytes. Synoviocytes B, isolated from synovial tissue of 19 RA patients and cultured in vitro for 3–6 passages, were stimulated with rhTNFα (10 ng/ml), rhIL-1β (1 ng/ml), rh IL-17 (10 ng/ml) or PMA (1 nM), a selective activator of protein kinase C (PKC), for 24 h. Taurine (Tau) or TauCl (50–500 µM) were added either simultaneously or 24 h after the stimuli. The concentrations of IL-6 and IL-8 were determined in culture supernatants using specific ELISAs. Proliferation of synoviocytes was estimated on the basis of ^{3}H-thymidine incorporation into these cells cultured in the presence of rh basic FGF (1 ng/ml) for 72 h. Cultured in vitro synoviocytes (2×10^4 cells) spontaneously secret low amounts of IL-6 (502 ± 73 pg/ml) and IL-8 (299 ± 103 pg/ml). In contrast, in the presence of TNFα, IL-1β, IL-17 or PMA, these cells synthesize substantial amounts of IL-6 (3160 ± 408, 9291 ± 881, 1336 ± 252 and 5087 ± 972 pg/ml) and IL-8 (7362 ± 797, 11653 ± 2019, 669 ± 176 and 8239 ± 1880 pg/ml), respectively. TauCl, but not Tau, inhibits cytokine- or PMA-triggered synthesis of IL-6 (IC_{50} ~ 225 µM) and IL-8 (IC_{50} ~ 450 µM) when added simultaneously with the stimul. However, IL-17-triggered production of IL-8 was not affected by TauCl. Inhibitory effect of TauCl on IL-6 and IL-8 synthesis by the cells pre-stimulated with IL-1β or TNF-α was weaker but still significant. Moreover, TauCl inhibits both the bFGF-induced and spontaneous proliferation of synoviocytes in a dose-dependent manner. Neither Tau nor TauCl affect cell viability. Our results show that a physiological factor, TauCl, blocks certain pathogenic functions of RA synoviocytes (proliferation, IL-6 and IL-8 synthesis) and thus may have therapeutic value. Since the inhibitory effect of TauCl is similar in both cytokine- and PMA-treated cells, we suggest that TauCl may affect signaling pathway dependent on the activation of PKC.

[Supported by grants from the Polish Ministry of Health and Welfare (No 24/98) and from the Institute of Rheumatology, Warsaw (No II/7).]

Taurine and hypotaurine in spermatozoa and epididymal fluid of cat

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Introduction. Taurine and hypotaurine are present in sperm cell and seminal plasma of numerous species [1] and have beneficial effects on sperm function [2]. Cat's tissues have a low cysteine sulfinate decarboxylase (CSN) and cysteine dioxygenase activity [3]. Consequently taurine is considered as an essential amino acid in cat [4]. In this study dosages of taurine and its precursors have been performed in spermatozoa and epididymal fluid of cats.

Materials and methods. *Sperm cell collection:* Motile spermatozoa were collected by flushing caudal epididymides. Testes and epididymides from sexually mature animals were obtained from Lyon Veterinary school, where cats were being castrated for behavioural disturbances. Tissues were transported to the laboratory in Earle medium at 30 °C and used within 1 h. The epididymides were placed in Earle medium at 37 °C in a Petri dishes, cleaned of irrelevant tissue and separated according to gross morphology into caput, corpus and cauda regions. Caudal epididymides were catheterized and flushed with Earle medium. Numeration and cell motility were assessed with a contrast phase microscope. After centrifugation cell pellets and supernatants were stored at –30 °C until use.

Dosage of taurine, hypotaurine and cysteamine: Elimination of proteins from the samples was performed by filtration (millipore UFC3 LGC 00). Amino acids were separated by ion exchange chromatography (Amino acid Analyzer Beckman 6300). Glucosaminic acid was used as an internal standard. Aminoacids

were detected by ninhydrin coloration and absorbance at 440 and 570 nm. In order to confirm identification of hypotaurine, aliquots of sperm extract and epididymal fluid were incubated with H_2O_2.

Results

Table 1. Taurine, hypotaurine and cysteamine concentrations (mean ± SEM) in spermatozoa (pM/10^6 cells), serum, and liquid of flushing of epididymides (nM/mL), (n = 4)

	Taurine	Hypotaurine	Cysteamine
Serum (nM/mL)	374.0 ± 73.6	Not detected	Not detected
Epididymide sperm cells	104.3 ± 30.7	891.6 ± 36.6	Not detected
Liqiud of flushing of caudal epididymides	998 ± 36.8	89.5 ± 8.9	Not detected

Conclusion. Hypotaurine and taurine are present at significant amounts in sperm cells, and epididymal fluid of cat. Hypotaurine has not been detected in serum. These results suggest that hypotaurine is synthetized by cat testis and/or epididymides. Cysteamine has never been detected in serum, sperm cell nor in epididymal fluid. This result suggests that the synthesis of hypotaurine in cat male genital tract is performed via the CSD pathway. Biosynthesis of hypotaurine by cat epididymal cells is under investigation.

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Taurine and pulmonary hemodynamics in sepsis

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Properties of taurine (TAU) and the reduction in plasma TAU found in sepsis have suggested a link between TAU deficiency and abnormal hemodynamics, but the relationship between TAU levels and hemodynamic patterns has never been assessed specifically. The study was carried out to perform such an assessment over a large group of measurements in a series of patients with post-traumatic sepsis.

Twohundredfortynine measurements of plasma TAU and other amino acids (AA) were obtained in 16 patients, with simultaneous measurements of cardiac index (CI) by the thermodilution method, mean blood pressure (MBP), pulmonary artery pressure (PAP) and respiratory index (RI). From these, systemic and pulmonary vascular resistance (SVRI, PVRI) and left and right cardiac work indices were obtained. Patients were receiving TPN with glucose (270 ± 70 mg/kg/h), fat (79 ± 48 mg/kg/h) and AA (59 ± 22 mg/kg/h) without exogenous TAU administration. Least-square regression statistical analysis was performed (Scheffé).

Significant inverse correlations were found between PAP or PVRI and TAU (r^2 = 0.28 and 0.19, respectively, $p < 0.001$), while MBP and SVRI were unrelated to TAU. Much weaker direct relationships were found between PAP or PVRI and other AA such as alanine, phenylalanine or tyrosine, and between CI and TAU ($r^2 \sim 0.10$, $p < 0.01$). PAP and PVRI were unrelated to AA clearances (except for TAU clearance, $r^2 = 0.33$, which reflected however only changes in plasma levels). Increasing PAP or PVRI, and decreasing TAU levels, tended to be correlated simultaneously with increasing RI ($r^2 = 0.14$ to 0.09, $p < 0.01$ for all) and worsening of respiratory dysfunction. In all cases requiring positive end-expiratory pressure (PEEP) greater than 10 cm H_2O, TAU level remained below 50 μM/L; plasma AA-grams in these cases showed that most AA levels tended to increase or to remain constant, contrary to the decrease found to TAU. TAU and PEEP together explained half of the variability of PAP.

This study provides evidence of a unique relationship binding together PAP and PVRI and plasma TAU levels in sepsis, and an association between decreasing plasma TAU and worsening of pulmonary dysfunction. More study is needed to relate these findings to vasoactive properties of TAU, to TAU-mediated protection against inflammatory processes rising PAP and PVRI, or to other mechanisms of co-variation of TAU and pulmonary hemodynamics.

Taurine in the motor nerve net of the jellyfish *Cyanea capillata*

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Two antisera against the sulfonated amino acid taurine were applied to subumbrella tissue of the jellyfish *Cyanea capillata*. Taurine-immunoreactive nerve nets were found in both the ectoderm and endoderm. The ectoderm had two morphologically and immunocytochemically distinct populations of neurons, the motor nerve net (MNN) which was immunoreactive to the taurine-like molecule, and the diffuse nerve net (DNN), which was immunoreactive to the neuropeptide Phe-Met-Arg-Phe-NH2 (FMRFamide). In the endoderm, immunoreactivity was found in the endodermal DNN. This localization was confirmed by double-labeling experiments, which also revealed that the endodermal DNN neurons may contain both taurine and FMRFamide-related peptide. Preliminary physiological experiments on epithelial-free preparations revealed uptake of ^{3}H-Taurine and release of detectable amounts of radioactivity during electrical stimulation of motorneurons. The results agree with earlier investigators finding an excitatory effect of taurine on jellyfish motorneurons in *Cyanea*.

Immunocytochemical localization of taurine in the fish retina under light and dark adaptations

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It is known that the retina contains an extremely high amount of taurine, which exhibits dramatic protective effects in photoreceptors exposed to light damage. As a most dominant osmolite, taurine may be released from photoreceptor outer segments by the light stimulation (Pasantes-Morales et al., 1973, 1998). We have previously found the lack of immunoactivity of taurine in the rod outer segments from the usually light-adapted fish such as the ayu *Plecoglossus altivelis,* lefteye flounder *Paralichthys olivaceus,* eel *Anguilla japonica* and goldfish *Carassius auratus* (Omura et al., 1997; Yoshimura et al., 1997; Inagaki and Omura, 1998). This finding prompted us to investigate the difference of immunocytochemical localization of taurine in the rod outer segments between the light- and dark-adapted states. In this study we used mainly the glass eel because of its rod-dominant retina, which exhibited untypical photomechanical movement.

Extremely intense immunostaining was found in the outer plexiform layer, the supranuclear region of photoreceptor cells, the cone outer segments and the rod inner segments. In the light-adapted state, the rod outer segments were not immunostained at all. In the dark-adapted state, while, plasma membrane of the rod outer segments was intensely immunostained. Similar difference in immunostaining was found also in the photoreceptor nuclei of the outer nuclear layer. Thus it was suggested that the lack of immunoreactivity in the rod outer segment may depend on the light adaptation and/or stimulation. Moreover, the distinctive immunocytochemical localization in photoreceptor cells suggested that taurine may be accumulated and/or synthesized in the supranuclear region and transported through the inner segment into the outer segment, being also involved in synaptic transmission in the outer plexiform layer.

(We are grateful to Prof. Manfred Ueck, Giessen, Germany, for generous suggestion, and to Prof. Norma Lake, Montreal, Canada, for sending the antibody.)

In vitro studies on the effect of taurine and glutamic acid on fowl sperm motility

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The aim of the study was to elucidate some details of those biochemical processes that might influence the motility of fowl spermatozoa *in vivo* in the hen's oviduct nearby the sperm storage tubules (SSTs). Taurine and glutamic acid – as special transmitter amino acids in the CNS – are known to be the most concentrated of the free amino acids in the oviductal fluid of hen during the egg formation cycle and their concentrations are independent from other amino acids taking part in protein synthesis.

Pooled, washed semen samples of White Leghorn roosters were used throughout the study. The analysis of motility was carried out objectively by videomicrograph. The addition of taurine to the washed fowl spermatozoa *in vitro,* within the range of 0–5 mM, did not appreciably affect the motility of either *intact* or *demembranated* spermatozoa. Motility was almost negligible at body temperature (40 °C) and maintained vigorous movement at room temperature (25 °C). Even in the presence of Ca^{2+} – one of the stimulators of sperm motility – before the addition of taurine, no stimulation or inhibition of motility was observed.

However, in the case of glutamic acid, as its concentration was increased from 0 to 5 mM, the motility of *intact* spermatozoa was reduced and inhibited both at 25 °C and 40 °C, even in the presence of Ca^{2+}, while the motility of *demembranated* spermatozoa was not inhibited.

The results suggest that glutamic acid, which affects presumably the plasma membrane and/or cytoplasmic matrix, can inhibit the motility of fowl spermatozoa. By this, it may have a role – among others – in the sperm storage mechanism as well, as it was demonstrated in high concentration in the environment of the SSTs. However, taurine – despite its highest concentration in the oviductal fluid – is not involved in fowl sperm motility. Its role in the reproductive processes of birds required further investigations.

Pool of free amino acids and their derivatives under conditions of excessive intake of taurine

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The intraperitoneal Tau administration in pharmacological active doses (1/10 LD50 per day) during 15 days leads to increase of nonessential amino acids concentrations (Thr, Lys) in blood plasma, increase of concentrations of Ala in the blood plasma and liver, activates an endogenous Tau syntheses in liver and practically does not change heart and skeleton muscles amino acid pool; activates glycolysis and utilization of amino acids carbon chains in tricarboxylic acid cycle in peripheral tissues and the whole brain. It also activates dopamine system in brain striatum and stem, changing a function of serotonin system in hypothalamus, including inhibition of both syntheses and degradation of the mediator.

The 15 days subconjunctival Tau administration in total amount of 1/10 LD50 causes an imbalance in the contents of both inhibitory and excitatory neurotransmitters in brain regions, as well as short-term increase of Trp concentration, however, practically not affecting on processes of formation of amino acids fund in peripheral tissues, that might be evidence of possibility of partial reproducing of the neurochemical effects of Tau under the given way of its administration.

Ionotropic Glutamate Receptors

Molecular mechanisms of alcohol action on NMDA receptors

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The actions of alcohol on the brain are mediated by its interaction with a variety of important neuronal receptors involved in cell signaling. Of particular importance is the finding that alcohol alters the function of several ionotropic receptors that are involved in shaping synaptic neurotransmission. Among neuronal receptors sensitive to alcohol are those activated by the excitatory amino acid neurotransmitter glutamate. Alcohol inhibits ion flux through both NMDA and non-NMDA receptors although there is considerable variability in the sensitivity of individual receptors to alcohol. Studies from this lab and others suggest that the alcohol sensitivity of NMDA receptors is influenced by the subunit makeup of the receptor as well as other factors which modulate receptor function via interaction with the C-terminus of NMDA subunits. For example, NMDA receptors containing the NR2A and/or NR2B subunits are generally more sensitive to inhibition to alcohol than those containing NR2C or NR2D receptors. In addition, the alcohol sensitivity of certain NMDA receptors is enhanced by a calcium-dependent process that requires the CO domain of the NR1 subunit. This modulation has characteristics that are similar to the calcium-dependent inactivation of NMDA receptors that is proposed to involve calmodulin and cytoskeletal clustering proteins. Finally, the alcohol sensitivity of certain NMDA receptors can be reduced by phosphorylation of NR2 subunits by Fyn tyrosine kinase. These results suggest that the alcohol sensitivity of NMDA receptors in brain neurons may vary in a dynamic fashion via interaction with key intracellular signaling processes.

[Supported by grants (AA09986 and AA00238) to JJW from the National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, USA.]

The role of NMDA receptors in the psychopharmacological responses to nicotine

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There is now substantial evidence that nicotine is the principal addictive component of tobacco smoke and that it has behavioural properties similar to those of other psychostimulant drugs of abuse. Both the locomotor stimulant properties of the drug and its ability to act as a reward in a self-administration schedule have been associated with stimulation of the dopamine (DA)-secreting neurones of the mesolimbic system which project from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc). Nicotine exerts its effects in the brain by acting on a family of neuronal nicotinic receptors. The stimulatory effects of systemic nicotine on mesolimbic neurones are mediated, predominantly at least, by receptors located on or close to the cell bodies in the VTA and the changes in DA release evoked by systemic nicotine reflect its effects on impulse flow to the terminal field.

The NAcc can be divided anatomically into two major subdivisions, the core and the shell, which probably subserve different functions. Stimulation of the DA projections to the shell of the NAcc are thought to mediate the rewarding properties of nicotine which reinforce its self-administration. The stimulatory effects of acute nicotine on DA release in this subdivision of the structure, measured using the technique of *in vivo* microdialysis, can be attenuated by drugs which block both nicotinic receptors and NMDA receptors for glutamate in the VTA. Thus, the response seems to involve the co-stimulation of both nicotinic and NMDA receptors expressed on the DA-secreting cells in this area of the brain. Stimulation of the NMDA receptors, expressed on these cells, preferentially increases the proportion of the cells exhibiting burst firing rather than influencing the overall firing rate of the cells. This conclusion is consistent with the dialysis results since enhanced burst firing of the neurones has a far greater effect on DA overflow in the terminal field than changes in the firing rate. The release of glutamate from nerve terminals is facilitated by the stimulation of a specific isoform of the nicotinic receptor, containing α_7 subunits, which are antagonised preferentially by methyllycaconitine. The administration of this antagonist also attenuates the effects of systemic nicotine on DA overflow in the NAcc shell, data which imply that the DA response to nicotine in depends upon co-stimulation of this isoform of the receptor located on glutamate terminals in the VTA.

The role of NMDA receptors in the regulation of the DA projections to the core of the NAcc is more complex. In drug-naïve rats, the prior injection of an NMDA antagonist enhances the effects of acute nicotine on DA overflow, measured using microdialysis. Repetitive daily injections of nicotine also cause sensitisation of its effects on DA overflow in this area of the NAcc. However, the administration of NMDA antagonists either during the pretreatment phase of the experiments or on the test day attenuate expression of the sensitised responses. These data suggest that both the development and expression of the sensitised DA responses depend upon co-stimulation of NMDA receptors. The core of the NAcc sends major projections to areas of the brain concerned with locomotor activity and it has been suggested that increased DA release in this area of the NAcc mediates the locomotor stimulant properties of psychostimulant drugs. Behavioural studies in these animals, however, suggest that the sensitised locomotor response, also observed in rats challenged with nicotine following pretreatment with daily injections of the drug, are not directly related to the enhanced DA overflow observed in the rats. The presentation will focus on an alternative hypothesis which proposes that the primary role of burst firing is increase the release of DA into an extra-synaptic compartment where it gains access to DA receptors, which subserve a different function, perhaps more closely related to the development of nicotine dependence.

(Results form the author's laboratory, discussed in the presentation, were obtained from studies supported by the VERUM Foundation and the Wellcome Trust.)

Ethanol inhibits NMDA-evoked release of various neurotransmitters at different potencies potentially related to NMDA receptor subunit assembly

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Rat brain cortex and striatum slices labeled with tritiated neurotransmitters and superfused with Krebs' buffer were used to study the effect of ethanol on N-Methyl-D-aspartate (NMDA)-evoked neurotransmitter release. We compared the effect of ethanol with ifenprodil, which has been shown in voltage-clamp experiments to inhibit NMDA-induced currents in recombinant NR1A/NR2B receptors at 4.5 times higher potency than NR1A/NR2A receptors [Williams (1993) Mol Pharmacol 44: 851–859] to yield functional evidence for different NDMA receptor subunit assembly in nerve terminals of different neurons or in different brain regions.

The NMDA-evoked release of the neurotransmitters [^{3}H]noradrenaline, [^{3}H]5-hydroxytryptamine, [^{3}H]GABA, [^{3}H]acetylcholine and [^{3}H]dopamine was concentration-dependently inhibited by ethanol. However, the inhibitory potency on [^{3}H]acetylcholine and [^{3}H]dopamine release (IC_{50} = 190 and 234 mM, respectively) in striatum was significantly lower than on [^{3}H]noradrenaline, [^{3}H]5-hydroxytryptamine, [^{3}H]GABA release (IC_{50} = 44, 51 and 72 mM, respectively) in the cortex. Ifenprodil inhibited the NMDA-evoked release of these neurotransmitters at a very similar potency order as compared to ethanol and the potencies of ifenprodil in inhibiting NMDA-evoked neurotransmitter release correlated well with those of ethanol ($r = 0.96$; $p < 0.001$). No differences in ethanol IC_{50} values were found for NMDA-evoked [^{3}H]noradrenaline and [^{3}H]GABA release between cortex and striatum. Whereas ethanol inhibition was complete (except for [^{3}H]acetylcholine and [^{3}H]dopamine whose concentration-response curves were cut off at 320 mM), maximum inhibition by ifenprodil was about 40%. In the presence of 100 nM ifenprodil the inhibitory potency of ethanol on NMDA-evoked release of [^{3}H]noradrenaline in the cortex was reduced from 44 to 266 mM but it did not affect the inhibitory potency of ethanol on dopamin- and acetylcholine release in striatal slices.

It is concluded that ethanol inhibits predominantly NR2B containing NMDA receptors which appear to be involved in the release of noradrenaline, 5-hydroxytryptamine, GABA and that ifenprodil could influence neuronal effects of ethanol *in vivo*.

The role of the NMDA receptor in ethanol withdrawal

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Ethanol, acutely, has been found to be a potent inhibitor of the function of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor in various neuronal preparations. Following chronic treatment of animals with alcohol to produce tolerance and physical dependence, the NMDA receptor is up-regulated in many brain areas. This adaptive change has been demonstrated with ligand binding studies and by quantitative immunoblotting of NMDA receptor subunit proteins, which are increased in a subunit- and brain region-specific manner. The increase in

NMDA receptor subunit protein levels occurs without corresponding changes in the respective steady-state mRNA levels. The up-regulation of the NMDA receptor appears to contribute to the occurrence of ethanol withdrawal seizures. The time course for these seizures is paralleled by the time course of receptor up-regulation, and prevention of NMDA receptor up-regulation by treatment of animals with gangliosides, concurrently with ethanol, results in reduced severity of withdrawal seizures. Chronic ethanol exposure of primary cultures of cerebellar granule neurons also results in an up-regulation of NMDA receptor number and function. This change produces increased susceptibility of the neurons to glutamate toxicity, and current studies indicate that repeated ethanol withdrawal episodes (but not chronic ethanol treatment *per se*) in mice also results in neuronal death. Thus, the up-regulation of the NMDA receptor following chronic exposure of animals (or humans) to ethanol may not only generate withdrawal seizures, but may also play a role in producing the neuronal damage seen in chronic alcoholics. This concept provides a basis for developing novel therapeutic approaches to not only reduce ethanol withdrawal signs, but also attenuate withdrawal-induced neuronal damage.

NMDA receptor subunit expression and function: Effects of ethanol

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Developmental changes in mRNA expression encoding NMDA receptor (NR) subunits and ethanol sensitivity of NR were investigated in primary cultures of mesencephalic (MES) neurones prepared from rats embryonic day 14. NR subunit expression and free intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were determined using RT-PCR and single-cell fura-2 microfluorimetry, respectively.

Total/mRNA isolation and RT-PCR were carried out according to standard protocols. Amplification products were analyzed by hybridization with digoxigenin-labelled DNA probes and CSPD chemiluminescent detection. Primer pairs flanking the alternatively spliced regions at the amino and carboxy terminus of NR1 were used, respectively, to estimate the ratios of the corresponding 3' and 5' splice variants. Amplification of the NR subunit 2A, 2B, and 2C cDNA fragments was performed with primer pairs reported by Audinat et al. [Eur J Neurosci (1994) 6: 1792].

NR1 subunit expression did not reveal significant differences in MES neurones kept 8, 10, or 14 day in vitro (DIV). NR1–4a was predominantly expressed at each stage. In cultures 8 DIV, the relative amounts of NR subunits 2A, 2B, and 2C corresponded to a ratio of approximately 1:2:1. The NR2C proportion increased with time, whereas both 2A and 2B decreased accordingly. NMDA (plus 10 μM glycine) induced a concentration-dependent increase in $[Ca^{2+}]_i$ increasing from DIV 8 to 14. Inhibition of the NMDA-induced enhancement caused by ethanol and ifenprodil, a preferential non-competitive NR1/2B receptor antagonist, decreased during this time interval from 55 to 18% and 75 to 49%, respectively.

In conclusion, the present data reveal developmental changes in NR subunit expression in MES neurones which are associated with an increase in NR function and a decrease in ethanol sensitivity.

Glutamate increase in the retina induced by retinal ischemia

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We observed prominent increase of glutamate in the vitreous space during reperfusion after 60 minutes ischemia in mammalian eyes. This excess amount of glutamate resulted in neural cell death in the retina, proven functionally and histologically. Succeedingly, our studies, employing cultured amacrine cells from embryonic rat eyes, also came to the same conclusion that ischemic changes in neural retina are induced by an increase in extracellular glutamate. Among the glutamate analogs, NMDA is responsive to this change in the retina. An influx of calcium through NMDA receptor channels activates nitric oxide synthase, inducing intracellular nitric oxide in selected (not all) amacrine cells. Nitric oxide reacts with free radicals in the cell and induces peroxinitrite, which is really toxic. This cascade triggered by ischemia is interrupted by extracellular zinc, magnesium, hemoglobin, and some NMDA antagonists. In terms of clinical application, there is a possibility that dihydroxyphenylalanine, superoxide dismutase, and catalase, as well as vitamins B6 and B12, are important candidates for administration before an ischemic attack for prevention of damage to the retinal neurons. Gene expression of nitric oxide synthase, IL-1, IL-6, TNF, TGF-beta in the ischemic retina was investigated in order to discover reaction substances common to ischemic change and inflammation.

Predominant expression of NMDA receptor subunit 2C in rat locus coeruleus neurones

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The expression pattern of mRNA encoding NMDA (N-methyl-D-aspartate) receptor subunits 2A, 2B, and 2C was examined by RT-PCR in single locus coeruleus neurones of Wistar rats, postnatal day 12–15, as well as in the locus coeruleus tissue of adult animals.

Noradrenergic neurones of the locus coeruleus were identified by noradrenaline (100 μM)-induced currents using the whole cell configuration of the patch-clamp technique. Subsequent to electrophysiological characterization by recording NMDA (100 μM)-activated current, the cytosol of the neurone was harvested and transferred into a PCR tube containing dNTP/pd$(N)_6$, DTT, and RNAse inhibitor. RT was carried out with Superscript™ II. Co-amplification of the 2A-C cDNA fragments was performed by nested hot-start PCR [Audinat et al. (1994) Eur J Neurosci 6: 1792; Flint et al. (1997) J Neurosci 17: 2469]. The PCR products were quantitatively analysed by slot-blot hybridization with digoxigenin-labelled DNA probes and chemiluminescence detection.

Nine out of 14 cells investigated exhibited co-expression of mRNA of each subunit, in which the amount of 2C largely exceeded those of 2A and 2B [A+/B+/C+ cells; expression values in % of total expression: 16 ± 3 (2A), 8 ± 2.9 (2B), and 76 ± 5 (2C)]. In three cells, subunits 2A (31 ± 14%) and 2B (69 ± 14%) but not 2C were detectable (A+/B+ cells). One cell each expressed only 2A (A+ cell) or 2B (B+ cell). The corresponding NMDA-induced currents (pA) measured were: –345 ± 65 (A+/B+/C+ cells), –145 ± 71 (A+/B+ cells), –60 (A+ cell), –122 (B+ cell). In the locus coeruleus tissue of adult animals the expression level of subunit 2C (51.9 ± 7%) exceeded those of 2A (32.3 ± 2%) and 2B (15.8 ± 6%).

In conclusion, the present data show that rat locus coeruleus neurones are heterogeneous with respect to their NMDA receptor subunit expression. Subunit 2C predominates in most neurones over 2A and 2B which appears associated with an increase in NMDA receptor function.

Role for tyrosine kinases and MAP kinase pathway in the modulation of glutamate release from hippocampal nerve terminals

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The mitogen activated protein kinase (MAPK) pathway has long been associated with the process of cell differentiation and long-term synaptic plasticity. However, recent reports have implicated the MAPK cascade and protein tyrosine kinases (PTK) in the modulation of short-term synaptic events, such as neurotransmitter release. In this study we investigated the role of PTK and of the MAPK cascade on glutamate release from hippocampal nerve endings. Genistein, a broad spectrum inhibitor of tyrosine kinases, inhibited the $[Ca^{2+}]_i$ response to 15 mM KCl and concomitantly decreased glutamate release evoked by the same stimulus, in a dose-dependent manner. Genistin, an inactive analog of genistein, did not affect significantly the KCl-evoked responses. In order to determine which Ca^{2+} channels were affected by PTK inhibition, we studied the effect of genistein on the $[Ca^{2+}]_i$ responses to KCl, in the presence and in the absence of specific blockers of voltage-gated Ca^{2+} channels (VGCC). Genistein showed a partially additive effect with the P/Q-type VGCC inhibitor ω-agatoxin IVA (ω-Aga IVA), and with the N-type VGCC blocker ω-Conotoxin GVIA (ω-CgTx GVIA), in inhibiting the release of glutamate and the $[Ca^{2+}]_i$ response to 15 mM KCl. Glutamate release evoked by 5 μM ionomycin was also inhibited by genistein, indicating that PTK also control glutamate release downstream of Ca^{2+} influx. Since the MAPK pathway can be activated by Ca^{2+} influx and following PTK activation, we investigated whether this cascade is involved in glutamate exocytosis. The MAPK kinase (MEK) inhibitor 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD098,059), decreased the release of glutamate evoked by either 15 mM KCl or 5 μM ionomycin. The $[Ca^{2+}]_i$ response to 15 mM KCl was also reduced by PD098,059, and this effect was partially additive with the inhibition of Ca^{2+} influx by blockers of the P/Q-type (ω-Aga IVA) and of the N-type (ω-CgTx GVIA) VGCC. PD098,059 further inhibited the $[Ca^{2+}]_i$ rise in the presence of L-, N- and P/Q-type VGCC inhibitors, as compared to the effect of all VGCC inhibitors in the absence of the MEK inhibitor. Immunoblot experiments, using an antibody that specifically recognizes the phosphorylated (active) form of MAPK, have shown that MAPK is active under resting conditions, and that its activation state is slightly increased following KCl depolarization. In the presence of PD098,059 the levels of active MAPK were significantly reduced. Our results suggest a role for both PTK and for the MAPK cascade in the modulation of glutamate release from hippocampal nerve endings, by acting upon calcium influx through P/Q-, N- and through non-characterized VGCC, and on the exocytotic machinery.

(Supported by PRAXIS XXI.)

Effect of endogenous glutamate on extracellular concentrations of taurine in striatum and nucleus accumbens of the awake rat: Involvement of NMDA and AMPA/kainate receptors

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The aim of the present study was to investigate the effects of endogenous glutamate on extracellular concentrations of taurine in striatum and nucleus accumbens of the awake rat. The glutamate uptake inhibitor L-*trans*-pyrrolidine-2,4-dicarbocylic acid (PDC) was used to increase the extracellular concentration of glutamate. Microdialysis experiments were performed at a flow rate of 2.5 μl/min. The amino acid content of 15-min dialysates was measured with HPLC coupled to a fluorometric detector. The perfusion of PDC (1, 2 and 4 mM) produced a dose-related increase of extracellular concentrations of glutamate and taurine in striatum and nucleus accumbens. PDC (4 mM) increased dialysate concentrations of glutamate from 1.07 ± 0.28 to 5.39 ± 0.70 μM and taurine from 1.08 ± 0.11 to 3.01 ± 0.32 μM in striatum, increasing glutamate from 0.29 ± 0.05 to 4.06 ± 0.41 μM and taurine from 0.65 ± 0.08 to 2.19 ± 0.23 μM in nucleus accumbens. Increases of extracellular taurine were significantly correlated with increases of extracellular glutamate, but not with PDC doses, which suggests that endogenous glutamate produced the increases of extracellular taurine in striatum and nucleus accumbens. The role of ionotropic glutamate receptors on the increases of taurine was also studied. In striatum, perfusion of antagonists of NMDA and AMPA/kainate glutamate receptors attenuated the increases of extracellular taurine. AMPA/kainate, but not NMDA receptors, also reduced the increases of extracellular taurine in nucleus accumbens. The results presented in this study show that glutamate-taurine interactions exist in striatum and nucleus accumbens of the awake rat.

New beta-hydroxyaspartate derivatives as glutamate transporter blockers

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Glutamate/aspartate transporters play important roles in maintaining the extracellular glutamate concentrations and in terminating excitatory neurotransmission. Inhibitors are essential for elucidating of the intrinsic properties and physiological roles of transporters. However, most of the known inhibitors are competitive substrates and induce transport currents. Therefore, blocker-type (non-transportable) inhibitors have been strongly required. Recently, we synthesised *threo*-β-benzyloxyaspartate (TBOA), a novel derivative of *threo*-β-hydroxyaspartate (THA), as a potent blocker of glutamate transporters.

TBOA inhibited the uptake of [^{14}C]glutamate in COS-1 cells expressing the human excitatory amino acid transporter (EAAT1, EAAT2 or EAAT3). The inhibitory effect of TBOA was much more potent than dihydrokainate and (2S,4R)-4-methylglutamate which are known as selective blockers of EAAT2. Electrophysiologically, TBOA induced no detectable inward currents in *Xenopus* oocytes expressing human EAAT1, EAAT2, or EAAT3. However, it significantly reduced the glutamate-induced currents, indicating the prevention of transport. The dose-response curve of glutamate was shifted by adding TBOA without a significant change in the maximum current. TBOA is, so far, the most potent competitive blocker of glutamate transporters.

L-glutamate is a high affinity substrate of the transporters whereas D-glutamate is poorly transported. On the other hand, both isomers of aspartate are known to be excellent substrates. In order to elucidate the stereoselectivity, we synthesized pure stereoisomers of TBOA and *erythro*-isomers. Among them L-*threo* isomer (L-TBOA) was most potent in each EAAT subtype, although all isomers acted as blockers.

Furthermore, TBOA did not show any significant effects on either the ionotropic or metabotropic glutamate receptors.

Therefore, TBOA should be a useful tool for investigating the physiological roles of transporters.

Developmental expression of the glutamate transporter EAAT-1, in the mouse retina

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Glutamate is the predominant excitatory neurotransmitter in the mammalian retina. Termination of glutamatergic synaptic transmission depends on rapid uptake of the released glutamate by a group of homologous proteins known as high-affinity glutamate transporters. Although the sites of expression of different glutamate transporter subtypes in the adult mammalian retina are known, information about the expression patterns of these transporters during development is lacking. We have determined the spatial and temporal expression pattern of the glutamate transporter, EAAT-1, in the developing eye of the C57/B6 mouse using *in situ* hybridization and immunocytochemistry.

In situ hybridization studies revealed high level expression of EAAT-1 mRNA in astrocytes before birth. In the retina, however, maximal levels of EAAT-1 mRNA were seen at P16. Subsequently, there was a reduction in EAAT-1 mRNA level. In the postnatal and adult retinas, EAAT-1 mRNA was found only in the Müller cells and astrocytes. In immunohistochemical studies, the first immunostaining of EAAT-1 protein was detected in E16 retinas, and the optic nerve showed the strongest immunostaining at this stage. In the postnatal retina, EAAT-1 immunostaining peaked around P15 and declined later.

In summary, our results suggest that the expression of EAAT-1 is down-regulated as the retina matures and the highest level of EAAT-1 expression coincides with the time of Müller cell maturation in the retina.

NMDA receptor blockade accelerates gastric emptying

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Rats increase meal size following administration of MK-801, a non-competitive NMDA receptor antagonist. MK-801 might enhance intake by accelerating the emptying of ingesta from the stomach, thereby delaying the onset of gastric satiation. We performed three related experiments to test this hypothesis. First, we measured the effect of MK-801 (100 μg/kg, IP) on 10-min gastric emptying of 15% sucrose or 0.9% NaCl. MK-801 significantly ($p < 0.05$) increased gastric emptying of NaCl (4.72 ± 0.4 ml) and sucrose (4.76 ± 0.5 ml) compared to vehicle injection (3.54 ± 0.3 ml and 2.93 ± 0.23 ml, respectively). Second, we examined the role of MK-801 on gastric emptying after either a small fixed volume (6 ml) or a larger *ad libitum* volume (~15 ml) of 15% sucrose. MK-801 accelerated emptying regardless of the volume ingested. Finally, we measured intake of 15% sucrose or 5 mM saccharin/0.1 M glucose in rats implanted with Silastic cuffs designed to reversibly occlude the pylorus. MK-801 significantly increased 30-min intake of sucrose if the pylorus was open, but not when it was closed. MK-801 had no effect on intake of the saccharin/glucose in either the pylorus open or closed condition. These results indicate that MK-801 enhances gastric emptying of either hypertonic, nutrient-rich sucrose or isotonic non-nutritive saline. Stomach distention does not seem to have a major role in MK-induced increase in intake. Rather, MK-801 increases food intake by accelerating gastric emptying, thereby reducing gastric mechanoreceptive satiety signals.

Regulation of growth hormone (GH) secretion by different glutamate receptor subtypes in the rat

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In recent years, it has been firmly established that glutamatergic pathways are a pivotal element in the hypothalamic circuitry involved in the control of pituitary function, and it is now well proven that the secretion of several anterior pituitary hormones is regulated by the excitatory amino acid (EAA) system. The actions of EEAs are mediated by different postsynaptic receptor subtypes, which include N-methyl-D-aspartate (NMDA), kainate (KA), 2-amino-3-hydroxy-5 methyl-4-isoxazol propionic acid (AMPA) and metabotropic receptors. In order to delineate the role of EAA neurotransmission in the control of GH secretion in the rat, we have characterized the effects of agonists and antagonist of glutamate receptors on GH release. Activation of NMDA, KA and AMPA receptors at different age-points resulted in clear-cut stimulation of GH secretion. However, age- and sex-dependent differences were detected in the pattern of response to the different agonists, as the ability of KA, but not of NMDA and AMPA, to stimulate GH release disappeared in the adulthood, and their relative potency was different between male and female rats. The stimulatory action of different glutamate receptor agonists was proven nitric oxide (NO)-dependent and not exerted at the pituitary level. In addition, the role of hypothalamic GH-releasing hormone (GHRH) in the stimulatory action of NMDA was evaluated. Immunoneutralization against endogenous GHRH or destruction of GHRH producing neurons did not result in complete elimination of NMDA-induced GH release, suggesting the involvement of signals other than GHRH in this response. Further, evidence was obtained on the modulation of the EAA system by gonadal factors in the control of GH secretion. As examples, gonadectomy increased KA-stimulated GH secretion in males and AMPA-induced GH release in females whereas neonatal estradiol treatment permanently impaired the ability of KA to elicit GH secretion in male rats. Finally, the physiological relevance of the EEA system in the control of GH secretion was assessed. Antagonization of NMDA receptors abolished pulsatile GH secretion whereas administration of antagonists of AMPA receptors decreased the amplitude of GH pulses. In conclusion, our data using the rat as animal model provide evidence for a relevant role of glutamate pathways in the control of GH secretion throughout the life-span.

Selective ligands for excitatory amino acid receptors

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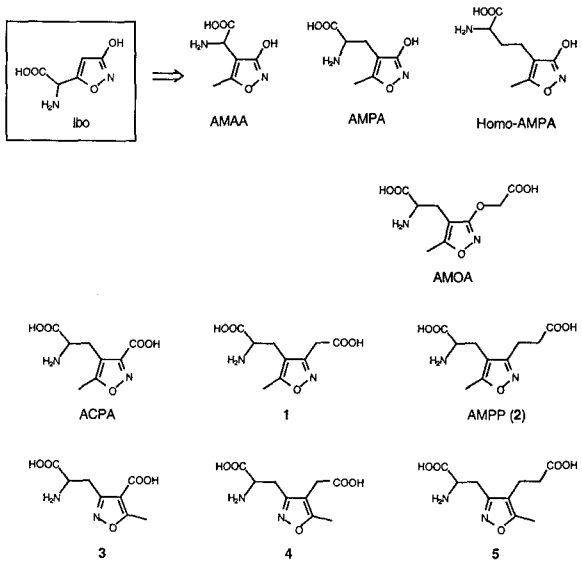
Receptors for excitatory amino acids (EAAs) are involved in the development of a number of acute and chronic neurologic disorders. Thus, selective ligands for EAA receptors are desired for pharmacological characterization and as potential therapeutic agents. EAA receptors are divided into two main classes, ionotropic and metabotropic receptors, which are both further subdivided into three subclasses. The ionotropic receptors mediate fast excitatory signals through NMDA, AMPA and kainic acid receptors, whereas metabotropic receptors mediate signals by production of second messengers through Group I, II and III receptors. A number of selective ligands have been developed using Ibotenic acid (Ibo) as a lead structure. The compounds AMAA, AMPA and Homo-AMPA show potent and selective activity at NMDA, AMPA and mGluR6 receptors, respectively. Further modification of the AMPA structure has lead to another even more potent AMPA agonist ACPA and to the AMPA antagonist AMOA.



ACPA and AMOA have formed the basis for the development of a series of compounds as tools for investigation of the structural requirements for AMPA agonists versus antagonists. The synthesis and pharmacology of compounds **1–5** will be described. These five structures cover activities ranging from inactivity, AMPA agonism to AMPA and NMDA antagonism.

Cerebral organic acid disorders induce neuronal damage via excitotoxic organic acids *in vitro*

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Introduction: We hypothesized that the age- and regional-specific brain damage observed in glutaryl-CoA dehydrogenase deficiency (GDD), which is one of the most frequent cerebral organic acid disorders, could be caused by overstimulation of NMDA receptors through the marker metabolites glutaric (GA) and 3-hydroxyglutaric (3-OH-GA) acids.

Material and methods: Primary neuronal cultures from chick embryo telencephalons were prepared as previously described [Pettmann B et al. (1979) Nature 281: 378–380]. Cultured neurons were incubated with 0.1–5.0 mM GA or 3-OH-GA together with different glutamate receptor antagonists (MK-801, ifenprodil, CNQX, L-AP3, L-NAME) and antibodies (polyclonal NR2A and NR2B antibodies) for 1–24 h. Cell viability was determined by trypan-blue exclusion method and LDH release. The expression of the NMDA receptor subunits NR2A and NR2B were determined by SDS-PAGE and Western blotting.

Results: 3-OH-GA and GA decreased cell viability concentration- and time-dependently. Only preincubation with MK-801, ifenprodil and NR2B antibodies totally prevented 3-OH-GA- and GA-induced neurotoxicity, whereas CNQX, L-AP3, L-NAME and NR2A antibodies failed to exert an protective effect. Expression of NR2B together with susceptibility to 3-OH-GA- and GA-induced neuronal damage increased from 2nd to 6th DIV.

Conclusions: The organic acids GA and 3-OH-GA induce neuronal damage through NMDA receptors (in particular the NR1/2B subtype), acting as false neurotransmitters. We concluded from our findings that the age- and regional-specific neuropathology in GDD is related to a high expression of the NR2B subunit in forebrain areas during a specific vulnerable period of brain development.

Novel and specific kynurenine pathway enzyme inhibitors from marine natural products

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Kynurenine, a metabolite of tryptophan along the "kynurenine pathway", is at a branch point of the pathway which can lead to the synthesis of both quinolinic acid (QUIN) and kynurenic acid (KYNA). These two metabolites interact with the *N*-methyl-D-aspartate (NMDA) receptors in a functionally opposite manner. KYNA is a non-selective antagonist of glutamate receptors, however QUIN is a selective agonist of NMDA receptors, and has been shown to act as a excitotoxic agent. High QUIN/KYNA ratio has been implicated in a variety of neurological diseases in which excitotoxic neuronal cell death is found e.g., AIDS related dementia, cerebral malaria, etc. Low QUIN/KYNA ratio may be achieved by inhibiting the key enzymes of this pathway (i.e. kynureninase and kynurenine 3-hydroxylase). This would potentially have neuroprotective effects. We have developed high throughput assays for kynurenine aminotransferase, kynureninase and kynurenine-3-hydroxylase which allows us to screen extracts from marine organisms for selective enzyme inhibitors. Active metabolites are purified, isolated and identified by HPLC, high-field NMR and mass spectral techniques. Extracts from a sponge of the *Aka* species were found to contain an inhibitor of kynureninase. We have recently purified and identified the active principle as being serotonin sulfate, and shown it to be a selective inhibitor of kynureninase, serotonin and 5-hydroxyindoleacetic acid being inactive. This finding may be suggestive of a novel interaction between the serotoninergic and excitatory amino acid pathways.

Homocysteine

Redox regulation at the homocysteine metabolic junction

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Homocysteine is a sulfur containing amino acid that is formed by the hydrolysis of S-adenosylhomocysteine, the spent form of S-adenosylmethionine, the major cellular methyl group donor. Elevated levels of homocysteine are correlated with two apparently unconnected pathologies: neural tube defects and atherosclerotic diseases. The two major metabolic avenues for the removal of homocysteine in mammalian cells are transmethylation and transsulfuration. The former is catalyzed by methionine synthase and by betaine homocysteine methyltrans-

ferase, but the latter enzyme has limited tissue distribution and is confined to the liver and kidney. Transsulfuration is catalyzed by cystathionine b-synthase and commits homocysteine to degradation and its ultimate removal as sulfate. However, one of the intermediates in this pathway is cysteine, precursor for glutathione, the major cellular redox buffer. Both methionine synthase and cystathionine b-synthase have redox active cofactors, cobalamin in the former, and heme in the latter. Studies from our laboratory have revealed that the activity of both enzymes is sensitive to the ambient redox potential under *in vitro* conditions. Thus, methionine synthase is less active under oxidizing conditions whereas the activity of cystathionine b-synthase is enhanced. Results from both *in vitro* and *in vivo* investigations on the role of redox on homocysteine metabolism will be presented.

Human betaine-homocysteine S-methyltransferase (BHMT): Characterization of the gene, nutrient modulation of its expression, and some properties of the recombinant enzyme

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BHMT (EC 2.1.1.5) is a cytosolic enzyme that catalyzes a methyl transfer from betaine to homocysteine forming dimethylglycine and methionine, respectively. BHMT is only expressed at significant levels in the kidney and liver. In 1996 we cloned a human liver cDNA encoding BHMT and have since focused on characterizing the human gene, characterizing the influence nutrition has on rat liver BHMT gene expression, and characterizing the properties of the recombinant human enzyme. The human BHMT gene spans 20 kilobases of DNA on chromosome 5q13.1-15, is composed of 8 exons, and all intron-exon junctions follow the GT-AG rule. Only one transcriptional start site has been found in liver, and we have functionally characterized the 5'-flanking region of BHMT for regions that confer promoter activity. Using various portions of the 5'-flanking region of the human BHMT gene fused to firefly luciferase, and transfecting these constructs into human hepatoma cells, we have identified the region between -202 and -1, relative to the transcriptional start site, as the only region that confers promoter activity. This region contains a TATA box centered 26 bp upstream of the transcriptional start site. Nutrition studies in rats have shown that methionine restriction causes a large induction of hepatic BHMT gene expression. The level of induction is proportional to both the level of choline in the diet and the severity of methionine deficiency. The mechanism responsible for the diet-induced changes in hepatic BHMT expression is currently under investigation. Using the recombinant human enzyme as a model, we have determined that BHMT is a zinc metalloenzyme. Site-directed mutagenesis studies indicate that Cys 217, Cys 299, and Cys 300 are zinc ligands. Homology searching indicates that the two regions of amino acid sequence flanking these essential Cys residues define a new family of zinc-dependent methyltransferases that use thiols or selenols as methyl acceptors.

Quantification of S-adenosylmethionine in whole-blood and plasma and its relation to homocysteine and other methionine metabolites

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S-adenosylmethionine (SAM) serves as the most important donor of methyl-groups in mammalian methylation processes and plays a key role in the regulation of the methionine metabolism, including transmethylation and transsulfuration pathways. Homocysteine (Hcys), the metabolic product of SAM, has attracted growing clinical interest over the last decade since even mildly elevated plasma levels are associated with an increased risk of atherosclerosis and vascular diseases. Despite intensive studies, however, the mechanisms of endothelial damage involving Hcys remain still unclear. The *in vivo* quantitative analysis of compounds important to methionine metabolism, their distribution in blood and tissue as well as their interrelations should provide more insight to elucidate the processes underlying premature atherogenesis. We present a new, rapid and highly selective high-performance liquid chromatographic (HPLC) method which allows the sensitive quantification of even very low SAM levels in whole-blood and in plasma. Plasma SAM levels of patients with stroke and controls were measured and compared to Hcys and folate levels with special regard to the presence of a common mutation (C677T) in the gene encoding for 5,10-methyltetrahydrofolate-reductase *(MTHFR)*. A correlation between low plasma SAM levels, low folate levels and high total plasma Hcys could be found in those patients homocygous for the *MTHFR*-mutation. As SAM is essential for the formation of taurine and glutathione, a low methylation potential in these subjects indicates a decreased antioxidative protection, aggravating the prooxidative potential of Hcys and thus inducing endothelial and neuropathic damage. Further investigations into the multiple determinants of the methionine metabolism and their distribution will soon be accomplished to provide a more complete overview of their complex interrelations and the crosslinks to vascular and neurodegenerative processes as well as the biochemical basis of possible prevention strategies.

Inhibition of serine hydroxymethyltransferase and cystathionine β-synthase with hydroxynorvaline induces neural tube defects in chicken embryos

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Introduction: Neural tube defects (NTD) in humans have a multifactioral aetiology. Some deficiencies in the folate metabolism have been positively linked to NTD. One-carbon units, utilized for the synthesis of biomethylating agents and DNA precursors, as well as for the methylation of homocysteine to methionine are mainly derived from L-serine, the natural substrate of both serine hydroxymethyltransferase (SHMT) and cystathionine β-synthase (CBS). We investigated the hypothesis that a deficiency in the activity of SHMT and/or CBS may be responsible for NTD observed in the chicken embryos and probably also in humans.

Experimental: Developing embryos (day 2: post-incubation) were exposed to hydroxynorvaline (HNV), a potential serine/threonine antimetabolite. Controls received sterile saline. Embryos were harvested on day 12 and investigated for congenital abnormalities. Liver SHMT and CBS activities were determined by means of modified radiometric assays. Chicken embryo fibroblasts (CEF) were treated with HNV in culture and DNA synthesis monitored with a fluorescence assay.

Results: A dose responsive incidence (13–18%) of NTD was observed in the HNF-treated embryos. Control embryos were all normal. Both SHMT and CBS appeared to be inhibited in liver extracts of HNV-treated embryos, while DNA synthesis was potently inhibited by HNV (K_I ~30 μM) in cultured CEF.

Conclusion: Inhibition of SHMT activity in the chicken embryo model mimicked an enzyme and resultant folate deficiency and subsequently caused the induction of NTD. The primary mechanism of this effect is probably associated with a restriction in the synthesis of DNA precursors.

Lowering of homocysteine concentrations in the general population: Is a combined B-vitamin supplementation superior to single vitamin administration and what doses are needed?

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Elevated plasma total homocysteine (tHcy) concentrations are considered to be a risk factor for vascular disease and fetal malformations such as neural tube defects. Recently, hyperhomocysteinemia has also been linked to cognitive disorders in the elderly. Supplementation with folic acid, vitamin B12 and B6 has previously been applied to lower tHcy. Most studies used high pharmacologic vitamin doses, were uncontrolled trials, or explored the tHcy-lowering properties of vitamins in certain population groups only. However, high-dose vitamin uptake may not be a suitable public health measure for the general population.

We aimed to elucidate the tHcy-lowering effect of low-dose B-vitamin administration, i.e. in the range of current recommended dietary allowances, to several groups of the general population (total $n = 615$). It was investigated if folic acid is a key factor with regard to a reduction in tHcy, or if synergistic effects occur in the presence of vitamin B12 and/or vitamin B6 together with folic acid.

In women of childbearing age it was shown that single folic acid supplementation was significantly less effective in lowering the tHcy concentration than combinations of folic acid and vitamin B6 or B12, or all three vitamins together. Consequently, a B-vitamin combination was used to supplement men and women of middle and old age. The observed mean relative reductions in tHcy after combined B-vitamin administration using similar vitamin doses varied between 12% and 16% in the supplemented groups. The extent of tHcy reduction was clearly dependent on the tHcy and plasma folate levels before treatment in all studies, with initially low plasma folate and high tHcy concentrations resulting in larger tHcy reductions after treatment.

Thus, for primary disease prevention in population groups of young and middle age, low-dose supplementation with a combination of folic acid, vitamin B12 and B6 may be recommended. In the elderly, the dose of vitamin B12 should be increased to a pharmacologic dose to counter the higher prevalence of an impaired vitamin B12 status in this age group and to ensure intracellular adequacy of both cofactors needed for an increased activity of methionine synthase. Further studies should elucidate the oral vitamin B12 dose required. However, results of currently ongoing intervention studies using vitamin supplementation to lower the risk of vascular disease have to be awated to justify large-scale public health measures in the general population. Current evidence on the role of tHcy in the etiology of vascular and other diseases, however, allows the supposition of a preventive role of increased uptake of folic acid, vitamin B12 and B6.

Glutathione/homocysteine ratio used as a marker for the assessment of coronary heart diseases

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Glutathione (GSH) is the primary intracellular antioxidant in humans. Oxidative cellular injury is postulated to be centrally involved in diverse processes including aging, cancer, and cardiovascular disease. Homocysteine (Hcys) concentrations have been associated with premature arterial disease in the North American and European Caucasian populations. Recently, it has been shown that elevated Hcys levels are an independent risk factor for atherosclerotic cardiovascular disease not related with hyperlipidemia, hypertension, diabetes, and smoking. In this study, we measured the glutathione (GSH)/Hcys ratio and plasma total Hcys concentrations in seventy-two Hispanic patients whom were hospitalized for coronary angiography. Results indicate that the mean plasma Hcys value in our patients with cardiovascular disease is lower (10.2 ± 0.4 μM) that other population reported in the US. Thus, this might be a possible explanation for the lower incidence of coronary disease in PR when compared with continental US. However, within our population, plasma total Hcys concentrations did not correlate with heart condition as measured by coronary angiography (normal = 10.9 μM, mild = 8.7 μM, moderate = 10.9 μM, severe = 10.0 μM; ANOVA = 0.29). On the other hand, we observed a significant correlation between GSH/Hcys ratio and heart condition (normal = 0.82, mild = 0.70, moderate = 0.51, severe = 0,51; ANOVA = 0.016). The main statistical difference was detected between normal and severe patients (0.82 vs. 0.51, $p = 0.007$). In addition, the GSH/Hycs ratio was statistically different between diabetic patients and non-diabetic individuals (0.49 vs. 0.67, $p = 0.007$). These findings may have multiple clinical ramifications including the prediction of the development of coronary disease using the plasma GSH/Hcys ratio.

Homocysteine thiolactone: Metabolic origin and protein homocysteinylation

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Although homocysteine thiolactone was obtained by chemical synthesis in the 1930s, the first indication of its biological significance came almost 50 years later with the discovery of enzymatic conversion of Hcy to Hcy thiolactone in error editing reactions of some aminoacyl-tRNA synthetases (AARSs). The non-protein amino acid Hcy is misactivated by several AARSs and forms an AARS-bound Hcy~AMP. Subsequent rejection of the Hcy~AMP intermediate involves an intramolecular reaction in which the side chain thiolate of Hcy displaces the AMP group from the activated Hcy, forming Hcy thiolactone as a product. The energy of the anhydride bond of Hcy~AMP is conserved in an intramolecular thioester bond of Hcy thiolactone. Consequently, Hcy thiolactone is chemically reactive and easily acylates any free amino groups, such as side chain lysine groups in proteins. Homocysteinylation leads to gross structural perturbations in proteins, manifested by the loss of enzymatic activity and/or denaturation. The metabolic conversion of Hcy to Hcy thiolactone, protein homocysteinylation, and resulting protein damage may underlie the involvement of Hcy in human pathologies, such as vascular disease and/or neural tube defects.

Mutated 5,10-methylenetetrahydrofolate reductase, hyperhomocysteinemia and risk on cardiovascular disease

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Moderate hyperhomocysteinemia (MHH) is a new risk factor for arteriosclerosis and thrombosis. About 10 to 30% of the normal population have high homocysteine levels contributing to an increased risk for arteriosclerotic and venous disease.

Main regulating enzymes of homocysteine metabolism are cystathionine β-synthase (CBS) and methylenetetrahydrofolate reductase (MTHFR). Heterozygosity for CBS deficiency is most likely not a cause for MHH in vascular disease. One of the causes of MHH is reduced MTHFR activity due to a C677T mutation in the coding region of MTHFR. This mutation causes thermolabile protein and reduced enzyme activity. Cases with this mutation will have elevated homocysteine in case their plasma fo-

late levels are low-normal. These elevated homocysteine levels can be normalized by administration of folate. Results of different studies of different designs will be presented.

Irreversible inhibition of lysyl oxidase by homocysteine thiolactone

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Lysyl oxidase, an enzyme secreted by vascular smooth muscle cells and fibroblasts, catalyzes the initiation of covalent crosslinkage formation within the extracellular matrix proteins, collagen and elastin. Homocysteine thiolactone, selenohomocysteine lactone and homoserine lactone were found to be competitive, irreversible inhibitors of lysyl oxidase, with a K_i values of 21 ± 3 μM, 8.3 ± 2.2 μM and 420 ± 56 μM, respectively. The first-order rate constants for inactivation (k_2) of the enzyme varied over a smaller range, ranging from 0.12 to 0.18 to 0.28 min^{-1} for the O-, Se- and thiolactones, respectively. Mutually exclusive labeling of the enzyme by [1-^{14}C]β-aminopropionitrile, [U-^{14}C]phenylhydrazine or [^{35}S]homocysteine thiolactone was observed. These labeling results together with the closely similar perturbations of the near UV-visible spectra of lysyl oxidase and of a model of its lysine tyrosylquinone cofactor by the thiolactone indicate that the lactones likely derivatize and reduce the active site carbonyl cofactor. Substitution with deuterium at the α-carbon of the thiolactone caused a deuterium kinetic isotope effect on k_2 of 3.2 ± 0.2, consistent with the involvement of rate-limiting α-proton abstraction during lactone-induced inactivation of the enzyme. The activities of plasma amine oxidase and diamine oxidase were only minimally reduced at concentrations of the sulfur or selenium lactones that fully inhibited lysyl oxidase. Thus, these lactones constitute a new category of mechanism-based inactivators selective for lysyl oxidase. Further, these results may relate to the development of connective tissue defects seen in homocystinuria.

(Supported by NIH Grants R37 AR 18880 and HL 13262.)

Homocysteine interacts with other NMDA receptor antagonists to induce developmental abnormalities

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Homocysteine induces developmental abnormalities of the orofacial, conotruncal and neural tube regions (neural crest and neural tube derivatives); folate prevented these defects if it reduced serum homocysteine [Rosenquist et al. (1996) PNAS USA 93: 15227–15232]. The ability to induce neural crest and neural tube abnormalities is a general function of antagonists of the NMDA type of glutamate receptor (NMDAR). We hypothesized that the ability of homocysteine to act as an NMDAR antagonist was the basis of its teratogenicity [Andaloro et al. (1998) Ped Res Pediatric Res 43: 1–7]. In support of this hypothesis, we found that homocysteine-induced neural crest and neural tube abnormalities were reduced significantly by NMDAR agonists, especially glycine [Rosenquist et al (1999) FASEB J (in press)].

According to this hypothesis, homocysteine and other NMDAR antagonists should interact additively or synergistically to increase the rate of occurrence or severity of congenital defects of the neural crest and neural tube. To test this, we treated embryos during the process of neural tube closure with homocysteine thiolactone to induce neural crest and neural tube defects in about 40% of survivors; homocysteine thiolactone in combination with 50 μl 0.1% ethanol, 0.005 nmol dextromethorphan, or 0.5 nmol dextromethorphan; or the ethanol and dextromethorphan solutions alone. Dextromethorphan and ethanol acted synergistically with homocysteine, inducing neural crest and neural tube defects at a higher level than predicted; 0.5 nmol dextromethorphan was harmless by itself, but it doubled the effect of homocysteine. These results show that homocysteine may interact synergistically with other NMDA receptor antagonists to exacerbate their respective teratogenic effects.

(Supported by U.S.P.H.S. grant RO1-HL-55940; the Anna and Ardith von Housen Endowed Professorship; the Johnson and Johnson Companies; and the Nebraska Health and Human Services System.)

Hyperhomocysteinemia and vitamin status of school children

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Multiple studies have shown that a moderately elevated plasma homocysteine concentration is an independent risk factor for atherosclerosis and thrombosis. Hyperhomocysteinemia is typically caused either by genetic defects of the enzyme cystathionine β-synthase or by nutritional deficiencies of vitamin cofactors. Testing for hyperhomocysteinemia may be useful to assess the nutritional status. Further investigations of mild hyperhomocysteinemia may clarify the etiology of this biochemical abnormality.

Total plasma homocysteine (tHcys), serum vitamin B12, serum folate, creatinine, respectively, were measured in blood specimen of healthy German school children (n = 256, age range 6–17 years) and their parents (n = 260, age range 26–50 years). The measurements are part of a prospective intervention study [Family Intervention Trial (FIT)] evaluating the nutritional status and risk factors of a large German population.

The results are shown in Table 1.

Table 1

	range (median value) children	
	male	female
tHcys [μmol/l]	1.9–11.0 (5.3)	0.6–11.4 (5.4)
Vitamin B12 [pg/ml]	317–1473 (682)	263–1658 (718)
Folate [ng/ml]	3.0–18.0 (11.4)	4.3–23.8 (10.2)
Creatinine [mg/dl]	0.21–1.00 (0.46)	0.32–0.81 (0.46)

	range (median value) adults	
	male	female
tHcys [μmol/l]	2.7–40.3 (10.5)	2.4–30.3 (8.8)
Vitamin B12 [pg/ml]	199–892 (438)	203–978 (443)
Folate [ng/ml]	2.6–19.5 (5.6)	2.3–27.4 (6.4)
Creatinine [mg/dl]	0.64–1.22 (0.90)	0.40–1.04 (0.70)

A mild homocysteinemia (tHcys >15 μmol/l) occurred in 7 percent of the adults in our study group. Correlations between folate, vitamin B12, creatinine and tHcys levels are discussed. Further studies are needed to clarify the age dependence of tHcys levels and the individual risk profile of deficient vitamin status.

(This study was supported by Bayerische Staatsregierung and Stiftung Kindergesundheit.)

Hyperhomocysteinemia in paediatric patients in treatment with antiepileptic drugs

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Aim: To study plasma total-homocysteine (tHcy) concentration in paediatric patients on antiepileptic treatment to detect a possible hyperhomocysteinemia caused by these drugs with antifolate action.

Patients: Plasma tHcy was determined in 100 children (age: 2 months–18 years) on antiepileptic treatment (carbamazepine, valproic acid, clonazepam or clobazam), regularly evaluated in the neurologic outpatient clinic. Blood for tHcy was collected in the course of antiepileptic drug monitoring. Children with history of stroke episodes, diabetes or nephropathy were excluded from the study. The epileptic children were distributed in three age groups to be compared with our reference values for similar ages (A: 2 months–10 years old, B: 11–15 years old and C. 16–18 years old). Reference values were established in our paediatric population by measurement of plasma tHcy in apparently healthy children who underwent pre-surgical analysis for minor surgery (n = 195). Hyperhomocysteinemia was defined as tHcy values above the 95th percentile of the distribution within the paediatric population (A: 8.0 µmol/L; B: 9.3 µmol/L; C: 10.8 µmol/L).

Method: Fasting plasma tHcy was determined by high performance liquid chromatography with fluorescence detection of the 7-fluorobenzo-2oxa-1,3-diazole-4-sulfonate (SBDF) derivatives (Vilaseca et al., 1997).

Results: Plasma tHcy concentrations were found significantly higher in the younger age groups (A and B) compared with our reference values for the same group ages (Table 1) [Mann-Whitney (a) $P < 0.000008$; (b) $P < 0.006$]. tHcy values were above the P_{95} of the reference values in 28% of the children undergoing antiepileptic treatment. The odds ratio for the risk of hyperhomocysteinemia in the children taking antiepileptic drugs was 7.2 (with 95% confidence interval: 3.3 to 15.6).

Epileptic patients	Age	tHcy (µmol/L) median	range
A (n = 64)	2 m–10 y	6.8 (a)	3.4–15.2
B (n = 23)	11–15 y	7.6 (b)	4.8–18.0
C (n = 14)	16–18 y	8.1	5.1–39.8
Reference values			P_5–P_{95}
A (N = 106)	2 m-10 y	5.8	3.7–8.0
B (N = 58)	11–15 y	6.6	5.1–9.3
C (N = 31)	16–18 y	8.1	5.7–10.7

Conclusion: Treatment with antiepileptic drugs is associated with a mild hyperhomocysteinemia in a high percentage of patients (28%), probably caused by the antifolate action of these drugs. The measurement of tHcy and the vitamins involved in homocysteine metabolism (folate, vitamin B12 and B6) in these patients is important because of hyperhomocysteinemia is a risk factor for vascular damage, and high tHcy concentrations may be easily corrected by pharmacological supplementation with folate.

Plasma homocysteine, brain MR lesions, and cognitive functions in elderly diabetic patients

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Purpose: The pathophysiological mechanisms of asymptomatic cerebrovascular disease, leukoaraiosis, and cognitive impairment which appears to be more prevalent in diabetic patients, remains unknown. To explore a possible contribution of plasma homocysteine to cerebral lesions supplied by subcortical small arteries or cognitive impairment, we examined the relationship between plasma homocysteine and cerebral lesions on brain MR images, or several cognitive performance tests in elderly diabetic patients.

Methods: We studied 250 elderly diabetic patients (mean age: 74 years) and divided into three groups based on neurological and brain MR findings: symptomatic cerebrovascular disease (CVD), asymptomatic CVD, and control group without any CVDs. The total score of brain T2 high intensity lesion was calculated by summing up focal scores in 24 brain areas.

Results: Diabetic patients with asymptomatic or symptomatic CVD had significantly higher homocysteine levels in plasma than those without any CVDs. Plasma homocysteine was also associated with the presence or number of small infarct-like lesions on MR images. In the diabetic patients without symptomatic CVD, high plasma homocysteine levels were associated with high total scores of T2 high intensity lesions or focal T2 high intensity scores in corona radiata or periventricular regions. The plasma homocysteine levels significantly negatively correlated with MMSE scores for diabetic men and digit symbol substitution sub-test of the WAIS-R for diabetic women.

Conclusions: Elevated plasma homocysteine could contribute to the development of asymptomatic CVD and T2 high intensity lesions on brain MR images possibly through small-vessel alterations, which may lead to the cognitive impairment in elderly diabetic patients.

Impaired glucose tolerance (IGT) is not a risk factor for disturbed homocysteine metabolism

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Elevated plasma homocysteine is suggested to be an additional risk factor for cardiovascular disease in both subjects with Typ 2 diabetes (T2D) and IGT. The aim of the present study was to investigate whether an insulin resistant/hyperinsulinemic situation in male diabetic and pre-diabetic subjects directly influences the homocysteine metabolism. Total fasting (tHcy) and post-methionine-load (PML-tHcy) homocysteine plasma levels were determined in 16 subjects with IGT, 13 subjects with T2D, and 15 normoglycemic controls (NGT). Homocysteine concentrations were measured by an immunoassay based on enzymatic conversion of tHcy to S-adenosylhomocysteine (SAH) followed by quantification with enzyme-labelled anti-SAH-antibodies. Fasting tHcy (IGT, 13.1 ± 4.6 µmol/L; T2D, 12.8 ± 4.0 µmol/L; NGT, 10.7 ± 4.4 µmol/L) and PML-tHcy (IGT, 46.5 ± 17.3 µmol/L; T2D, 41.1 ± 6.8 µmol/L; NGT, 38.0 ± 9.7 µmol/L) showed no differences between the groups. Correlation analysis showed a direct relationship between fasting tHcy and PML-tHcy ($r = 0.636$; $p < .01$). In addition, fasting tHcy and PML-tHcy correlated directly with creatinine ($r = 0.489$; $p < .01$; $r = 0.339$; $p < .05$) and fasting proinsulin ($r = 0.395$; $p < .05$; $r = 0.386$; $p < .05$), respectively. Multiple regression analysis showed only a direct relationship between fasting tHcy and creatinine. No relationships have been found between fasting tHcy and PML-tHcy, respectively, and indicators of an insulin resistant/hyperinsulinemic state, e.g. fasting plasma glucose, HbA1c, insulin, proinsulin, microalbumin, triglycerides, total cholesterol, LDL- and HDL-cholesterol. Summarizing, our data suggest that homocysteine is not a risk factor in pre-diabetic and diabetic subjects without late complications. However, fasting tHcy levels tend to be elevated after onset of diabetic nephropathy, indicating the dependence of homocysteine concentrations likely on renal function in these subjects.

Prevalence of hyperhomocysteinemia and of elevated plasma levels of other sulfhydryl compounds in patients with dilated cardiomyopathy

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A number of experimental and clinical studies have clearly demonstrated a correlation between a mild increase of plasma homocysteine (Hcy) and several cardiovascular diseases; furthermore, alterations in the redox status of Hcy rapidly affects and is related to the redox status of other plasma aminothiols.

In this study we determined fasting plasma levels of total Hcy, cysteine (Cys) and cysteinyglycine (CysGly) in 44 patients affected with dilated cardiomyopathy with mild, moderate or severe cardiac impairment, evaluated according to NYHA functional classes.

Plasma levels of Hcy (mean value ± SD: 24.9 ± 11.9 μmol/l), Cys (342 ± 72.4 μmol/l) and CysGly (98.04 ± 82.2 μmol/l) were significantly higher ($p < .0001$ for all groups) in dilated cardiomyopathy patients than in 36 healthy age- and sex-matched controls (7.5 ± 2.8 μmol/l, 118.6 ± 31.2 μmol/l and 17.8 ± 7.4 μmol/l) for Hcy, Cys and Cys Gly, respectively).

Hcy increase did not correlate with NYHA stage; however, linear regression analysis showed a significant correlation between progression of cardiac impairment and both Cys and CysGly concentrations ($r = 0.6$, $p < .0003$ and $r = 0.81$, $p < .0005$, respectively).

In patients with cardiovascular diseases, elevated plasma levels of Hcy and especially of Cys and CysGly represent a major mechanism of insult to myocardical structures; indeed, the extracellular increase of these substances is probably a consequence of the formation of mixed didulfides between these substances and proteins containing free sulfhydryl groups, with a consequent alteration in protein conformation and enzyme activity, ultimately leading to an impairment of myocardial cell function. Furthermore, a decreased metabolic bioavailability does not allow the plasma excretion of both Hcy and other aminothiols. Thus, elevated plasma Hcy levels may be considered as a direct consequence of the cardiovascular disease itself and/or of some of the complicating conditions related to it.

To prevent vasculotoxicity from hyperhomocysteinemia, give affected children vitamin B_{12}, give fertile females folate and give all > ages 50 daily oral 25 to 100 μg vitamin B_{12}

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All B-vitamin-correctable hyperhomocysteinemias are examples of genetic nutrition (Genetic Nutrition, AP Simopoulos, et al., Macmillan, NY, NY 1993). In each case, the appropriate vitamin counteracts a phenotypic (clinical disorder) expression of a genotypic predispositions. The genetic defect in infants and children is mutation in their cystathione synthase genes; in fertile females it is mutations in their 5, 10-methylenetetrahydrofolate reductase (MTHFR) genes, which can also produce neural tube and other birth defects (TKAB Eskes, Nutr Rev 56: 236–244; 1988). Past age 50, it is genetic predisposition to insidiously progressive gastric atrophy, first producing inability to absorb B_{12} (due to loss of gastric acid and enzymes), and then inability (due to subsequent loss of gastric intrinsic factor) to absorb more than 1% of any oral dose of crystalline vitamin B_{12}. (In: Present Knowledge in Nutrition, 7th Ed. Eds: EE Ziegler, LJ Filer, Jr. ILSI Press, Washington, DC 1996, pp 191–205) B_{12} deficiency occurs by median age 65 ~50% of elderly, producing hyperhomocysteinemia in 60% of them (Flynn et al., J Am Coll Nutr 16: 258–267; 1997). With the Flynn group (U Missouri-Columbia) and the R Green group (U Cal-Davis), we are in 1999 testing, in many elderly, the proposition that low serum folate is due to intestinal malabsorption produced by B_{12} deficiency, and low red cell folate is due to inadequate B_{12} to allow folate to enter red cells and be retained therein, and how much of their B_{12} deficiency is due to *H. pylori* in the stomach.

Membrane Transport

Amino acid transport by 4F2-heterodimers

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System L is the major Na^+-independent amino acid transporter of mammalian cells. It is constituted by the type II membrane protein 4F2 which is covalently linked to the polytopic membrane protein LAT1 via a disulfide bridge. The transport pore of the transporter is most likely constituted by LAT1, whereas 4F2 is necessary for the translocation of the complex into the plasma membrane. The 4F2 protein does not only interact with LAT1, but also with a number of other amino acid transport proteins, namely y^+LAT and some other yet unidentified transport proteins. In oocytes expressing 4F2 alone at least four different transport activities can be detected.

The 4F2/LAT1 heterodimer was functionally expressed in *Xenopus laevis* oocytes and its transport properties were analysed by flux measurements and two-electrode voltage clamp technique. Expression of 4F2/LAT1 resulted in a rapid increase of a Na^+-independent neutral amino acid antiport activity, with all characteristics of system L, and an associated cation-conductance. Replacement of Na^+ by NMDG suppressed the currents almost completely. Currents were not affected in the presence of substrates of system L or its inhibitor BCH. Both, transporter and channel activity increased with the same time course after injection of the 4F2/LAT1 mRNAs. Expression of 4F2 alone induced a small conductance after expression for five days. It is suggested that the 4F2/LAT1 heterodimer forms a larger complex with an oocyte-endogenous non-selective cation-channel, which is similar to the non-selective cation channel (NCS) of mammalian cells.

Cloning of a cDNA that codes for the human cationic amino acid transporter-3 (hCAT-3)

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The family of mammalian cationic amino acid transporters (CATs) comprises of at least five related carrier proteins encoded for by four different genes. From human cells, full length cDNA

clones for CAT-1, -2A, -2B and -4 have been isolated. Here, we report the cloning of a cDNA encoding for the fifth member of the human CAT family, hCAT-3. CAT-3 has recently been identified in rat and mouse [Hosokawa H et al (1997) J Biol Chem 272: 8717–8722, Ito K, Groudine M (1997) J Biol Chem 272: 26780–26786]. In adult animals, CAT-3 seems to be exclusively expressed in the brain. Using reverse transcription and polymerase chain reaction, we first amplified a 895 bp hCAT-3 cDNA fragment from mRNA of a human teratocarcinoma cell line (NT2) that can be differentiated into neuronal cells. To obtain a full length cDNA for hCAT-3, we then screened cDNA libraries from different regions of the human brain. From 11 libraries containing more then 470,000 individual clones, only one positive clone was identified. Analysis of the nucleotide sequence revealed that this clone encodes for the 236 C-terminal amino acids of hCAT-3. The missing 5' sequence of the hCAT-3 cDNA was subsequently identified by rapid amplification of cDNA ends (RACE) using mRNA from NT2 cells. The amino acid sequence deduced from the complete cDNA is 83.4% and 81.6% identical to rat and mouse CAT-3, respectively. It contains one consensus sequence for N-glycosylation and several consensus sequences for phosphorylation by serine/theorine and tyrosine kinases. We are currently studying the expression of hCAT-3 in different human tissues and cell lines.

Mammalian peptide transporters and the secret of their substrate specificity

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Proton dependent electrogenic transporters for di- and tripeptides have been identified in bacteria, fungi, plants and mammalian cells. They all show sequence independent transport of all possible di- and tripeptides as well as of a large variety of peptidomimetics. We used the mammalian peptide transporters PEPT1 and PEPT2 as models to define the molecular basis for their multisubstrate specifity. By employing computational analysis of possible substrate conformations in combination with transport assays using transgenic yeast cells and *Xenopus laevis* oocytes expressing PEPT1 or PEPT2, the minimal structural requirements for substrate binding and transport were determined. Based on a series of medium chain fatty acids bearing an amino group as a head group (ω-amino-fatty acids, ω-AFA), we show that electrogenic transport by PEPT1 requires as a minimum the two ionized head groups separated by at least four methylen groups. Consequently, a >500 pm <630 pm distance between the tow charged centers (carboxylic carbon and amino nitrogen) is sufficient for substrate recognition and transport. Removal of either the amino group or the carboxy group in ω-AFA maintained the compounds affinity for interaction with the transporter but abolished the capability for electrogenic transport. Additional groups in the ω-AFA backbone that provide more hydrogen bonding sites appear to increase substrate affinity but are not essential. Amino- and carboxy groups in close vicinity prevent the interaction of substrates with the carriers thus excluding a priori all amino acids from binding even when they have the proper backbone such as lysine. The information provided here do a) explain the capability of the peptide carriers for sequence independent transport of thousands of different substrates and b) set the molecular basis for a rational drug design to increase for example the absorption of peptide based drugs mediated by PEPT1.

Molecular biology of transport systems for neutral amino acids

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Recent cloning studies have identified several transport systems that function in the transport of neutral amino acids. These transporters fall into three categories: Na^+- and Cl^--coupled transporters (PROT and GLYTs). Na^+-coupled transporters (ASCT1 and ATB^0/ASCT2) and heterodimeric transporters ($b^{0,+}$, y^+L and L). PROT is specific for proline and is expressed in the brain but not in the intestine and kidney. The latter two tissues do however possess a proline transport system, but apparently PROT is not responsible for this transport process. GLYTs are specific for glycine and have also been studied primarily in the brain. However, GLYT1 exhibits a broader tissue expression pattern and may be responsible for the transport of glycine in the intestine, kidney and placenta. ASCT1 and ATB^0/ASCT2 are widely expressed in animal tissues. ASCT1 is specific for alanine, serine, and cysteine whereas ATB^0/ASCT2 has a broader specificity for neutral amino acids including glutamine. Both transporters function as amino acid exchangers. ATB^0/ASCT2 potentially functions in the glutamate/glutamine cycle known to occur between the neurons and astroglia in the brain and between the placenta and liver in the fetus. ATB^0/ASCT2 is also a receptor for the RD114/type D retroviruses. This transporter is a potential candidate for the human amino acid transport defect Hartnup disorder. $b^{0,+}$ is a Na^+-independent transport system for cystine and neutral/basic amino acids, expressed principally in the intestine and kidney. It is also an obligatory amino acid exchanger. It functions as a heterodimer and one of its subunits (rBAT) has been cloned. Defects in the function of $b^{0,+}$ cause cystinuria. y^+L and L are expressed in several tissues. Both systems are heterodimers. 4F2hc is a common subunit for both systems. y^+LAT and LAT are the other subunits specific for y^+L and L, respectively. The individual subunits of the heterodimeric complexes of y^+L and L do not exhibit detectable transport function on their own, but dimerization between the subunits constitute the functional transport systems. y^+L mediates Na^+-independent transport of basic amino acids and Na^+-dependent transport of neutral amino acids. L mediates Na^+-independent transport of neutral amino acids. y^+L is also an obligatory amino acid exchanger. Defects in the function of y^+L cause lysinuric protein intolerance.

The role of betaine and taurine as organic osmolytes in the mammalian kidney

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Betaine and taurine have been implied to play a significant role in the volume regulation and osmotic adaptation in a variety of organisms and species. In the mammalian renal inner medulla where urinary concentration and urinary dilution cause dramatic changes in the extracellular osmolarity, both amino acids participate in osmoregulation of the kidney cells.

In the thick ascending limb of Henle's loop (TALH) betaine is one of the predominant osmolytes. Betaine is taken up across the luminal membrane but is also synthesized within the TALH cells. Betaine synthesis increases in parallel with the extracellular osmolality. At the same time plasma membrane permeability mediating betaine efflux decreases. Thus in TALH cells in culture an increase of betaine content results.

In MDCK cells – a cell line derived from more distal parts of the nephron – betaine uptake occurs at the basolateral cell pole via a sodium and chloride dependent transport system (BGT1). In these cells BGT1 is under osmotic control as is the betaine release pathway. Similar mechanisms regulate also intracellular taurine content.

In inner medullary collecting duct cells, which form the final segment of the nephron, only taurine efflux is osmotically controlled. Here the efflux channel is located basolaterally and is shared by chloride, taurine, and myoinositol.

Thus a segment-specific involvement of the two amino acids in cell volume regulation can be demonstrated, thereby not only their relative importance as osmolyte varies but also the way in which the intracellular content is adjusted to the extracellular osmolarity.

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Osmoregulatory amino acid transport, cell volume and control of cell function

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Owing to the high water permeability of most cell membranes, any osmotic gradient across the cell membrane leads to the respective water movements and alterations of cell volume. Obviously, excessive cell swelling disrupts the structural integrity of the cell. Beyond that and possibly more important, alterations of cell volume modify the hydration state and thus the function of cellular proteins such as enzymes. To avoid untoward alterations of cell volume cells employ a wide variety of cell volume regulatory mechanisms including ion transport across the cell membrane as well as alteration of cellular organic osmolyte content by transport and metabolism. The major organic osmolytes are the polyols sorbitol and inositol, the methylamines glycerophosphorylcholine and betaine as well as amino acids and amino acid derivates such as glycine, glutamine, glutamate, aspartate and taurine [for review see Lang et al (1998a, b)]. Upon cell shrinkage, cells gain osmolarity by activation of Na^+/H^+-exchanger, Na^+, K^+, $2Cl^-$-cotransport, Na^+-channels and organic osmolyte accumulation. Upon cell swelling, cells loose osmolarity by activation of KCl-cotransport, K^+- and Cl^--channels as well as osmolyte release. The volume regulatory mechanisms participate in diverse cellular functions, such as transport, metabolism, migration, excitability, cell proliferation and cell death. Notably, cell swelling stimulates protein synthesis and inhibits proteolysis, effects reversed by cell shrinkage. Hormones such as insulin and glucagon exploit the cell volume regulatory mechanisms to govern protein metabolism. Insulin swells hepatocytes by activation of Na^+/H^+ exchanger and Na^+, K^+, $2Cl^-$-cotransport and thus exerts its antiproteolytic action. Glucagon shrinks hepatocytes by activation of ion channels thus stimulating proteolysis. The effect of the hormones on proteolysis are mimicked by equivalent alterations of cell volume and abolished by reversal of cell volume changes. Similarly, TGFβ-1 exerts its antiproteolytic and proteinsynthetic effect in part by altering cell volume. TGFβ stimulates the expression of the cell volume regulatory kinase h-skg, which in turn stimulates cell volume regulatory NA^+, K^+, $2Cl^-$-cotransport. Again, the effect of TGFβ is mimicked by osmotic cell swelling and reversed by inhibition of Na^+, K^+ $2Cl^-$-cotransport. Stimulation of cell proliferation is paralleled by activation of Na^+/H^+-exchanger and/or Na^+, K^+, $2Cl^-$-cotransport, apoptotic cell death by activation of cell volume regulatory Cl^--channels, taurine release and inhibition of Na^+/H^+-exchanger. Pharmacological evidence points to a primary role of these cell volume regulatory mechanisms in cell survival and cell death.

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Peptide transport

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PEPT1 and PEPT2 are H^+-coupled peptide transported that mediate the transmembrane transport of dipeptides and tripeptides in animal cells. These two transporters differ primarily in substrate affinity and tissue distribution, but they exhibit considerable similarity in the operational mechanism. PEPT1 is a low-affinity transporter and is expressed predominantly in the intestine and, to a much small extent, in the kidney. PEPT2 is a high-affinity transporter and is expressed in the kidney and the brain, but not in the intestine. While the physiological role of PEPT1 in the maintenance of protein nutrition is easily recognizable, the physiological role of PEPT2 is not readily apparent. PEPT1 is receiving increasing attention in recent years for its enormous potential as an oral drug delivery system. The promiscuity of PEPT1 in terms of substrate specificity makes it an ideal candidate for this purpose. Since the expression of PEPT1 is primarily restricted to the intestine, animal models with disrupted PEPT1 gene will provide a valuable tool to understand the physiological function of this transporter in protein assimilation. Disruption of the function of a homologous low-affinity peptide transporter in *C. elegans* results in significant delay in growth, suggesting a nutritional function for this transporter. PEPT1-knockout mice will be very useful in elucidating the nutritional role of this transporter in detail. Murine PEPT1 consists of 709 amino acids and exhibits 92% and 83% sequence identity with rat PEPT1 and human PEPT1, respectively. The murine PEPT1 gene, isolated from a BAC ES MOUSE genomic library, is comprised of 23 exons. All exon-intron boundaries conform to the consensus donor/acceptor sequence. A targeting vector for homologous recombination to generate PEPT1-knockout mouse has been constructed in pKO Scrambler vector. The insert contains ~13 kbp (exons 9–18) of the murine PEPT1 gene in which exon 11 is disrupted with a neomycin resistance gene. Exon 11 codes for the region containing the transmembrane domain 7. This region has been shown to be essential for PEPT1 activity as assessed from the function of PEPT1-PEPT2 chimeras. The targeting vector has been transfected into ES cells derived from the 129/SvEv mouse strain and transfectants identified by a positive-negative selection scheme using neomycin and gancyclovir. A single ES clone containing the targeting construct at the target locus has been identified by PCR and Southern analysis. Further studies in the generation of the PEPT1-knockout mouse are in progress.

Analysis of the NO: Arginine pathway in tumor growth and metastasis using knockout mice

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The research to be presented directly tests the role of L-arginine and one of its metabolic products nitric oxide, in breast tumor growth and metastasis. Several recent reports indicate nitric oxide (NO) acts as a potent inhibitor of angiogenesis, tumor cell growth, and metastatic spread. Other reports indicate a stimulatory role for NO. NO is produced by a family of nitric oxide synthase (NOS) enzymes; L-arginine is its exclusive and immediate precursor. NO is produced in large amounts by an inducible form of NOS (iNOS or NOS2) that is under strict transcriptional control and encoded by a single gene. iNOS activity is dependent upont the *transport* of an *exogenous* (circulating) source of L-arginine and is rate-limited by the availability of extracellular L-arginine. The other NOS enzyme forms are encoded by distinct genes, they are constitutively expressed and their activity is regulated by intracellular Ca^{++} flux using *intracellular* L-arginine. The constitutively expressed Ca^{++} regulated enzymes

produce smaller amounts of NO that function to control blood pressure and neural transmission. Conflicting reports on the role of NO in breast cancer growth and metastasis led us to study this question using a mouse strain that produces multifocal breast adenocarcinomas in 100% of females that metastasizes to the lung of 100% of the mice by 3.5 months. This PyVmT transgene was introduced into mice lacking iNOS (via targeted mutation) to determine whether the rate of growth and spread of breast cancer is affected by iNOS activity and/or dietary L-arginine. The analysis of arginine defined diets in iNOS deficient animals permitted an analysis of dietary arginine that is separate from the effects generated by the production of large amounts of NO via this enzyme. Since exogenous arginine is *required* for iNOS enzyme activity, and the arginine transporter CAT2 is co-regulated with iNOS, we also ablated the Cat2 gene by homologous recombination and tested the effect of this genetic function on tumor progression. We determined that CAT2 is required for L-arginine influx into macrophages to produce NO. The mouse models provides a genetic approach to directly and conclusively test the function of L-arginine transport and NO production on breast cancer growth and metastasis and whether dietary L-arginine alters the frequency and occurrence of metastasis in this model system. Our findings will be presented.

Activation of cationic amino acid transport in vascular endothelial and smooth muscle cells in oxidative stress

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In view of the importance of nitric oxide (NO) as a modulator of vascular tone under basal conditions and in disease and chronic inflammation [1], co-activation of cationic amino acid transport via system y^+ (CATs) by bacterial endotoxin, proinflammatory cytokines and atherogenic lipids plays a key role in maintaining supply of L-arginine for sustained production of NO via inducible iNOS.

Our studies in cultured macrophages and vascular smooth muscle cells provided convincing evidence that L-arginine transport and NO production were closely coupled in cells activated with pro-inflammatory cytokines [2, 3]. Activation of arginine transport by LPS was blocked by cycloheximide but, unlike the time-dependent increase in NO production, was insensitive to inhibition by dexamethasone. In these studies NO synthesis was critically dependent on extracellular L-arginine (K_i ~30 μM) and inhibited in the presence of either L-lysine or L-ronithine. Studies in rat aortic smooth muscle cells deprived of serum for up to 48 h have established that TNF-α and IL1β can synergise to induce arginine transport, nitrite production and iNOS and CAT-2 mRNA, all of which were abolished by treatment of cells with cycloheximide [4, 5]. Although cytokines do not appear to alter CAT-1 mRNA levels in quiescent smooth muscle cells, other studies in serum-deprived rat aortic smooth muscle cells have conformed that stimulation of arginine and lysine transport by angiotensin II increases mRNA levels for CAT-1 and CAT-2 transporter isoforms [6]. Oxidative stress induced by lysophosphatidylcholine has been shown to initially (2 h) decrease cationic amino acid transport in aortic smooth muscle cells, which subsequently increases over the next 6–24 h [7]. Stimulation of ornithine transport was associated with a transient increase (2 h) in mRNA expression for both CAT-1 and CAT-2 isoforms, with CAT-2 message elevated ~20-fold and CAT-1 message increased only ~3-fold.

In contrast to our findings in aortic smooth muscle cells [2], dexamethasone apparently increases arginine transport in pulmonary endothelial cells [8]. Moreover, in porcine pulmonary artery endothelial cells, LPS apparently stimulates system y^+ activity through an autocrine release of TNF-α and IL-1, since pretreatment of cells with TNF-α antibodies or an IL-1 receptor antagonist attenuated the actions of LPS [8]. In human umbilical vein endothelial cells TNF-α induces a time-dependent (4–24 h) increase in mRNA levels for CAT-2B (but not CAT-1), which correlate with elevated nitrite accumulation [8]. Thus, a variety of external stimuli associated with oxidative stress appear to modulate expression of CAT transcripts and NO production in vascular cells.

(Supported by the British Heart Foundation and Tommy's Campaign.)

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Transport mechanism for oligeopeptides and oligopeptide drug targeting constructs: Implications for distribution, elimination and cell-specific delivery in the whole organism

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Small therapeutic oligopeptides (2–12 amino acids), designed for interaction with cytokine and growth factor receptors, unfortunately, are rapidly removed from the body. Efficient glomerular filtration and carrier-mediated membrane transport processes are involved in their clearance. By coupling of such peptides to macromolecules, elimination via these pathways is prevented and exposure to the receptors aimed at can be largely improved. Some of these constructs undergo receptor-mediated endocytoses and can be used as carriers to deliver associated drugs to various cell types in the body.

However, the application of (glyco)-peptide drug carriers can be severely corrupted by down-regulation of the receptor-make up of the target cells in the diseased state. We therefore designed a new type of polypeptide carrier, homing at receptors that are known to be highly upregulated in the pathological target tissue.

For this purpose we designed ligand peptides (minimized proteins) representing the receptor-recognizing domains of PDGF and collagen type VI, aimed at receptors that are highly expressed, particularly on activated hepatic stellate cells (HSC). This myofibroblast-type of cell largely contributes to connective tissue expansion during liver fibrosis. Drug carriers for the stellate cell have not been reported before.

Methods: Cyclic octapeptide moieties (n = 10.12) with affinity for the two receptors were coupled to HSA (pPB-HSA and pCVI-HSA, respectively). Receptor binding experiments confirmed binding of these ligand peptides to their receptors *in vitro*. The organ distribution of pCVI-HSA and pPB-HSA was determined 10 min after iv injection of tracer doses in normal and fibrotic rats, 3 weeks after bile duct ligation. Hepatocellular distribution was scored after double-immunostaining of the liver sections with an antibody against the designated hepatic cell type in combination with anti-HSA IgG.

Results: 62 ±6% of the dose of pCVI-HSA accumulated in fibrotic livers at 10 min after injection, of which the major part was taken up in HSC. 48 ±9% of pPB-HSA accumulated in fibrotic rat livers and this carrier was also mainly taken up by HSC. Much smaller amounts of both constructs were taken up in normal rat livers, predominantly in other cell types.

Conclusion: Minimized proteins attached to soluble or particle type of macromolecules represent a novel carrier modality of which selective body distribution is induced by the disease process to be targeted. They can be utilised as receptor antagonists and at the same time can deliver therapeutic agents to the desired site of action.

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Cystinuria, lysinuria and cystinuria

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Cystinuria is a common recessive disorder of renal re-absorption and intestinal absorption of cystine and dibasic amino acids. Two types of cystinuria are distinguished on the basis of the cystine and dibasic aminoaciduria of the obligate heterozygotes: in Type I, heterozygotes are silent, whereas in non-Type I heterozygotes there is a variable degree of urinary hyperexcretion of cystine and dibasic amino acids (incompletely recessive inheritance). Mutations in *SLC3A1,* encoding the protein rBAT, cause cystinuria Type I, but not cystinuria non-Type I. The gene causing the latter Type has been assigned to a 1.3 Mb region on 19q12–13.1

Expression-cloning was used to identify rBAT as a protein which induces amino acid transport system $b^{0,+}$ in oocytes. rBAT shows homology with the heavy chain of the cell surface antigen 4F2 (4F2hc; also named CD98). 4F2hc induces system y^+L and L amino acid transport activities in oocytes. Structural and functional evidence suggested that rBAT and 4F2hc are heterodimeric amino acid transporters. In the last few months, four cDNAs (LAT-1, LAT-2, y^+LAT-1 and y^+LAT-2) have been identified as light subunits of 4F2hc. These subunits co-express system L (LAT-1 and LAT-2 isoforms), and system y^+L (y^+LAT-1 and y^+LAT-2 isoforms) with 4F2hc. Interestingly, mutations in the y^+LAT-1 transporter cause Lysinuric Protein Intolerance (LPI), an inherited aminoaciduria due to defective renal re-absorption of dibasic amino acids. The structural and functional similarities between 4F2hc and rBAT suggest that a member of this family of subunits might be the subunit of rBAT needed to fully express the amino acid transport system $b^{0,+}$ activity, and a good candidate for non-Type I cystinuria. Recently, we identified a new member *(SLC7A9)* of this family. Co-transfection of *SLC7Ap* and rBAT in COS cells brings rBAT to the plasma membrane and results in the expression of amino acid uptake activity. *SLC7A9* is expressed in kidney, liver and small intestine, and it is localized to the cystinuria non-Type I locus. Cystinuria-specific mutations of *SLC7A9* have been found in non-Type I patients of diverse origin. All these results demonstrate that mutations in *SLC7A9* cause non-Type I cystinuria, and suggest that *SLC7A9* may encode the light rBAT subunit ($b^{0,+}$ AT).

Signal transduction pathways involved in the modulation of system A for neutral amino acid transport

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System A for neutral amino acid transport activity is regulated by amino acid availability and medium osmolarity. Hypertonicity triggers an increase in system A activity, which involves a regulatory mechanism that is different from that responsible for the amino acid starvation response, and which may be the result of the activation of pre-existing system A carrier proteins. Osmotic up-regulation of system A transport activity has been found in all cell lines tested so far, including fibroblasts, renal epithelial cells, hepatoma cells and, more recently, a variety of cell lines derived from human pancreatic tumors. The signal transduction pathways involved in the regulation of sytem A by amino acid starvation and hypertonicity are not known. Recent work from our laboratory has focused on the activation of several members of the MAP kinase family, including the extracellular signal-regulated kinases (ERKs) and p38. System A response to amino acid starvation and hypertonicity has been studied in a fibroblast cell line, CHO-K1 (wild type, WT), and two independent somatic cell mutants derived from CHO-K1 cells, ala^r4, which has lost its ability to respond to amino acid starvation, and ala^r4-H3.9, which is resistant to hypertonicity.

Inhibitors of tyrosine kinases, such as genisteine and DHCM, did not block the response of system A activity to hypertonicity in CHO-K1 cells. Similarly, treatment of the cells with phorbol esters (TPA) did not alter the hypertonic response, although it markedly decreased basal uptake rates. Wortmanin, an inhibitor of PI3Kinase, blocked the starvation-mediated response but did not alter the osmotic regulation of system A. SB202190, an inhibitor of p38, did not affect the ability of this transport system to respond to amino acid starvation and hypertonicity, whereas the inhibition of ERK by PB98059 completely blocked the adaptive response to amino acid starvation, but did not alter the increase of system A activity following hypertonic shock. Hypertonicity induced p38 phosphorylation in both WT cells and the hypertonicity-tolerant mutant ala^r4-H3.9. Amino

acid starvation did not result in any significant change in p38 phosphorylation. ERK1 and ERK2 were highly phosphorylated in the hypertonicity-tolerant mutant in the basal state, when compared with the WT. After hypertonic shock, ERK1 and ERK2 were rapidly phosphorylated in the WT, whereas in the ala^{r}4-H3.9 mutant, only ERK2 phosphorylation increased as a result of the anisotonic stress. The ERK inhibitor PB98059, which blocks the adaptive response to amino acid starvation but is unable to inhibit the osmotic regulation of system A, significantly decreased ERK1 and ERK2 phosphorylation.

It is concluded that: the osmotic regulation of system A may not rely upon tyrosine kinase activation and involves signal transduction pathways which are different from that responsible for the adaptive response to amino acid starvation; the stress-activated kinases (ERKs and p38) do not appear to be involved in the osmotic regulation of system A, whereas the phenotype of the hypertonicity-tolerant mutant ala^{r}4-H3.9 cannot be explained by the basal overphosphorylation of ERK1 and ERK2. These data, along with recent observations from other laboratories that typical osmoprotective genes, such as aldose reductase, are induced independently of stress-activated kinases, suggest that multiple pathways are involved in the control of osmotic-responsive genes. Indeed, other transporters that are up-regulated after hypertonic shock, such as BGT1 (betaine transporter), do require p38 kinase activity for induction, which further supports the heterogenous molecular basis of hypertonicity-mediated induction of plasma membrane transporters.

Amino acid transport in the renal proximal tubule

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Vectorial transcellular transport of amino acids in the renal proximal tubule is mediated via functionally and structurally different amino acid transporters at both sides of the epithelial cells. Several kidney preparations, a variety of cultured renal epithelial cells and expression models have been used to study renal amino acid transport. Na^+-dependent and -independent transporters, Cl^--dependent and H^+-gradient driven transporters and amino acid exchangers are present in renal epithelial cells. Some of these transporters have been functionally analyzed in detail and the molecular structure is known, whereas structure and function of others still remain unclear. Regulation of renal amino acid transport has only recently been under investigation. This review summarizes more recent information on amino acid transport in the renal proximal tubule emphasizing functional and regulatory aspects.

Hormonal control of renal amino acid transport

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Previous experiments indicated a key role of dexamethasone (DEX) and triiodothyronine (T3) in maturation of renal amino acid transport. To investigate the influence of these hormones and of epidermal growth factor (EGF) on renal amino acid transport in adult rats, we used clearance and micropuncture techniques. Since tubular amino acid reabsorption is very effective, the carrier systems were overloaded by administration of high doses of neutral (glutamine, leucine, alanine) and basic amino acids (arginine), and taurine. The fractional excretion of bolus and endogenous amino acids into the urine was measured by HPLC. In addition to control rats, 5/6-nephrectomized (5/6-NX) rats as a model of chronic renal failure, and bile duct ligated (DL) rats as a model of hepato-renal syndrome were investigated. The results indicate differential regulation of the tubular carrier systems. DEX reduced fractional excretion of bolus amino acids glutamine and leucine in control, in 5/6-NX and in DL rats, whereas taurine reabsorption was only stimulated in both models of impaired kidney function. In controls only the leucine reabsorption was increased significantly after T3 pretreatment. In 5/6-NX and DL rats T3 reduced the fractional excretion of all bolus and various endogenous amino acids. An EGF pretreatment was also suitable to improve amino acid reabsorption in control rats, but, compared to DEX and T3, the stimulatory effect was only of moderate degree. To determine the site of DEX action along the nephron, we microinfused radiolabelled ^{14}C-glutamine and ^{14}C-leucine into different nephron segments and determined the fractional excretion relative to ^{3}H-inulin. Pretreatment with DEX enhanced glutamine uptake in the proximal convoluted tubule, but not in later parts of superficial nephrons. The leucine reabsorption was also enhanced in later parts of the nephron. We conclude that renal amino acid transport is under differential control of glucocorticoids, T3 and growth factors, which might act on the level of gene expression of the carrier proteins. Inhibition experiments with the γ-glutamyl-transpeptidase inhibitor acivicin showed participation of this enzyme in enhanced glutamine uptake after DEX pretreatment.

Mechanisms of iodothyronine and tryptophan transport

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Iodothyronines (including the thyroid hormones T_3 and T_4) are large, iodinated tyrosine derivatives which retain a functional amino acid moiety. Thyroid hormone (TH) action and metabolism require hormone transport across cell membranes. Iodothyronine transport into cells is inhibited by a wide variety of substances, including certain amino acids (notably tryptophan), bilirubin and drug conjugates. The aromatic amino acid Trp is an important precursor for neurotransmitter and hormone synthesis and an essential dietary constituent. There is evidence for a close functional link between transport of iodothyronines and tryptophan in erythrocytes, hepatocytes (by System T in both cases) and astrocytes (by System L). Recent studies have revealed that several members of a new family of amino acid permeases (including LAT, E16, IU12) exhibit activation of System L amino acid transport only when co-expressed with 4F2hc glycoprotein. These permeases (light-chain) interact covalently with 4F2hc (heavy-chain) to produce a functional, heteromeric "transporter unit" in the cell membrane. Both T_3 and T_4 are substrates of System L transport mediated by heterodimers of 4F2hc and IU12 Ic permease co-expressed in *Xenopus* oocytes. T_3 and tryptophan exhibit reciprocal inhibition of their 4F2hc-IU12 induced uptake and T_3 uptake (Km of 2.3 μM) is also inhibited by excess rT_3 and BCH (synthetic System L substrate). IU12 is an early T_3-response gene, up-regulated during amphibian development, which may be involved in the signal transduction pathway of T_3-induced metamorphosis. 4F2hc-Ic permease pathways may be major routes for transport and exchange of TH across vertebrate cell membranes. TH and sulfated derivatives are also substrates of organic anion transporters Ntcp and oatp1-3. T_3/T_4 transport at the cell membrane may be facilitated by direct interactions between hormone receptors and amino acid transporter proteins. Modulation of such interactions may be important for control of T_4/T_3 metabolism during altered thyroid status.

(Work supported by MRC, BBSRC, The Wellcome Trust and University of Dundee.)

Amino acid transporters associated with surface glycoproteins

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Amino acid transport across cellular plasma membranes depends on (co)-transporters and exchangers with overlapping specificities that function in parallel. Recently we and others have identified the first mammalian, Xenopus and platyhelminth members of a new family of permease-related amino acid transporters. The members of this family require association with a cell-surface glycoprotein for surface expression and thus to exert their transport function across the plasma membrane. Two such associated surface glycoproteins have been identified because they induce amino acid transport when expressed in *Xenopus* oocytes: 4F2hc/CD98 is a protein with quite ubiquitous expression, in particular in proliferating cells and at the basolateral membrane of some transporting epithelia. rBAT is structurally related to 4F2hc but is mainly expressed at the apical surface of small intestine and kidney proximal tubule. These glycoproteins display a single evident transmembrane segment with an intracellular NH_2-terminus. As yet, we have analyzed the function and localization of 4 glycoprotein-associated amino acid transporters (gpa-AT) which covalently bind to the 4F2h glycoprotein. These highly lipophilic proteins share a topology prediction of 12 transmembrane domains. The NH_2-terminus is intracellular and the cysteine residue which forms the disulfide bond with 4F2hc has been identified in the second putative extracellular loop. Two of these gpa-AT (LAT2 and y^+LAT1) are essentially localized to the basolateral membrane of some epithelia and are considered to be involved in amino acid (re)absorption. The two others (LAT1 and y^+LAT2) have a broader tissue distribution. gpa-At's appear to function as amino acid exchangers and display L-type (LAT1 and LAT2) or y^+L-type (y^+LAT1 and y^+LAT2) transport specificities. We have shown that a additional member of this family ($b^{0,+}$AT) specifically associates with apical rBAT instead of basolateral 4F2hc and produces $b^{0,+}$-type amino acid transport. This transport and the 4F2hc-associated epithelial y^+LAT1 display the functions and localizations expected for the transporters known to be defective in cystinuria and lysinuric protein intolerance, respectively.

Differences in transperitoneal membrane transport among amino acids according to their molecular charges on peritoneal dialysis

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Peritoneal dialysis (PD) is widely used for uremia therapy. On PD, not only wasting metabolites retained due to renal dysfunction but necessary substances like amino acids (AA) are removed by transperitoneal membrane transport (TMT). To clarify TMT differences among AA according to their isoelectric point (pI), 2L of 2.5% glucose dialysate was dwelled in peritoneal city for 4 hours in 22 patients with chronic renal failure and plasma and dialysate concentrations of 8 mino acids, leucine (Leu), isoleucine (Iso), ethionine (Met), asparagine (Asn), glutamine (Gln), asparatic acid (Asp), glutamicid (Glu) and lysine (Lys), which have similar molecular weights between 130 and 150 daltons were measured, and the dialysate/plasma concentration ratios (D/P) of these AA were calculated.

Results: The D/P of Asp and Glu were much lower than those of other AA. D/P of Glu was significantly higher than those of other AA and showed the highest value. D/P of Leu and Iso were significantly lower than Met, and there were no significant differences in D/P between both Met and Lys and Leu and Iso.

	Asp	Glu	Asn	Gln	Met	Leu	Iso	Lys
pI	2.77	3.22	5.41	5.65	5.74	5.98	6.02	9.74
D/P	0.06	0.16	0.67	0.70	0.62	0.55	0.56	0.60
(SD)	(0.03)	(0.06)	(0.17)	(0.16)	(0.15)	(0.16)	(0.17)	(0.15)

Conclusion: Transperitoneal membrane transport of AA is influenced by molecular charge, and the movement of negatively charged AA is most remarkably retarded.

Kinetics of 3-[^{123}I]Iodo-L-α-methyl tyrosine (^{123}I-IMT) transport in rat astrocytes and C6 glioma cells

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Introduction. 3-[^{123}I]Iodo-L-α-methyl tyrosine (^{123}I-IMT) is used for diagnosis and monitoring of brain tumours by means of single-photon emission tomography (SPET). Up to now, little is known about the transport system for ^{123}I-IMT into brain tumour cells. It is assumed that ^{123}I-IMT is transported by a specific carrier for large, neutral amino acids (L-system). In this study we characterized ^{123}I-IMT transport in the rat C6 glioma cell line and compared it to that of non-transformed astrocytes of neonatal rats.

Methods. First the time course of ^{123}I-IMT uptake into C6 glioma cells and astrocytes was examined for a range of 1 to 60 min. In addition, ^{123}I-IMT uptake rates with varying concentrations of ^{123}I-IMT (2.5–50 μM) in the medium were quantified to assess the kinetic parameters of ^{123}I-IMT transport. Furthermore sodium-dependent and sodium-independent competition of ^{123}I-IMT with other amino acids, which are known to be transported by the L-system, was investigated.

Results. ^{123}I-IMT showed similar uptake rates into rat C6 glioma cells and astrocytes with an asymptomic approximation to a steady-state level. The analysis of the uptake rate of ^{123}I-IMT with different concentrations was concordant with a single uptake system in C6 glioma cells and astrocytes. The Michaelis constants K_m of ^{123}I-IMT were similar for C6 glioma cells (26.2 ±1.9 μM) and astrocytes (18.8 ±1.8 μM). However, maximum transport velocities V_{max} differed significantly between C6 glioma cells and non-transformed astrocytes (3.5 ±0.2 nmol/mg protein/min and 0.6 ±0.3 nmol/mg protein/min, respectively). Sodium-independent uptake of ^{123}I-IMT amounted to 77% and almost 100% of total uptake in C6 glioma cells and astrocytes, respectively. The neutral amino acids L-leucine and 2-aminobicyclo-[2.2.1]-heptane-2-carboxylic acid (BCH), the model substrate of the L-system, significantly reduced the uptake rate of ^{123}I-IMT in C6 glioma cells and astrocytes by more than 80%.

Conclusion. The results described above are consistent with a main uptake of ^{123}I-IMT by rat C6 glioma cells and astrocytes through the sodium-independent L-system. In both cell types ^{123}I-IMT showed similar K_m values but about six times greater transport velocities in C6 glioma cells compared to non-transformed astrocytes.

Modulation of amino acid uptake and release in synaptosomal preparations by hyperforin

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The acylphloroglucinol derivative hyperforin has recently been identified as one of the potential antidepressant component of the therapeutically used extracts of Hypericum perforatum. Initially it was demonstrated to be the major synaptosomal biogenic amine reuptake inhibitor present in the extracts. Soon it became evident, however, that hyperforin is also an almost equipotent inhibitor for the reuptake of GABA and glutamate. Later in vivo studies using push-pull canula and superfusion technique demonstrated that although hyperforin did not modulate the release of GABA, aspartate, serine, arginine, taurine or 5-hydroxyindol acetic acid, it did facilitate the release of glutamic acid and other studied biogenic amines. These later observation led us investigate the potential effects of hyperforin on the synaptosomal release of glutamate and other amino acids and to clarify the mechanisms involved in these effects of the agent. In vitro incubation techniques using purified rat cortex synaptosomal preparations were used for these purpose.

Hyperforin (0.1–3 μM) concentration dependently enhanced the release rate of endogenose glutamate from the synaptosomal preparations and this effect of the agent was observed even in calcium free medium containing EGTA. Calcium measurements using Fura-2 technique revealed that synaptosomal calcium concentration is concentration-dependently (0.1–3 μM) increased by hyperforin and that this effect is also independent of calcium concentration in the medium. Using a specially developed filteration technique and HPLC analysis method for several amino acids we confirmed that hyperforin enhances the release of glutamate, aspartate and GABA but not those of various other amino acids. Efforts made till now to clarify the mechanism(s) revealed that transporter proteins, protein kinases and the mitochondrial compartment of the synaptosomes are most probably not involved in the observed effects of hyperforin. Several circumstantial evidences indicate, however, that modulation of calmodulin mediated processes and volume regulated ion channels are closely associated with the mode(s) of action(s) of hyperforin.

These observation not only reconfirm that the mechanism involved in the antidepressant like efficacy of hyperforin is not similar to the till now known antidepressants, but indicate also that modulation of the storage and release of amino acid neurotransmitters via intracellular mechanisms could be involved in its unique pharmacological activity profile.

Insulin resistance as a consequence of aspartate and asparagine supplementation

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Aspartate (asp) and asparagine (asn) supplementation increases the muscle glycogen concentration and also the time to exhaustion in rats. Recently, we published that the supplementation of those amino acids (45 mg/kg of body weight per day) for five days increases the muscle glycogen content and decreases the glucose transport in epitrochlearis muscle incubated with 2-deoxyglucose. The present paper investigates the effect of asp/asn supplementation (45 mg/kg of body weight per day) for seven weeks on the insulin resistance measured by euglicemic clamp (4 μU of insulin/kg body weight per minute) in rats. The animals weighting around 110 g received asp/asn in the drinking water. The first euglicemic clamp occurred at the first day before the amino acid supplementation started. The animals were kept for seven weeks and the euglicemic-clamp procedure were repeated at the 5th and 7th week. The results, expressed by mg of glucose infused by kg of body weight (BW) per minute, indicate that the supplementation for seven weeks reduces the insulin sensitivity close to 50% (control 21.83 +0.82, 5 weeks 20.07 +1.88 and 7 weeks 11.70 +2.11 mg/kg BW per minute, $p < 0.01$). This effect occurred probably by some modification on the intracellular process of glucose transport translocation since the tyrosine kinase activity didn't show any difference between groups (control and supplemented) thus allowing to conclusion that asp/asn has some effect upon the process involving the glucose transport vesicle docking to the membrane.

[Supported by FAPESP (93/4334–5).]

Physiology

Relationships in metabolism of BCAA and glutamine

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Enhanced catabolism of skeletal muscle, increased oxidation of BCAA and increased utilisation and synthesis of glutamine are characteristic metabolic features of SIRS caused by severe infection, trauma injury and cancer. Glutamine is mainly synthesized in skeletal muscle and together with alanine constitutes the major portion of amino acids released from muscle to the blood stream. The main donor of nitrogen for synthesis of glutamine and alanine in muscle are branched-chain amino acids (BCAA; valine, leucine and isoleucine). A series of experimental studies using male albino rats has been conducted to provide more information about pathophysiological and therapeutic significance of BCAA and glutamine in SIRS.

The results showed that endotoxemia activates catabolism of BCAA in skeletal muscle [Clin Nutr (1996) 15: 91–93]. However, the suppression of BCAA catabolism associated with conversion of BCKA to BCAA and with release of BCAA to the blood stream occurs in hepatic tissue [Am J Physiol (1997) 273 (Endrocrinol Metab 36): E1052–1058; Metabolism (1998) 47: 681–685]. We hypothesize that this response of hepatic tissue enables the body to prevent rapid loss of essential BCAA and decreases the speed of muscle wasting in SIRS. Other studies have shown that exogenous glutamine or alanyl-glutamine decrease protein breakdown and leucine oxidation both in intact post absorptive controls, in endotoxemia and after irradiation [Am J Physiol (in press); Metabolism (in press)]. In another study, infusion of ammonia salts caused a significant decrease in BCAA levels in plasma and skeletal muscle and an increase in leucine oxidised fraction [J Hepatol (in press)]. These results collectively indicate the increased flux of nitrogen between BCAA and glutamine which enables the body to synthesise remarkably high amounts of glutamine and to prevent the loss of essential BCAA.

Amino acid and protein metabolism after bilateral nephrectomy in rats

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The aim of the study was to evaluate the changes in protein and amino acid metabolism in acute renal failure. Acute renal insufficiency was induced by bilateral nephrectomy (BNX). The control group consisted of sham operated rats. Twenty four hours after surgery the effect of BN on protein metabolism (protein turnover, proteolysis, protein synthesis) and metabolism of branched chain amino acids (oxidation, incorporation in body proteins) was evaluated using primed constant intravenous infusion of L-[1-^{14}C]leucine (1.9 μCi/ml).

BNX induced a significant increase in plasma levels of urea, creatinine, phosphates and potassium and decrease in pH, bicarbonate, BB, BE and trigylceride. In BNX animals decreased plasma levels of Thr, Ser, Asn, Glu, Gln, Met, Tyr, Trp, Lys and Arg were observed while Tau increased. There was also a significant decrease in protein synthesis in skeletal muscle and a decrease in whole-body protein breakdown ($p < 0.01$), protein synthesis ($p < 0.01$) and leucine clearance ($p < 0.001$). The most exciting observation was a marked increase in leucine oxidized fraction ($P < 0.001$) in BNX animals which was caused mainly by the decreased rate of leucine incorporation in body proteins.

We conclude that BNX causes a significant decrease in the whole body protein turnover associated with an increase in leucine oxidized fraction. We suppose that this response can explain rapid wasting of lean muscle tissue in acute renal failure.

(The study was supported by the grand No. 306/98/0046 of the Grant Agency of the Czech Republic.)

Glutamine metabolism in stressed patients

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Burn injury, sepsis and trauma lead to profund alterations of protein metabolism including increase in protein turn-over, increased release of amino acids by muscles and increased metabolism of these substrates in splanchnic areas in order to support accelerated gluconeogenesis and acute phase protein synthesis. These alterations are mainly under the control of hormones (cortisol and glucagon) and mediators such as tumor necrosis factor.

Glutamine (GLN) is a major precursor of glucose in gluconeogenesis and in addition is the main energetic substrate for rapidly multiplicating cells such as enterocytes and cells of the immune system when activated. Also, GLN controls protein synthesis and catabolism in muscle and the liver. In addition, GLN is the precursor and glutamate required for synthesis of glutathion which plays an important role protecting splanchnic areas against free radicals. Finally, stressed patients have a tendency to metabolic acidosis which is counteracted by an increased GLN metabolism in the kidney in order to support ammoniagenesis.

Enhanced GLN requirements in stressed patients are supported by increased *de novo* synthesis by muscles and lungs. Also, as a part of this adaptation process, the liver switches from a GLN-consuming organ to a GLN-producing organ.

However, when the stress is very intense and/or long-lasting, rate of GLN consumption becomes far above rate of synthesis and this leads to an exhaustion of GLN reserves at the plasma and tissue levels. As a consequence, there is an increase in muscle protein catabolism, an intestinal failure including increased permeability to endotoxins and bacteria, and a dysimmunity state. Taken together, these alterations favour septic complications which will worsen the prognostic.

Hence, counteracting GLN depletion appears to be a major therapeutic goal for patients in intensive care unit.

Glutamine and immune function

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Glutamine is utilised at a high rate by cells of the immune system in culture and is required to support optimal lymphocyte proliferation and production of cytokines by lymphocytes and macrophages. Macrophage-mediated phagocytosis is influenced by glutamine availability. Hydrolysable glutamine dipeptides can substitute for glutamine to support in vitro lymphcoyte and macrophage functions. In man plasma and skeletal muscle glutamine levels are lowered by sepsis, injury, burns, surgery and endurance exercise and in the overtrained athlete. The lowered plasma glutamine concentrations are most likely the result of demand for glutamine (by the liver, kidney, gut and immune system) exceeding the supply (from the diet and from muscle). It has been suggested that the lowered plasma glutamine contributes, at least in part, to the immunosuppression which accompanies such situations. Glutamine or its precursors has been provided, usually by the parenteral route, to patients following surgery, radiation treatment or bone marrow transplantation or suffering from injury. In most cases the intention was not to stimulate the immune system but rather to maintain nitrogen balance, muscle mass and/or gut integrity. Nevertheless, the maintenance of plasma glutamine concentrations in such a group of patients very much at risk of immunosuppression has the added benefit of maintaining immune function. Indeed, the provision of glutamine to patients following bone marrow transplantation resulted in a lower level of infection and s shorter stay in hospital than for patients receiving glutamine-free parenteral nutrition. Animal studies have shown that inclusion of glutamine in the diet increases survival to a bacterial challenge.

Leucine and methionine metabolism during administration of methionine free diet

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Growth of most malignant cells are dependent on methionine supplement. Indeed, methionine free nutrition slowed tumor growth in rats. We investigated leucine and methionine metabolism in tumor bearing rats receiving methionine free nutrition, as compared with standard diet. Fractional synthesis rate of tumor, liver, and muscle in rats receiving methionine free nutrition was measured, using a constant infusion of 1-^{14}C-leucine. The rate of tumor protein breakdown was measured by the method of Istfan et al. We measured endogenous methionine production by a constant infusion of ^{3}H-methionine technique, using equation of Steel. ^{3}H-methionine incorporation to the tumor protein was measured in mice receiving methionine free diet. Methionine free TPN increased whole body protein breakdown rate and this was associated with an increase in methionine production. Tumor weight was decreased by administration of methionine free TPN as compared with standard TPN solution, because tumor protein breakdown rate was increased. Tumor protein synthesis rate, on the other hand, was unaltered by administration of methionine free TPN. Methionine free diet enhanced ^{3}H-methionine incorporation into the tumor protein as compared with standard diet. These results suggested that methionine free nutrition slowed tumor growth by an enhancement of tumor protein breakdown, and tumor protein synthesis was not reduced because endogenous methionine entered into the tumor cells. In

based on the above results, we used methionine as a carrier of the anticancer drug to tumor. Mitomycin was conjugated with methionine by peptide bond, using carbodidimide. Methonine-mitomycin conjugate injection with supplementation of methionine free diet reduced tumor growth as compared with standard diet plus methionine-mitomycin conjugate in mice. This is a novel approach to target mitomycin to the tumor.

The nutrition and the development of amino acid levels during the first year of life

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Plasma amino acid levels show considerable dynamic changes during the first year of the life. This trend is also influenced by the nutrition of the children. We can divide the first year of the life to three periods.

1. 0–10 days. This part of the life is the starting time for function of all the homeostatic systems. The same time is period of the "tuning" between above systems. The catabolism is connected with fast consumption of glycogen and mobilization no-esterificated fatty acids during the first three days. We can see rising of these amino acids, which have intracellular levels much higher than plasma levels. It evidences for currently proteolyses. Above situation is typical for glutamin and prolin. Essential amino acids decrease in this period. Part of them is used for the gluconeogenesis, the rest is oxidised in the Krebs circle.

The newborn's weight begins to rise after the third day of life. The decreasing of branch chained amino acids is stopped, glycin gradually decreased and on the other side increase levels of arginine and alanine. The influence of the nutrition (mother milk, formula milk etc.) is in this period very low.

2. Period to the 6 months. The changes in amino acid levels continues in this period, but with smaller rate. However, they strongly depend on the nutrition. We must follow separately amino acid levels in children fed by mother milk and by artificial milks etc. The great changes are especially for taurin branch chained and aromatic amino acids.

3. Period from 6 months to 1 year. This is time of the consolidation of the amino acid levels. Results reflect various diseases or problems in the nutrition. Further greater changes become as far as in adolescent age.

Investigation of amino acid spectrum in plasma and tumorous tissue of children with neuroblastoma and nephroblastoma

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We analyzed free amino acid profile in tunorous tissue and blood plasma of children (age 1–14 years) suffering from neuroblastoma and/or nephroblastoma (Wilms' tumor of kidney) by the use of high-performance liquid chromatography. The results demonstrate that there is a significant difference in content of free amino acids of the considered tumors. Neuroblastoma contains about a half amount of total free amino acids in comparison with nephroblastoma. (59.78 ±44.88 vs. 103.03 ±43.37 μmol/g). Nevertheless, the percentage of water (humidity) of both kinds of tumorous tissue is very similar (85.1 vs. 81.0%). The Fisher index is also similar in both kinds of tumors.

Of the single amino acids, *in neuroblastoma* there is more significantly ($p < 0.01$) *decreased* average content of *taurine, histidine, arginine,* less decreased ($p < 0.05$) are serine, asparagine, glutamate, tryptophan and lysine, on the other hand higher *values,* oppositely to nephroblastoma (not significant), had been measured in case of threonine and ornithine. However, the concentration of *free amino acids in blood plasma* is in both kinds of tumors practically even. (2378 ±632 vs. 2311 ±292 μmol/l) as well as *Fischer index* (3.765 vs. 3.318). It was found significant differences in content of free amino acids among ganglioneurinoma (a benign tumor) and neuroblastoma. This study may be helpful to the application of imbalanced amino acids for correction of metabolic disturbances in patients suffering from malignancy.

Can amino acids loss during dialysis therapy of chronically renal insufficient patients be avoided?

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Introduction: Under dialysis therapy the classical symptoms of uremic patients with chronic renal insufficiency can be eliminated or reduced but the late adverse effects of uremia like changes in metabolism, changes of immunological potentials, changes in endocrinological and regulatory processes cannot be excluded. A central problem in the treatment of those patients is the tremendous disturbance in the metabolism of proteins, peptides and amino acids which are the main reason of morbidity and letality under these conditions. Besides the very strong stimulation of catabolism the supporting dialysis therapy itself has substantial additional load not only by the loss of pathological change of metabolic functions of the ill kidneys but also in addition by the loss of proteins and especially amino acids during the therapeutic procedure.

Study design, materials and methods: Our presentation demonstrates the study with the high aim to avoid the loss of amino acids by compensating the natural gradient of concentrations between blood and dialysis fluid for amino acids. To achieve this effect with avoidance of amino acid loss during dialysis therapy and with quantification of the effects, investigations were done in 10 patients with chronic renal insufficiency under continuous dialysis therapy in 10 consecutive treatment sessions. Before and after the dialysis we measured the micromolar concentrations and the percentage profile of each amino acid in plasma to quantify the changes in the amino acids profile in plasma by the dialysis itself. The instrumental equipment for the dialysis treatment is a closed system which allows to measure quantitatively the amino acid changes also in the dialysate fluid. To eliminate the natural gradient between plasma and dialysate fluid the amino acids' composition within this compartment, the fluid was composed in the same micromolar concentration and in the same distribution profile of each amino acid in the fluid like the state in plasma under physiological conditions. The amino acid composition in the dialysate fluid in the fresh prepared solution and at the end of the dialysis were measured. We have constructed the amino acid profile in the dialysate fluid equivalent to reference ranges which were measured in 300 healthy blood donors, we follow the hypothesis that even in the case of imbalances in plasma the application of a physiological pattern in the dialysate fluid the concentration gradient produce a physiological pattern in blood. The analyses are performed with low pressure liquid chromatography with postcollum derivatization with ninhydrin which allows a quantitative determination of the physiological amino acids in plasma and in the dialysate fluid. The documentation and the graphic interpretation of the results follows new concepts and allows the differentation between balanced or imbalanced states. The reference range was determined for 23 amino acids with micromolar concentrations and the presentage profile which are strongly determining the cellular support and waste elimination.

Results: The main results are given as median of amino acid concentrations in plasma before and after dialysis ranging from

2650 ±300 µmol/l to 2928 ±294 µmol/l for total content. The concentration for each amino acid and the determination of the variation is given for all amino acids. The equivalent results are given for the dialysate fluid before and after dialysis. The main result is the reduction of imbalances of these chronic impaired patients which show that not only the imbalance in plasma disappears or is reduced but also the natural outflux and loss of amino acids is dramatically reduced.

Conclusion: The gradient free dialysis of chronic renal insufficient patients reduces the imbalances of amino acids in the compartment of blood plasma which is responsible for the cellular uptake of amino acids. The most important result is that no further amino acid loss are provoked if instead of the traditional open dialysis therapy a gradient free control of amino acid status in these patients is applied.

Protein and amino acid metabolism during space flight

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Space flight is associated with a chronic loss of protein from the muscles with anti-gravity functions which are located in the trunk and legs. Protein turnover is increased early inflight followed by a substantial decrease (45%) with increasing time in space (3+ months). The changes in protein metabolism are due to both the effects of space flight and the dietary intake of the individual astronaut/cosmonaut. The changes in protein turnover are accompanied by changes in amino acid metabolism. Analysis of the plasma amino acids and has proven to be a useful tool for investigating human protein metabolism. They can provide an index of substrate availability for protein synthesis. In addition measurement of the urinary 3-methylhistidine (3-MeH) excretion provides an estimate of muscle protein breakdown. To investigate these changes we measured the plasma amino acid distribution patterns before, during and after flight on the space shuttle together with the urinary 3-MeH excretion. The plasma samples were collected from the four payload crew of the 1993 SLS2 shuttle mission. Samples were taken 45, 15 and 8 days before flight, inflight on days 2, 8 and 12 after launch, post flight on the day of landing and again 6, 14 and 45 days after landing. Results: Most of the changes found pertained to the essential amino acids, particularly the branched chain amino acids (BSAA). (i) The plasma aminograms for inflight days 8 and 12 were very similar and both aminograms were very different from that of flight day 2. Flight day 2 was not different from the preflight ground control. (ii) With increasing time in space, there was an increase in the concentration of leucine and isoleucine in the plasma ($p < 0.05$). (iii) The concentrations of the total essential amino acids and the BCAA in particular were decreased on the day of landing ($p < 0.05$), (iv) 3-MeH excretion was unchanged on this mission although it has been reported to be increased on other missions.

Protein depleted mice: Limiting amino acids in protein turnover

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Amino acid catabolism is a major component of the obligatory N loss in protein deprived animals. Some of this catabolic loss is due to the utilization of amino acids as metabolic precursors of other N-containing molecules. The loss of non-essential amino acids can be minimized by their replenishment from other N sources. The catabolism of any essential amino acid, however, is amplified but the loss of other amino acids, because their major fate is protein turnover. Since protein synthesis requires all 20 amino acids in adequate proportions, the irreversible loss of any essential amino acid prevents the reincorporation of an equivalent amount of the other 19, which must then be catabolized. Thus, when any essential amino acid is utilized as a metabolic precursor in protein depleted animals, *it becomes limiting in protein turnover.*

In this study we attempted to define the limiting amino acids in protein depleted mice. We showed that Met, Phe and Trp, and to a lesser extent the branched-chained amino acids, as well as their keto-derivatives, had a protein sparing effect measured by the oxidation of protein-bound leucine, urea excretion and hepatic protein degradation.

These anabolic effects could be obtained whether the amino acids or the keto derivatives were administered intraperitoneally, intragastrically or subcutaneously. The effect of subcutaneous injections demonstrated in the portal circulation was not necessary.

The anabolic effects could be obtained with relatively small amounts of the key amino acids (7 µmol each). A trebling of this amount provided little additional benefits.

(Supported by NIH grant R01 DK43833.)

Biochemical basis of domoic acid-induced neurotoxicity

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Domoic acid (DA), a shellfish toxin, produced GI distress, followed by a deficit in short-term memory in patients who consumed steamed blue mussels *(Mytilus edulis).* Three patients died as a result of mussel intoxication. The mechanism(s) by which DA induces neurotoxicity is not fully defined. Results of our studies demonstrate (i) that the mussel-induced biotoxicity was directly proportional to DA level in mussel, (ii) that the toxin was highly concentrated in mussel stomach, which was engorged with phytoplankton containing predominantly diatom *Nitzschia pungens* which was the source of DA, and (iii) that DA was depurated from toxic mussel with time, and (iv) that DA was purified by extraction of mussels with chloroform: methanol mixture, followed by column ion exchange- and high performance liquid-chromatography. Intracellular level of Ca^{2+} ($[Ca^{2+}]i$) was elevated by DA, and by glucose deprivation and removal of Na^+. Effect of glucose deprivation was dependent on incubation time. Sodium fluoride, which inhibits glucose metabolism, in the presence of glucose elevated $[Ca^{2+}]i$. Glucose metabolism and production of energy, i. e. ATP are critically important for the regulation of Ca^{2+} homeostasis in brain. DA also binds to brain membranes, which was inhibited by extracellular ATP, and by blockers of Ca^{2+} and Na^+ channels. These results indicate a relationship between DA-induced neurotoxicity, glucose metabolism and ATP production, and Na^+ and Ca^{2+} transfer across brain membranes.

The role of branched amino acids and tryptophan in post-operative and chronic fatigue

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The neurotransmitter 4-hydroxytryptamine (5-HT) is involved in fatigue and sleep. Its precursor, tryptophan, binds to albumin in the blood. Increased mobilisation of plasma free fatty acids (FFA), which also bind to albumin, leads to more free tryptophan (FT) and thus to increased brain 5-HT which may cause central fatigue. The branched chain amino acids (BCAA) com-

plete with tryptophan for entry into the brain across the blood-brain barrier, thus elevated plasma FT results in an increased plasma concentration ratio of these amino acids (p[FT:BCAA]). Central fatigue is implicated in chronic fatigue syndrome (CFS) and post-operative fatigue.

Plasma albumin, FT, total tryptophan and BCAA were measured before and after major surgery; and in CFS patients before, during and after maximal exercise on a bicycle ergometer. Ethical permission was obtained for all studies. In CFS patients, plasma FT was higher pre-exercise than in controls ($p < 0.05$) but did not change during or after exercise. Plasma FT increased in the controls at maximal exercise ($p < 0.02$), peaking at 5 min post-exercise ($p < 0.001$), returning to baseline at 60 min. The baseline p[FT:BCAA] appeared 31% higher ($p < 0.1$) in the CFS patients than the controls and remained at similar levels during and after exercise. The apparent failure of these patients to change the p[FT/BCAA] during exercise may indicate increased sensitivity of brain 5-HT receptors as demonstrated in other studies (Cleare et al, 1995).

During post-operative recovery in both elderly and coronary artery bypass graft patients (CABG), plasma FT concentrations were significantly increased by 61% and 37% respectively, compared with baseline levels; the p[FT:BCAA] was also increased (Yamamoto et al, 1997). Plasma albumin concentrations were significantly decreased after surgery in both elderly (28%) and CABG patients (20%). In a recent pilot study, an increase in fatigue (measured by the Profile of Mood States questionnaire) observed after major surgery showed a trend towards correlation with plasma FT. Provision of BCAA has improved mental performance in endurance athletes (Blomstrand et al, 1997). BCAA supplementation might help to counteract the effects of an increase in plasma FT, and thus improve the symptoms associated with post-operative or chronic fatigue.

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Roles for intracellularly synthesized glutamine in intestinal epithelium

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Although several studies demonstrate beneficial effects of exogenously supplied glutamine (Q) via the enteral or parenteral route in stressed individuals, very little information is available regarding the potential role for intestinal epithelial derived Q. Previous studies from our laboratory have shown that the small intestine has the capability to synthesize Q via glutamine synthetase (GS) and that this enzyme demonstrates development-related changes in expression and activity. This enzyme and its mRNA are preferentially localized to the proliferative crypt region. In the present experiments, methionine sulfoximine (MS) inhibition of Q synthesis in proliferating (IEC-6) and differentiating (Caco-2) cells was used to determine a role for intestinally synthesized Q. In uninhibited IEC-6 cultures, proliferation markers (cell number, protein and DNa accumulation and synthesis) showed a dependence on extracellular Q over a concentration range of 0.06 to 1.06 mmol/L, with apparent half-maximal responses of 0.46 mmol/L extracellular Q. In contrast, these measures of proliferation in GS-inhibited cultures required 1.06 mmol/L Q for half-maximal response and were reversed by supraphysiologic concentrations of extracellular Q. Q-uptake was not affected. Inhibition of GS in Caco-2 cells decreased sucrase-isomaltase activity, trans-epithelial electrical impedance, microvillus formation and intercellular tight junction integrity. These findings suggest important roles for intestinal GS in small intestinal epithelial proliferation and integrity, which we hypothesise are due to GS-derived-Q-mediated synthesis of hexosamines, nucleotides and proteins. We speculate that natural inhibitors of this enzyme could be deleterious to normal intestinal function.

Branched-chain amino acid aminotransferase activity decreases in sheep skeletal muscle during development

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Branched-chain amino acid aminotransferase (BCAT, EC 2.6.1.42) catalyses the reversible transamination of branched-chain amino acids (leucine, isoleucine and valine). Two isoenyzmes [mitochondrial (BCATm) and cytosolic (BCATc)] have been identified in mammals. BCAT specific activity decreases in sheep tissues during the development. Our goal was to characterize mechanisms involved in that decrease, which is specific to the ovine species. We focused our study on skeletal muscle which is, due to its mass, the main in vivo site of BCAA transamination.

BCAT specific activity has been quantified in diaphragma, longissimus dorsi, semi-mebranosus, semi-tendinosus and tensor fascia latae from fetus (-6 d, n = 6), new born (1 d, n = 6), preruminant (16 d, n = 5) and ruminant (59 d, n = 5) lambs. It varied from 1.0 ±0.6 (tensor fascia latae from weaned animals) to 8.1 ±1.0 nmol/min/mg prot (longissimus dorsi from fetus). The type of muscle and the age have a significant effect on BCAT specific activity (two-way anova, $P < 0.05$). BCATc and BCATm specific activities have been evaluated from immunoprecipitation data obtained with a specific antiserum anti-sheep BCATm. The type of muscle and the age have a significant effect on BCATc and BCATm specific activities too. However, the magnitude of the decrease is much higher for BCATc than for BCATm. We conclude that BCATc is mainly responsible for the decrease in BCAT activity in sheep skeletal muscle during the development and that the regulation of BCATc and BCATm expressions involves distinct mechanisms.

Concentration of amino acids in patients with colorectal carcinoma

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Introduction: Each catabolic condition appears to have its own reproducible intracellular pattern – The extracellular concentration of amino acids does not exactly reproduce the real amino acids pool – Muscle tissue is considered to be ideal for measuring of amino acids concentration, because it is by far most abundant cellular tissue, it is relatively uniform as to cellular composition, the biochemical changes are known to accompany circulatory and nutritional disorders in this tissue.

The aim of study: Evaluate intracellular and extracellular concentrations of amino acids in patients with colorectal carcinoma and to compare them to concentrations of amino acids in healthy tissues.

Patients and methods: *Patients.* 1996–1998
50 adult patients with localized colorectal carcinoma
22 women and 28 men,
age 35–80 years, mean age 62,76 ±12.95 years
Nutritional status of patients. BMI 22.47 ±3.19.
Total plasma protein 66.48 ±7.51 g/l – albumin 33.63 ±4.69 g/l – 10 patients lost more than 5 kg in the period of 1 year before the operation.

Analysis. Amino acids were determined by HPLC – High performance liquid chromatography.

Controls. For plasma aminogram – age and sex matched 20 healthy volunteers – for tumorous tissue (intracellular aminogram) – abdominal muscle.

Perspectives: The better understanding of amino acid concentration in tissue could help us for better composition of artificial nutrition for patients with malignant disease. – The correlation with normal values might be used in the future as the type of new tumorous marker.

Cranial electrical stimulation in the therapeutic utilization of amino acids: Assessment and treatment for the electromagnetic dimension to health

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CES increases the conversion of amino acids to neurotransmitters. Its particular mode of operation is as a corrective measure for brain dysrhythmia. Brain rhythm influences the development of psychiatric conditions as well as poses as a trigger that induces physical manifestations of illness. Through the positioning of the stimulating electrodes, CES connects the diurnal rhythm of head and heart through the vagus nerve. It thereby functions as an anti-dysrhythmic on a whole-body level.

Proper application of CES therapy depends on the accurate assessment of the extent of brain dysrhythmia. Brain Electrical Activity Mapping (BEAM) is a diagnostic well-suited diagnostic to evaluate the brain's neurobioelectrical status. Through QEEG and evoked potential measurements, a series of full-colour images are produced, permitting the confirmation of disease states including anxiety, depression, and insomnia – in addition to epilepsy, psychiatric conditions, head trauma, memory loss and Alzheimer's, Parkinson's, personality disorder, and dysfunction of the lobes of the brain.

Once proper diagnosis is made, an effective treatment program, the foundation of which is amino acid therapy, may be prepared. Key amino acids essential for neurotransmission and brain health include:

- Phenylalanine: in pain perception
- Tyrosine: as an anti-depressant
- Tryptophan and melatonin: in anxiety, depression, and sleep disorder
- Taurine: as anti-seizure
- Glutamate amino acids: for brain function as well as promoting growth hormone levels; gamma aminobutyric acid (GABA) in natural free-form and the medication gabapentin for anxiety, seizure, depression, and mood swings
- Serine: role in psychiatric disorders, mood, and memory.

CES is the Electromagnetic Doorway to good health. It works as an important adjunct in programs invoking nutrients, hormones, and medications. Its multiplicity of health benefits include: induction of relaxation response; exertion of control over frontal lobe behaviour; modulating hormone levels; a possible antidote to electromagnetic field radiation. When amino acid supplementation is coupled with CES therapy, this combination is a potentially potent therapeutic regimen for anti-aging.

Relationship between MAO activity and DNA content of various normal and abnormal tissues of humans and animals

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DNA content appears to be a good reference substance to express the activity of many biochemicals including enzymes of various tissues. However, it is not true as DNA of abnormal tissues varies significantly.

Biological amines are deaminated in the presence of monoamine oxidase in various tissues. The activity of these enzymes varies significantly and so do the DNY content which has been reported in our three studies. The purpose of this investigation is to establish the relationship between MAO and DNA of various normal and abnormal tissues.

MAO-activity was determined using colorimetric method and radiochemical method while DNA-content was analysed using colorimetric method.

In this study, MAO-activity and DNA-content of the cirrhotic human livers show 20.7% and 163.4% than those of normal respectively. In the second study, MAO-activity and DNA-content of livers of rats maintained on a pantothenic acid deficient diet show 65.5% and 146.7% than those of normal respectively. In the third study, MAO-activity and DNA-content of the uteri of ovariectomized rats show 18.0% and 204.8% than those of normal respectively. These preliminary observations suggest an inverse relationship between MAO-activity and DNA-content of various abnormal tissues. Further, the analysis of MAO-activity and DNA-content of various other abnormal tissue might serve as a reliable marker during deteriorative conditions.

The relationship between the effects of amino acids and pancreatic enzymes

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The anti-inflammatory effect of amylase remains even after passing this enzyme through the gastrointestinal wall. Consequently this activity is maintained after the decomposition of amylase on lower components.

Common features of cysteine and amylase exist. Both accelerate the passage of gelatine solution through a viscostalagnometre, prevent the gelling of this solution, and influence the iodine coloration of starch.

The anti-inflammatory reaction was studied firstly on rat paw, which was injected with 0.1 ml of 6% dextran solution. The tested group (10 rats) received a single dose of cysteine (10 mg/1 ml) administered once by gastric sond. The control group (11 rats) received 1 ml of physiological saline. Measurement in the 90th minute from the start of the test showed that the reduction of the size of the oedema was 36.2% larger in the cysteine group than in the case of the controls.

Secondly granuloma was induced by injecting 0.5 ml of carragenin solution in the dorsum of rats. The tested group (10 rats) received cysteine 10 mg/1 ml by gastric sond, the control group (10 rats) 1 ml of physiological saline once a day for 5 days. The granuloma was then isolated and weighed. In the cysteine group, the granuloma was 51.4% lighter than in the controls.

The anti-inflammatory properties of cysteine constitute a promising similarity with amylase, raising the question whether the liberated amino acids from amylase can assume the function of the destroyed enzyme.

Effect oft L-tryptophan on the recovery of carbon tetrachloride-induced chronic liver injury in rats

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We have reported that L-tryptophan (Trp) administration to rats with carbon tetrachloride (CCl_4)-induced chronic liver injury alleviates the liver injury by maintaining liver protein synthesis. In order to further clarify the therapeutic effect of Trp on CCL_4-induced chronic liver injury, we examined the effect of Trp on the re-

covery of the liver injury in rats. Male Wistar rats aged 6 weeks were injected with CCl_4 (1.0 ml/kg) or saline every day for 2 weeks. The animals were further injected with Trp (50 mg/kg) or saline erveryday for 2 weeks. Transaminases (AST and ALT), albumin, and trigylceride (TG) were assayed in sera. Protein, TG, tryptophan 2,3-dioxygenase (TDO), and lipid peroxide (LPO) were assayed in livers. Rats injected with CCl_4 for 2 weeks showed increased AST and ALT activities and decreased TG concentration in the serum and increased TG and LPO contents and decreased TDO activity in the liver, but no changes in serum albumin concentration and liver protein content. After 2 weeks of saline injection, the rats with chronic liver injury showed an incomplete recovery of the increased AST and ALT activities and decreased TG concentration in the serum and the increased TG and LPO contents and decreased TOD activity in the liver. Two weeks of Trp administration to the rats with chronic liver injury caused an almost complete recovery of these changes. These results indicate that Trp stimulates the recovery of CCl_4-induced chronic liver injury in rats by enhancing improvements of disrupted hepatic TG and Trp metabolisms and enhanced hepatic lipid peroxidation.

A role for D-serine in the Central control of blood pressure?

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NMDA receptors in the medulla and spinal cord are thought to be involved in the regulation of blood pressure. It is now fairly well established that the NMDA binding site and the strychnine-insensitive glycine binding site are tightly coupled allosteric domains of the NMDA-receptor channel complex. Kynurenic acid is an *endogenous* antagonist and D-serine is an *endogenous* agonist at the NMDA-glycine site. Twe have therefore examined the distribution of (a) kynurenine amino-transferase (KAT) which is responsible for the synthesis of kynurenic acid and (b) D-amino acid oxidase (DAO) which is an enzyme of D-serine catabolism and can thus be used as an inverse marker for D-serine. KAT and DAO were localised by immunohistochemistry and histochemistry respectively in the brain and spinal cord of Wistar Kyoto rats. KAT like immunoreactivity (KAT-li) was observed in both glia and neurons throughout the brain and spinal cord. DAO activity, however, was found only in glial elements and only in the cerebellum, medulla and spinal cord. The nucleus of the solitary tract (NTS), the rostral and caudal ventrolateral medulla (RVLM and CVLM) and the intramediolateral column (IML) of the spinal cord showed staining for KAT-li but were lacking in DAO activity.

The differential distribution of KAT-li and DAO taken together with the absence of DAO but the presence of KAT-li in certain nucleii of the CNS, indicates a strong interaction between D-serine and kynurenic acid at the NMDA-glycine site in areas that are integral to the central control of blood pressure.

Analysis and Geochemistry

New perspectives on ancient proteins through matrix-assisted laser desorption ionization mass spectrometry

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The small quantity of material retained in fossils coupled with the inability to obtain sequence information by classical Edman degradation are two of the most difficult problems in structural characterization of ancient proteins. We purified picomole quantities of the bone protein osteocalcin (OC) using a microbore reversed phase high performance liquid chromatography method and subsequently characterized this material by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). The presence of OC in modern and ancient bones is suggested by SCD-polyacrylamide gel electrophoresis (SDS-PAGE) and radioimmunoassay (RIA). A band consistent with the molecular weight of OC is indicated by SDS-PAGE of material isolated from 800 yr BP and 12,000 yt BP bones. Concentrations of OC estimated by RIA are 0.2 to 450 ng/mg of bone for samples between 800 and 53,000 yr BP. The molecular weight of in-tact OC in modern samples was determined by MALDI-MS and protein sequences were obtained by peptide mass mapping and a novel derivatization approach with post-source decay analysis. MALDI-MS data for three ancient samples with RIA confirmed osteocalcin (800 yr BP, 12,000 yr BP and 53,000 yr BP) indicate peaks with a molecular mass within the range of modern OC.

Stable nitrogen, carbon and sulfur isotope compositions of proteinaceous materials and their amino acid constituents

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The stable carbon, nitrogen and sulfur isotope compositions of proteins and their amino acid constituents are powerful probes with respect to delineating biosynthetic pathways and reconstructing food webs. The information that can be obtained from each element is unique, owing to differences in source resolution and to distinct isotope fractionations associated with their respective incorporation into the biosphere. The bulk tissues of plants have stable carbon isotope compositions that reflect their respective photosynthetic pathways (e. g. C-3 vs C-4) and these carbon isotopic signatures are retained with increasing trophic position (± a few per mil). However, owing to the complexity of biosynthetic processes, individual amino acid constituents of plant and animal protein exhibit distinct, but predictable differences in carbon isotopic composition. Nitrogen enters the biomass in several forms (e. g. N_2, NH_4^+, NO_3^-), and enrichments in ^{15}N with increasing trophic level affords a unique probe for distinguishing herbivores from carnivores. As in the case of carbon, amino acid constituents of an organism's protein exhibit a predictable range of stable nitrogen isotope compositions. Unlike carbon and nitrogen, sulfur is more directly incorporated into biomass protein (e. g. cysteine, methionine) with minimal fractionation. Because of the unique isotopic signatures of marine and terrigenous sulfur, it is possible to distinguish and quantify the relative contributions of the primary food sources to coastal organisms based on their stable sulfur isotope compositions. Ex-

amples are presented to demonstrate the power of using three stable isotope probes (carbon, nitrogen, sulfur) for the study of proteinaceous materials and their respective amino acids to reconstruct ancient diets, trophic levels and marine vs terrigenous food sources for coastal communities.

Comparison of different methods for the determination of total homocysteine, phenylalanine and leucine

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The standard amino acid analysis (AA) by ion exchange chromatography is a valuable tool for the diagnosis of inborn errors of metabolism. However, for the monitoring of certain diseases, like phenylketonuria (PKU), maple syrup urine disease (MSUD), or homocystinuria, the method has certain advantages, but many disadvantages. The amount of plasma that is needed for the analysis is relatively high, and venous puncture is inapropriate for home monitoring. In addition, for the determination of total homocysteine (tHcy), plasma has to be separated from the blood cells almost immediately after puncture.

In almost all neonatal screening programs, capillary blood collected and dried on filter paper is used for the analysis. Because this is a very simple sampling procedure, methods using dried blood, were compared with methods using plasma.

For the determination of phenylalanine 4 different methods were compared: (1) Standard amino acid analysis by means of ion exchange chromatography; (2) The ISOLAB NCS phenylalanine determination kit, based on fluorescence enhancement of a phenylalanine-ninhydrine reaction product by the dipeptide L-leucyl-L-alanine; (3) the Quantase kit (Tantase Neonatal Diagnostics) for enzymatic determination of phenylalanine, and (4) the Guthrie Test as a bacterial inhibition assay (BIA). For the determination of leucine, the plasma amino acid analysis was compared with the ISOLAB NCS leucine determination test kit. For the determination of tHcy, the procedure of the AXIS ELISA test kit was modified to fit for dried blood samples, and the values were compared with plasma tHcy measured by GC/MS. For phenylalanine the different methods correlated well, with the restriction that the BIA was linear only up to 800 µmol/l.

For the monitoring of MSUD it seems that AA is the best method, because the NCS kit does not discriminate between leucine and isoleucine.

Determination of tHcy from dried blood samples (db) gave reliable results compared to plasma tHcy determined by GC/MS, with a mean different of –0.9 µmol/l (plasma – db) and a SD of 2.88 µmol/l. The mean value for tHcy in a newborn population was 4.3 ±3.0 µmol/l.

Determination of amino acids by ESI-tandem mass spectrometry

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Background: Routine methods for amino acid determination, e. g. ion exchange chromatography with ninhydrin detection are usually time consuming, expensive and not suitable for high throughput analysis of a large number of samples. Tandem mass spectrometry with atmospheric pressure electrospray ionisation (ESI-MSMS) has recently been shown to be suited to screen human new-born blood samples for the presence of inborn errors of metabolism. We describe here the analytical procedure for the quantitation of 16 amino acids out of 3 µl of whole blood within an instrument running time of 3 min.

Methods: A blood spot from a filter paper equivalent to 3 µl of blood was punched out and transferred to a 96 well microtiter plate. After addition a set of 10 stable isotope labelled amino acids as internal standards (500 to 2,500 pmol/sample) amino acids were extracted with 150 µl of methanol. The dried residue was derivatised with butanolic hydrochloric acid (56° C, 20 min) and subjected to MSMS analysis (PE-SCIEX API 365 LC/MS/MS).

Results: Neutral loss scanning of 102 D was suitable for the quantitation of threonine, serine, proline, histidine, alanine, aspartic acid, glutamic acid, methionine, tyrosine, phenylalanine, isoleucine/leucine and valine. Glycine was detected by a loss of a 56 D fragment whereas a 119 D loss was suitable for the measurement of citrulline, ornithine, arginine and lysine. Specific problems encountered: due to their identical molecular weight isoleucine and leucine could not be quantitated separately, and, due to its instability, glutamine and asparagine were found to be decarboxylated to its respective acids. Apart from these findings, there was in general a good correlation between the results of ESI-MSMS and ion exchange chromatography. Determination was linear over concentration range tested (20 to 100 µmol/l) and intraassay and interassay coefficients of variation were in the range of 10 to 15%.

Conclusion: ESI-MSMS proved to be a highly sensitive, linear and sufficiently precise method for the quantitative determination of amino acids in physiological samples. This method can thus be used for large scale screening applications when speed and cost effectiveness are mandatory.

Determination of amino acids in microdialysis fluid and comparison to plasma values

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Background: Microdialysis is a new technique for the monitoring of low molecular weight substances in interstitial fluid by means of a continuously perfused artificial capillary with a semipermeable membrane placed into various tissues. Its clinical usefulness had been demonstrated for bedside monitoring of e. g. glucose, lactate, glycerol and glutamate. In the present study we investigated the feasibility of microdialysis to determine tissue concentrations of amino acids. In addition, subcutaneous tissue amino acid concentrations of five new-borns were assessed and compared to plasma values.

Methods: The suitability of the microdialysis catheter was evaluated by placing the catheder into Ringer buffer or into a 50 g/l plasma protein solution containing 32 amino acids at a final concentration of 150 µmol/l. The in vitro recovery of amino acids in the microdialysis fluid was measured. In vivo data were obtained from five new-borns where microdialysis fluid and in parallel a blood sample was obtained for amino acid determination. For the purpose of comparison the fraction of non protein bound amino acids was determined in the patients plasma by ultrafiltration.

Results: All amino acids tested crossed freely the microdialysis as well as the ultrafiltration membrane with recoveries close to 100%. In the microdialysate collected from the patients a distinct amino acids composition was found as compared to plasma. Mean subcutaneous tissue concentrations were lower as compared to plasma for taurine, serine, alanine, aspartate, glutamate and ornithine and higher for valine, isoleucine, leucine, methionine, phenylalanine, tyrosine and arginine. These finding indicate net uptake or release from subcutaneous tissue.

Conclusion: Microdialysis offers a convenient and minimal invasive way to study tissue amino acid composition thus being

a promising tool for the study of amino acid metabolism in vivo. Net flux of amino acids into subcutaneous or adipose tissue may give important information on the nutritional state in critically ill patients. However, the relevance of subcutaneous tissue amino acid composition for clinical purposes remains to be established.

Fully automated amino acid analysis with the KNAUER Automatic Amino Acid Multi-Analyzer

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The most powerful methods in amino acid analysis for the clinical field and for food and nutrition analysis are OPA-(pre-column-) [1] and Nihydrin-(postcolumn-)analysis [2]. While OPA offers a very good resolution even for critical separations as for glutamine and asparagine, the classical Nihydrin method allows a stable, highly reproducible and accurate measurement for almost any matrix.

With the Multi-Analyzer KNAUER offers an Automatic Amino Acid Analyzer which – due to its enhanced modular HPLC concept – can be used for high accurate amino acid analysis [3] and for additional compounds like organic acids, keto acids, polyamines (biogenic amines) or antibiotics, antiepileptics and further clinical relevant parameters.

The fully software controlled instrument can be outlined as precolumn analyzer as well as postcolumn analyzer for step or binary gradient methods.

The completely modular construction allows a specific recognition of any hardware part through the system software, i. e., the appropriate detector can be choosen from a set of detectors running. Postcolumn coil can be switched in and off from the solvent line quickly. A scope of applications with this new modular concept will demonstrate its powerful usage in today's multi-method HPLC laboratory.

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A new method for the quantitative determination of protein of bacterial origin on the basis of D-aspartic acid and D-glutamic acid content

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In the past years several methods have been developed for the determination of the proportion which is of microbial origin of the nitrogen-containing substances passed from the rumen into the abomasum or the small intestine. Recently, on examining the D-amino acid content of foodstuffs, particularly milk and milk products, it has been observed that, in addition to D-Ala, D-glutamic acid (D-Glu) and D-aspartic acid (D-Asp) can also be detected in similar quantities, primarily in products which have links with bacterial activity. This gave rise to the idea of examining the DAPA, D-Glu and D-Asp content of bacteria extracted from the rumen of cattle and that of chyme from the same cattle, in order to determine the type of relation existing among these three components, and to establish whether D-Asp and D-Glu can be used in the estimation of protein of bacterial origin. On determination of the DAPA, D-Asp and D-Glu content by means of amino acid analyser and high performance liquid chromatography of duodenal chyme from five growing bulls and of ruminal bacteria from the same bulls, the following values were established. For chyme (and, in brackets, for ruminal bacteria) r value calculated by means of linear regression was 0.778 (0.758) between DAPA and D-Asp, and 0.703 (0.808) between DAPA and D-Glu. The r values between the crude protein content of ruminal bacteria and the markers examined were found to be the following: DAPA, 0.737, D-Asp, 0.7254; D-Glu, 0.614. In the model experiment performed for the re-obtaining of values for protein of bacterial origin the theoretical values were determined on the basis of D-Asp and D-Glu and values approximately 10% higher than the theoretical value on the basis of DAPA. It is therefore recommended that in addition to DAPA these other two amino acids be included among the bacterial protein markers.

Age estimation based on the concentration of D-aspartic and D-glutamic acid of teeth

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In order to ascertain the correlation between D-amino acid content of teeth and the age of the individual, 22 teeth with different ages were collected from the dental clinic of Pannon Agricultural University Faculty of Animal Science Kaposvár, Hungary. The age of the individuals was between 17 and 62 years. The D-aspartic acid and D-glutamic acid of these teeth was determined by Hitachi-Merck LaChrom high performance liquid chromatography using precolumn derivatization by OPA/TATG. The correlation between the age and the D-amino acid content [D/L ratio or ln (1+D/L)/(1-D/L)] was determined by linear regression. The correlation can be seen in the Fig. 1 and 2. It was established that, similarly to the results published in the literature, there was very close correlation between the age and the D-aspartic acid content. The coefficient of correlation (r) was higher than 0.99 in both cases. Very similar correlation was also obtained for D-glutamic acid. In both cases the r was higher than 0.98–0.99. A conclusion can be drawn that besides D-aspartic acid D-glutamic acid can be used for age estimation of the individual at death, but this conclusion needs to be checked by many other investigations. It is supposed that there could be correlation between the age and the other amino acids which are very sensitive to racemization (histidine, phenylalanine, tyrosine or perhaps cystine, cysteine and threonine) but to ascertain this correlation is the mission of the future.

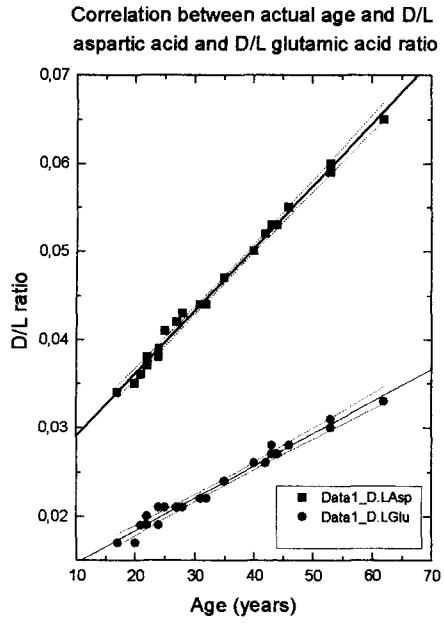

Fig. 1

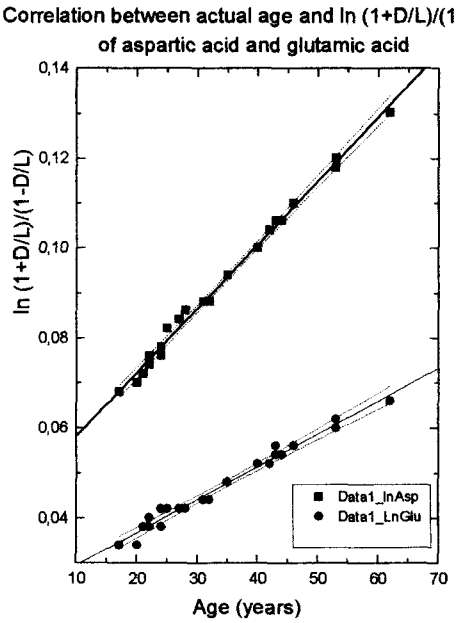

Fig. 2

Analysis of amino acids neurotransmitters in the brain – location, release and total concentration

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Removal of retinal input from a restricted region of adult visual cortex leads to a substantial reorganisation of the retinotopy within the deprived zone. However, the molecular mechanisms contributing to this reorganisation are poorly understood.

Excitatory neurotransmitter, glutamate and inhibitory neurotransmitter, γ-aminobutyric acid (GABA) have been suggested to be involved in the plastic changes. To investigate the function of amino acid neurotransmitters in the adult visual cortex during the reorganisation, several analysis methods have been developed: 1) Immunocytochemistry methods were performed to localise the changes of glutamate, aspartate and GABA-positive neurones in cortical layers II–VI of area 17; 2) an *in vivo* microdialysis method has been designed to sample amino acids release in the extracellular space of area 17 in awake cat. Aspartate, glutamate and GABA in microdialysate were analysed by two microbore HPLC-ED methods; 3) to measure the total (intra- and extracellular) concentration of amino acids neurotransmitters, tissue sample were obtained from 200 μm cryostat section and 2×2 mm^2 grey matter containing the six cortical layers were dissected out of area 17. 13 amino acids in tissue extracts were determined by another microbore HPLC-ED method.

A neurotransmitter mechanism for visual cortical reorganisation of adult cat was developed from the results obtained by all methods.

The molecular basis of hypertension: the ring cluster formed by the amino acid triad Tyr[4]-His[6]-Phe[8] in angiotensin II triggers activity

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The accumulated experimental evidence for Angiotensin II supports a bioactive conformation characterised by a charge relay system between Tyr hydroxyl, His imidazole and Phe carbocylate, analogous to that found in serine proteases [Blow et al (1969) Nature 221: 377], as well as a ring cluster of the triad key amino acids Tyr[4], His[6], Phe[8] which appears to be responsible for activity. Thus conformational analysis using modern 2D NMR techniques in receptor-simulating environments has shown proximity of the three key amino acids sidechains and the formation of tyrosinate has been demonstrated by nanosecond time resolved tyrosinate fluorescence studies. Comparative nuclear magnetic resonance studies of the backbone structure between peptide agonists and antagonists have shown that only agonists display ring clustering and form a charge relay system. In addition the proposed conformation overlays the recently discovered nonpeptide ANG II receptor antagonist Losartan and analogs when molecular modelling techniques and superimposition studies are applied. Finally, the charge relay conformation is supported by the design and synthesis of a novel constrained ANG II cyclic analogue [Sar[1], Lys[3], Glu[5]] ANG II, which possesses biological activity when tested in the rat uterus assay and in anaesthesized rabbits. This potent cyclic analog was designed to have as a major molecular feature the integrity of the ring cluster. Based on structure activity relationships which demand the presence of Phe, Tyr and His for ANG II to possess biological activity it can be inferred that the ability to form a ring cluster and consequently a charge relay system may be the key stereoelectronic molecular features of ANG II for exerting biological activity.

Comparison of the large-scale protein identification in proteomics by amino acid composition analyses and mass spectrometry

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Proteomics is a technology-driven science, studying differential protein expression with the goal to discover novel drug targets and diagnostic tools. It is comprised of two steps, separation of protein mixtures by two-dimensional electrophoresis and identification of the proteins, mainly by mass spectrometry or amino acid composition analysis. Of the mass spectrometric techniques, matrix assisted laser desorption ionization mass spectrometry is the fastest and most often used for serial protein identification. Proteins are usually analyzed directly from the gels, following in-gel digestion with specific proteases. When low-salt-concentration buffers are used, no desalting is required, so that about 800 samples can be analyzed by one person daily. The use of internal standard peptides to correct the measured peptide masses, usually results in an unambiguous protein identification. Amino acid analysis is a classical analytical method, which enjoys a revival nowadays, mainly due to its application in the large-scale protein identification. The method requires a

relatively inexpensive instrumentation and can be practically established in any biochemical laboratory, still it requires skilled personnel. It is independent of the buffer composition and of post-translational modifications of proteins. However, it is slower than matrix assisted laser desorption ionization mass spectrometry and a higher ambiguity is associated with the identifications.

Development of a chiral HRGC method for rapid quantification of amino acids enantiomers in food matrices by ethylchloroformate derivatization in aquoeus media

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The importance of amino acids enantiomers in biological, biochemical, clinical, and food chemistry requires the development of suitable methods both for qualitative and quantitative determinations. The main problem in gas chromatography (GC) determination of amino acids is the need for derivatization, commonly involving laborious and multi-step procedures, thereby loosing the advantage of speed of GC, itself.

Following the amino acid derivatization procedure introduced by Husek in 1991, and the works of Abe and co-workers, we have investigated, in the present study, a more extended approach for the quantification of amino acids enantiomers, using heptafluorobutanol and ethylchloroformate.

After careful adjustment of the reagents proportion and of the chromatographic conditions, the separation of all the protein amino acids, except for arginine, could be completely resolved within 50 min, on a Chiralsil-L-Val column. Except for Pro, all eluted amino acids were completely separated into their enantiomeric pairs. For all amino acids derivatives, excluding Glu, the D-enantiomer eluted faster. The within-day reproducibility of the derivative formation showed relative standard deviation lower than 5% on average and the derivatives formed were stable for more than one week. The calibration curves were conducted using *p*–chlorophenylalanine as the internal standard. A linear relationship was obtained for all the amino acids with correlation coefficients being above 0.99 in the range 0.02–2 µmol.

The method was applied to several food matrices namely yoghurt, coffee and vinegar, following the classical approach of protein precipitation followed by ion-exchange clean-up. The results of the amino acid composition showed good agreement with literature results.

D-Amino acids as tracer for bacterial alterations of dissolved organic matter

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Fixed nitrogen is a principle limiting nutrient for primary production in Arctic waters. Inorganic nitrogen concentrations are low in shelf surface waters, yet fixed nitrogen persists in dissolved organic matter. Small rapid cycling compounds such as free amino acids, amines and urea are fundamental to microbial and planktonic ecology, but only account for a minor fraction of the total dissolved organic nitrogen (DON). Most DON stored into the oceans surface resides in other nitrogenous substance, of which only a minor part is identified on a molecular level. The remaining part is suggested to consist of hydrolysis-resistant or amide-containing molecules. Using DL-amino acids a tracer I report evidence that the vast majority of DON has been altered by microbial processes.

In marine waters, high D/L-ratios were found for total dissolved amino acids. Several explanations has been suggested for the occurrence of D-amino acids in the environment. However, the principal biochemical source of D-amino acids is peptidoglycan, the main component of bacterial cell walls. Peptidoglycan is a heterogeneous biopolymer formed of amino-sugar chains, which are cross-linked by peptide bridges. In these peptide, D-amino acids such as D-Ala, D-Glu, D-Asp and D-Ser are found, besides some nonprotein amino acids. D-Ala is the most suitable tracer for peptidoglycan, due to its principal incorporation and its relatively consistent D/L ratio in this material. Using general structural information of peptidoglycan and proteinacous material and the D/L ratio of Ala, the contribution of peptidoglycan to the amino acid pool can be estimated. In marine samples the contribution ranged from 36% for the shelf areas to 44% in the surface waters of the Laptev Sea, where the proportion increased with depth up to 75%. In contrast to the dissolved organic matter, the particulate material contained only a minor amount of peptidoglycan.

The high proportion of peptidoglycan in sea water is in strong contrast to the common opinion that dissolved amino acids in sea water are dominantly derived from algal sources. The results presented here give evidence that DOM released by phytoplankton is rapidly turned over by bacteria, which are subsequently removed by protozoans and viruses. While the soluble content of bacterial cells is grazed, the structural biopolymers such as the bacterial cell walls remain and are enriched in DOM. Due to the incorporation of D-amino acids, peptidoglycan may be more resistant against the attack of common hydrolytic enzymes and thus be part of the refractory DON-pool.

Copper(II)-L-carnosine complexes. Kinetics of monomer-to-dimer conversion as studied by NMR and EPR

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L-Carnosine (β-alanylhistidine) is present at concentrations of $1*10^{-3}$–$4*10^{-3}$ mol.dm^{-3} in skeletal and nasal olfactory epithelium where it is supposed to play role in the regulation of the transport of copper. The importance of metal ions in many biochemical reactions is well known. Specifically, the dipeptidases, including carnosinase, require divalent metal ions for their activity. This is due, in part, to the ability of the metal to simultaneously coordinate the substrate and the enzyme. Therefore the knowledge of metal dipeptide chelate structures is necessary for the elucidation of this phenomenon. Several reports have been published on copper(II)-carnosine complexes in aqueous solution. Evidence for different structures and species has been obtained. The results depend not only on the experimental conditions (especially the ligand-to-metal ratio), but also on the technique used. One observes generally the formation of the dimer Cu_2-(L-Carnosine)$_2$, but in excess of carnosine, the EPR study shows the possibility of the formation of the tetramer Cu-(L-Carnosine)$_4$. Above pH5, our NMR and EPR studies reveal an equilibrium between monomers (paramagnetic species) and dimers (diamagnetic species due to copper spin-spin coupling through space). At first, the paramagnetic species is formed; consequently the H_2 and H_5 proton signals of the imidazole cycle are drastically broadened; then the system approaches slowly an equilibrium which is reflected on the spectra by a narrowing of the lines as the concentration of the diamagnetic species increases. The evolution of the linewidths and chemical shifts with time allow to follow the kinetics of the conversion of the initial monomers into dimers when the ligand-to-metal ratio exceeds 2. For example, at pH7 and a ligand-to-metal ratio of 10 the time necessary to attain equilibrium is 2 hours. Higher temperatures favour the formation of the dimer.

Characterization of a series of tetrapeptides analogues of AS-I phytotoxin by HPLC on a porous-exchange support and by capillary electrophoresis

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A series of tetrapeptides analogues of AS-I phytotoxin synthesized either in solution or by the solid phase technique, as previously reported [1,2] were identified by HPLC on porous-exchange support [3] and by capillary electrophoresis. The absorption behaviour of the tetrapeptides was studied onto a polymer-layer type anion-exchanger, poly(vinylimidazole) (PVI) absorbed and cross-linked on a porous silica support of large porosity (100 nm) particle diameter. The anion-exchange capacity of the adsorbent (0.63 mequiv/g of silica) was determined by frontal chromatography by equilibrating the column with a solution of 1 mol/L NaCl in a 0.02 mol/L Tris buffer (pH 7) and displacing the absorbed ions with a solution of 0.5 mol/L $NaNO_3$ in the same buffer. The results showed that the separation of peptides was comparable to the reverse phase separation. Capillary electrophoresis separations were performed on a Beckman P/ACE system 2100 Instrument (Fulleston, CA, USA) controlled by a computer and equipped with uncoated fused capillaries (120 cm, id 75 μm). Electrophoresis buffer was 40 mM phosphate acid pH 7.0. The applied voltage was 25 kV and the detector was positioned near the cathode. Elution of peptides was monitored at 200 nm. The retention time characteristic for each peptide studied on the capillary electrophoresis and their calculated pI are given below:

Peptide	Retention time	pI (calculated isolelectric point)	Mode of injection
Hse-Val-Gly-Glu	1.86	3.550	hydrodynamic
His-Val-Gly-Glu	2.29	5.313	>>
Cys-Val-Gly-Glu	3.12	3.550	>>
Val-Ser-Gly-Glu	2.44	3.550	>>
Thr-Ser-Gly-Glu	3.00	3.550	>>
Tyr-Ser-Gly-Glu	2.75	3.550	>>

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Interaction of dihydroxyboryl amino acids with ols or carboxylic acids studied by zone electrophoresis and application of the interaction to isolation of the amino acids

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The complex of p-dihydroxyborylphenylalanine (p-BPA) with fructose has been used for the boron neutron capture therapy because of its improved solubility into a body fluid. During studies of the physicochemical nature of p-BPA, a specific interaction of p-BPA with oxalate ions was found by using a zone electrophoresis in 1991. Since then, we have been studying the interaction of p-BPA with various kinds of organic carboxylic acids (oxalic, succinic, malonic, tartaric, malic, acotinic, trimesic, citric and isocitric acid) which were used as a supporting solution. The interaction largely depended on the stereochemical and electrostatic nature of the acis.

Among them, oxalic, citric or isocitric acid strongly interacted with p-BPA. The complex of p-BPA formed with oxalic, citric or isocitric acid electrophoretically migrated towards the anode under acidic conditions. As usual amino acids migrate towards the cathode under acidic condition, the complexation above mentioned means the possibility of specific isolation of dihydroxyboryl amino acids from the usual amino acids. We applied the complexation reaction to a mixture of p-BPA, phenylalanine and tyrosine, and successfully isolated p-BPA from both the amino acids.

Thermochemical properties of L-α-amino acids solutions in urea-water mixtures

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L-α-amino acids as structural components of proteins participate in all the physiological processes of living cell. Therefore, many physical and chemical studies have been done to elucidate interaction mechanisms between amino acids and organic compounds in cell fluids.

In aqueous solutions amino acids are found as zwitterion $R\text{-}CHCOO^{-}NH_3^{+}$ which differ from others only in side groups R-.

The aim of the presented study was to measure the enthalpies of dissolutions of L-α-amino acids (glycine, alanine, amniobutricy acid, valine, leucine, proline, cysteine, methionine, serine, threonine, aspargine and glutamine) in water and aqueous solutions of urea at the temperature 298.15 K. Based on the obtained results, standard solutions enthalpies of L-α-amino acids in the examined solutions were determined. Using McMillan-Mayer's [1] theory, modified by Desnoyers [2], these values were used to find the enthalpic heterogeneous interaction coefficient h_{AU}, which characterise the mutual interaction between the amino acid zwitterion and urea molecule in water. Intermolecular interaction in solutions, represented by the enthalpic interaction coefficients, take place with the participation of the solvent molecules.

Therefore, it seems to be interesting to exam the dependence between enthalpic coefficients h_{AU} and values of parameter describing hydrophobicity of side chains of studied amino acids. Moreover, in order to get an additional information about factors which have influence on an interaction enthalpic coefficients values, I have analyzed several parameters using multiple regression. These parameters like: hydrophobicity, polarizability, number of hydrogen bonds and apparent molar volumes, describe different properties of side groups in the molecule of studying L-α-amino acids.

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Trypsin from chum salmon and its behaviour for inverse substrate; comparison with bovine and SG trypsin

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Previously we reported that "inverse substrates" behave as specific substrates for trypsin and trypsin-like enzymes. Inverse substrate, *p*-amidinophenyl or *p*-guanidinophenyl ester, is so designed that non-cationic acyl moiety was introduced into the trypsin active site by means of cationic leaving group. These acyl trypsin intermediates are expected to play a key role in trypsin-catalyzed peptide synthesis, and their applicability was reported in our previous work.

It is a general belief that poikilothermic organisms (ectotherms), such as fishes, living in cold environments have compensated for reduced catalytic rates at low temperatures by evolutionary adaptation of existing enzymes. Enzymes from cold-adapted species tend to have a higher catalytic efficiencies than their mammalian counterparts. Thus, trypsin from cold-adapted species could be expected highly efficient catalyst of enzymatic peptide synthesis. Chum salmon *(Oncorhynchus keta)* is a major salmon species in Japan. Chum salmon is born in the river at water temperature lower than 8 °C, and grown up in North Pacific Sea. We are interested in the catalytic efficiency of the trypsin from the chum salmon.

Chum salmon trypsin was isolated as follows: Pyloric ceaca from chum salmon were separated from other intestinal components and homogenized in Tris buffer (pH 7.8). After stirring, the homogenate was centrifuged at 12,000xg for 60 min. The supernatant was fractionated with ammonium sulfate, and the 30–70% fraction of ammonium sulfate was treated with cold acetone. The obtained precipitate was chromatographed on Benzamidine Sepharose® 6B, and ion exchange chromatography (DEAE Sepharose® and CM Sepharose®). Finally, at least 2 types of anionic trypsin and 1 type of cationic trypsin were separated.

Kinetic parameters were determined with typical substrates, such as BAPA and TAME. One of the anion type trypsin, which was major chum salmon trypsin, has 10 times higher activity than bovine and SG trypsin for both substrates in 25 °C. Moreover, chum salmon trypsin indicated that the activity was maintained at 5 °C. Kinetic parameters in inverse substrate were also compared to those of bovine and *Streptmyces griceus* (SG) trypsin.

The short-term regulation of glutaminase in the rat small intestine

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The activity of the key enzyme in glutamine hydrolysis, glutaminase (E.C. 3.5.1.2) was measured in rat enterocyte mitochondria in the presence of various compounds. Glutaminase activity was measured in the absence and presence of its best-known activator, phosphate.

Potassium bicarbonate, potassium sulphate, and ammonium sulphate were all found to have stimulatory effects on glutaminase activity. The presence of 10 mM phosphate markedly increased the stimulatory effects of potassium sulphate and potassium bicarbonate. At 1 mM concentrations of both ammonium chloride and ammonium sulphate, glutaminase activity was inhibited by almost 50% in presence of 10 mM phosphate. Increasing concentrations of ammonium sulphate beyond 1 mM managed to overcome these inhibitory effects with a 1.6 fold increase in glutaminase activity at 20 mM ammonium sulphate when compared to activity in the presence of 10 mM phosphate alone. Higher levels of ammonium chloride did not result in any increase in glutaminase activity indicating that it was the ammonium ion that was inhibiting glutaminase.

We therefore suggest that the ammonium ion is a potent inhibitor of intestinal glutaminase activity.

Interpreting the origins of extraterrestrial amino acids based on their stable isotope compositions

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It has been recognized for more than 30 years that the distribution and stereochemistry of amino acids in carbonaceous meteorites may provide valuable information concerning organic synthesis at the time of and perhaps prior to the formation of our solar system. However, all carbonaceous meteorites that have impacted the Earth are exposed to its biosphere. Thus, distinguishing amino acids indigenous to these meteorites from potential contaminants subsequent to impact continues to be a serious challenge. We have developed analytical methods for determining the stable carbon and nitrogen isotope compositions of individual amino acids enantiomers in complex mixtures at their natural abundance levels ($nmolg^{-1}$) in carbonaceous meteorites. With respect to the Murchison meteorite, our results clearly indicate an extraterrestrial origin for protein and non-protein amino acids. This is because the stable carbon and nitrogen isotope values for these components range from +5 to +28‰ and from +37 to +184‰, respectively, clearly enriched in ^{13}C and ^{15}N relative to amino acid constituents of biogenic materials on Earth. Having established that amino acids in Murchison are extraterrestrial in origin, the challenge that lies ahead is that of exploiting their respective isotopic signatures to constrain their mechanisms of formation. In theory, all rate-dependent chemical reactions should result in products that are depleted in ^{13}C and ^{15}N relative to the substrates from which they are synthesized. For biogenic systems, the amino acid constituents of all organisms exhibit a range of $\delta^{13}C$ and $\delta^{15}N$ values, reflecting isotopic fractionations associated with their respective syntheses. Similarly, preliminary results indicate that instinct isotopic fractionations result from laboratory-simulated abiotic syntheses of amino acids. It is hypothesized that the differences in isotope compositions of amino acids in the Murchison meteorite can be used to reconstruct their modes of origin, provided that they have retained their respective isotope integrities over the time elapsed subsequent to their synthesis.

Amino acid racemization, peering into the black box

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Amino acids racemization kinetics are reconsidered in terms of our knowledge of the ultrastructure of biominerals and the composition of their organic fraction. It is argued that the conventional view of racemization kinetics in molluscs is inadequate to explain the phenomenon observed in shells, notably in the later stages of decomposition in which the rate is slower than the rate of racemization of free amino acids. Estimates of rats of hydrolysis are recalculated using an extension of our previously published polymer degradation model. Patterns of release of free amino acids suggests rate constants spanning between two and three orders of magnitude. We argue that the slowest rate constant can either be interpreted as a pool of very stable peptide bonds or the result of decomposition within a closed system containing limited water.

The pattern of so called "leaching in" bones eggshells and mollusc shells is reconsidered in terms of their differing ultra-

structures. It is concluded that the pore systems in the respective biominerals (including those developed during decomposition of the organic matrix) account for the observed differences in rate of flux. Estimated diffusion coefficients of amino acids at the temperatures used in laboratory studies are less than an order of magnitude greater than those in fossils, whereas rates of hydrolysis and racemization are 5–6 orders of magnitude higher. The problems encountered by underestimating the impact of diffusion on racemization kinetics during artificial diagenesis are explored. It is argued that the intra-crystalline protein fraction is effectively a closed system and will provide more predictable racemization kinetics, although hydrolysis of this fraction may display second order kinetics.

Amino acids racemization kinetics in bear teeth dentine. Application to pleistocene fossil bear aminochronology

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Fossil bear remains are very common in the Pleistocene and Holocene palaeontological record of Europe but in spite of that large amounts of bone and tooth remains were found, their dating is still problematic because they are out of ^{14}C dating method range, and bone and dentine are open systems that make unreliable U-series dating method use. Recently the Electro Spin Resonance Dating Method has been employed with promissory results.

The Biomolecular Stratigraphy Laboratory of the Madrid School of Mines has been analyzing amino acid racemization in bear tooth dentine since 1993 but the former results were erratic because of the complexity of the collagen derived protein chains, polipeptides, dipeptides and free amino acids racemization rates. With a 3,500 Dalton membrane dialysis use we have been able to obtain homogeneous results being possible to establish two fossil bear aminozones: the former is the Middle Pleistocene *Ursus deningeri* (ca. 300 ka B. P.) and the latter the Upper Pleistocene *Ursus spelaeus* (between 100 and 25 ka B.P.). In some cases bear remains are hominid associated.

In order to estimate absolute ages and paleotemperatures of fossil bears and to approach the kinetic equation of aspartic acid and glutamic acid racemization we have exposed modern bear *(Ursus americanus)* dentine samples to elevated temperatures in a stove in sealed tubes with inert (N_2) atmosphere. Ultra clean water and ultra clean quartz sand were added.

The results of our kinetics heating experiments, after sample 3,500 Dalton dialysis, show that there is an excellent time-aspartic acid correlation, also being possible to observe the "apparent kinetics reversal" also observed in heating experiments on mollusca samples.

Aspartic acid racemization constant when we adjust to a first order reversible kinetics ecuation:

$2k_Lt = C+1n\ [1+D/L)/(1-D/L)]$ where $K = k_D/k_L = 1$

For the first 200 hours experiment results $k_L = 0.010 \pm 0.001$ (95%, 2 δ); being $K_L = 0.0008 \pm 0.002$ (95%, 2 δ), for the whole experiment time (ca. 1600 hours).

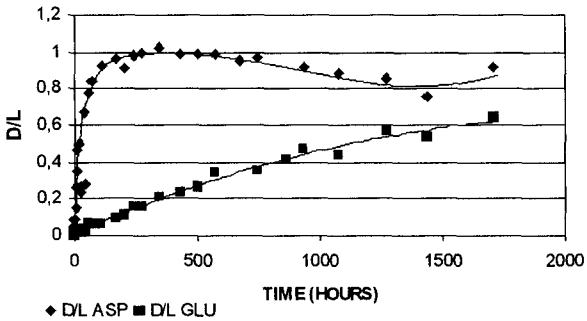

Fig. 1

Glutamic acid racemization rate is lower than the aspartic acid one and after 1600 hours of heating only 0.6 racemization ratio was reached.

Conservation of the basic pattern of cellular amino acid composition in biological evolution

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We showed that the cellular amino acid composition obtained by amino acid analysis of whole cells differs in organisms such as eubacteria, protozoa, fungi and mammalian cells. However, the basic pattern of cellular amino acid composition was relatively constant in all organisms examined in the previous study.

In the present study, the archaeobacteria and plant cells were examined, because their data are considered important in understanding the relationship between biological evolution and cellular amino acid composition. The cellular amino acid composition differs in carrot, *Torenia fournieri* and *Cymbidium,* but the basic pattern was quite similar. The cellular amino acid compositions of the archaeobacteria examined *(Archaeoglobus fulgidus, Pyrococcus horikoshii, Methanobacterium thermoautotrophicum* and *Methanococcus jannaschii)* differs from each other, but they are quite similar to those determined from codon usage data based on the complete genomes. This fact strongly suggests that codon formation preceded protein formation. A comparison of the cellular amino acid composition with the codon usage based on the complete genomes suggests that primitive life had plural origins. Thus, the cellular amino acid composition reflects biological evolution. It is thought that the primitive life form that appeared on earth at the end of prebiotic evolution might have also had a similar-cellular amino acid composition.

Turnings of cysteine and histidine catalized by xanthine oxidase

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Xanthine oxidase turns hypoxanthine to xanthine and then to uric acid. It has dimer structure and consists tow FeS centres, one FAD molecule and one Mo(V) atom per monomer. The mechanism of action of the rat liver xanthine oxidase was studied on highly purified preparation [1]. It was established that its FeS-centre forms superoxide. $FADH_2$ turns it to H_2O_2 and reduces FeS-centre. Mo(V) changes its valency to Mo(VI) and splits H_2O_2 to $OH^•$ and OH^-. $OH^•$ from two xanthine oxidase monomers form H_2O_2. Mo(VI) binds with OH^- and transfers only $OH^•$ to the substrate [2]. It was established that cysteine and histidine are isosteric xanthine oxidase inhibitors. Dehydrogenated cysteine takes $OH^•$ from Mo(VI)-OH and turns to casteinesulfenate that oxidises by H_2O_2 to cysteinesulfinate (Fig. 1).

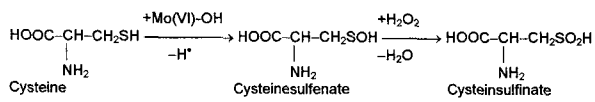

Fig. 1

Histidine can be oxidized by xanthine oxidase to 2-oxyhistine as a substrate (Fig. 2).

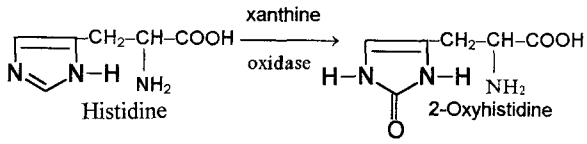

Fig. 2

The speeds of formation of cysteinsulfinate and 2-oxyhistidine in the reaction systems consisted 18.8 pmols of xanthine oxidase and 4 mkmols of xanthine, and corresponding inhibitor constants are presented in the Table 1.

Table 1

Amino acid	Quantity in the reaction system, mkmols	Speed of oxidation mkmols x min per 1 xanthine oxidase molecule	Ki, mM
Cysteine	0.25	0.090 ±0.12	0.34
Histidine	1.0	0.85 ±0.093	0.49

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Calculation of the amino acid structure of xanthine oxidase allosteric centre

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It was established that corticosteroids bind with xanthine oxidase allosteric centre and inhibit the enzyme [1]. Also DDT and polychlorodibenzodioxins (PCDDs) are xanthine oxidase allosteric inhibitors too, but more powerful than corticosteroids. It was shown that corticosteroids bind with xanthine oxidase only with the help of hydrogen binds. But in the case of DDT and PCDDs also the interactions between their aromatic cycles and the enzyme aromatic cycles take place. Using Hukkels approach and QSAR data [2] of amino acids, corticosteroids, DDT and PCDDs the amino acid structure of xanthine oxidase allosteric centre was calculated. Established, that NH-group of histidine imidasol, serine OH-group and tyrosine OH-groups form hydrogen binds with the inhibitors. Aromatic cycles of tyrosine and phenilalanine interact with the aromatic cycles of DDT and PCDDs. Amino acid rests of xanthine oxidase allosteric centre bound with 4,9-dichlorodibenzodioxin (with corresponding electron charges) are presented in the Fig. 1.

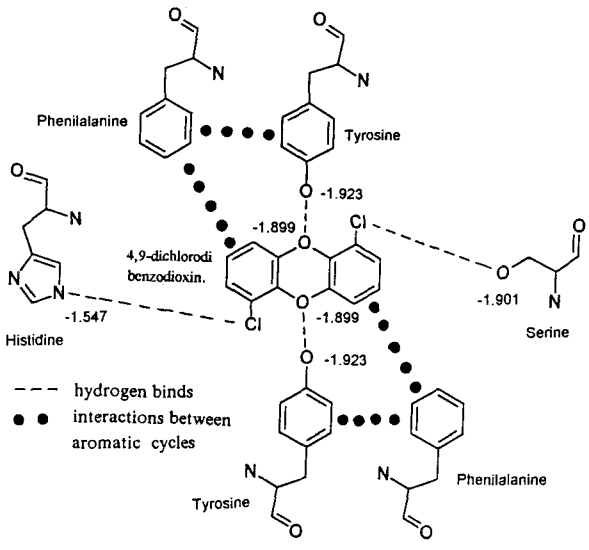

Fig. 1

Xanthine oxidase was recommended as the experimental model for screening of chemicals for their possible effects as endocrine disrupters that bind with the receptors of different steroid hormones.

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Glutamate and aspartate as spacers for aromatase purification by affinity precipitation

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Different enzymes can be purified with the help of affinity precipitation. It was established that spherical agarose covalently bound with substrates through spacers can be used as sorbent for affinity precipitation of corresponding enzmyes [1].

Very effective method of the aromatase purification from the animal's tissues was elaborated on this base. Spherical agarose was covalently bound with 16-methyltestosterone through spacers glutamate and aspartate (Fig. 1).

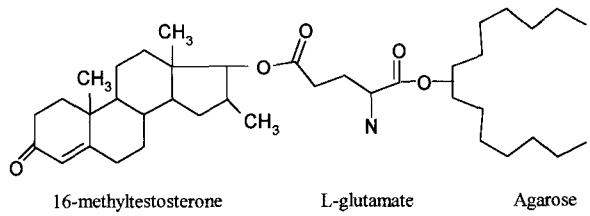

Fig. 1

For purification of aromatase microcosms from the animal's tissue (ovary, uterus, breasts or placenta) should be eliminated with the help of differential centrifugation. Cytochromes P450 should be extracted from the microcosms by sodium cholate and precipitated by the cold (–18 °C) acetone. The precipitate should be soluted in the bidistilled water and aromatase should be precipitated on the sorbent for affinity precipitation (pH 7.4) and eluted by 0.1 M tris-HCl-buffer (pH 8.0) consisted 1 M NaCl and 0.1 mkM 16-methyltestosterone [2].

Though glutamate molecule is longer than aspartate molecule the aromatase affinity precipitation with the spacer glutamate has the same effect that one with the spacer aspartate. The experimental data is presented in the table of purification of aromatase form the rat ovaries (Table 1).

Table 1

Stage of purification	Albums, mg	General activity, nmols/min	Enzyme outlet, %
Homogenate	96	643	100
Elimination of microsom albums by sodium cholate	6.612	594	92.4
Acetone precipitation	3.565	592.3	92.1
Affinity precipitation	0.268	528.15	82.14

Stage of purification	Specific activity, nmols/min per 1 mg of albums	Purificational degree, times
Homogenate	1.34	0
Elimination of microsom albums by sodium cholate	16.63	12.41
Acetone precipitation	29.04	21.67
Affinity precipitation	299.94	223.84

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Amino acids from natural sources in reactions of rhenium clusters formation

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Hydrolizates of some tissues, free amino acids and small peptides are known as unexhaustible sources of nitrogen containing and optically active protein predecessors. Some of them are used in medicine as unmodified mixture (e. g. cerebrolysine) and a lot of the chemically changed natural mixtures is used too.

We have studied the reaction between free amino acids gotten from rape maize grain, roots and shoots of maize seedlings and fresh bull brain with the salts of octaclorodirhenat (III) anion under reflux in aprotonic solvents. Changing of the colour of the reaction solution from blue to green and increasing of stability in aqueous solutions were observed.

Each of the sources is characterized by special amino acid composition. Kinetics of reaction was controlled by thin layer chromatography of amino acids as any other method of control is impossible. Visible spectroscopy was used to check formation of the complex and to identify its structure. Quadruple bond Re-Re is registered in electronic spectra in sorption area 14,000 cm^{-1} (14 kK). Substitution of chlorine by an amino acid radical led to shift of the band to shorter wave area and to decreasing of intensity of the band. Thus three types of substituted rhenium complexes with carboxylic groups of amino acids were registered: cis-dicarboxylates of dirhenium tetrachloride, tricarboxylate of dirhenium trichloride and tetracarboxylate of dirhenium dichloride. Some amino acids (Thr, Gln, Asn, Ser) were the most preferable complex ligands. It is possible to stop the reaction in order to obtain certain type of a complex.

Interaction of dihydroxyboryl amino acids with old or carboxylic acids studied by zone electrophoresis and application of the interaction to isolation of the amino acids

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The complex of p-dihydroxyborylphenylalanine (p-BPA) with fructose has been used for the boron neutron capture therapy because of its improved solubility into a body fluid. During studies of the physiochemical nature of p-BPA, a specific interaction of p-BPA with oxalate ions was found by using a zone electrophoresis in 1991. Since then, we have studied the interaction of p-BPA with various kinds of organic carboxylic acids (oxalic, succinic, malonic, tartaric, malic, acotinic, trimesic, citric and isocitric acid) which were used as a supporting solution. The interaction largely depended on the stereochemical and electrostatic nature of the acids.

Among them, oxylic, citric or isocitric acid strongly interacted with p-BPA. The complex of p-BPA formed with oxalic, citric or isocitric acid electrophoretically migrated towards the anode under acidic conditions. As usual amino acids migrate towards the cathode under acidic condition, the complexation above mentioned means the possibility of specific isolation of dihydroxyboryl amino acids from the usual amino acids. We applied the complexation reaction to a mixture of p-BPA, phenylalanine and tyrosine, and successfully isolated p-BPA from both the amino acids.

Sulphur-Containing Amino Acids

Derivatization of cysteine and related compounds for capillary electrophoresis and liquid chromatography with the use of 2-halopyridinium salts

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Biological thiols, such as homocysteine, cysteine and glutathione, are products of sulfur metabolism, they are reactive and ubiquitous and have been extensively studied in various biological systems. Other biological thiols are derivatives of these compounds. Chemistry of sulfhydryl groups dictates their function in health and disease. From a biological point of view, pK, redox potential, and capacity for free-radical formation are the keys to thiol biochemistry and, therefore, to both the noxious properties and cytoprotective functions of biological thiols. The determination of thiol-containing compounds in biological material is important for biochemical and clinical studies. To analyse total thiol content disulfides have to be reduced in order to restore their thiol counterparts, which are then subjected to derivatization and determination. Derivatization is almost always performed in order to protect reactive sulfhydryl function and to improve detectability and separation properties. This talk will outline 2-halopyridinium salts as biological thiols pre-column derivatization reagents with respect to their reactivity, specificity, spectroscopic characteristics and especially their applicability to high performance capillary electrophoresis and high performance liquid chromatography analysis. The proposed derivatization reaction takes advantage of the high nucleophility of thiols; they displace rapidly in aqueous solution the halogen atom at position 2 in the pyridine ring of pyridinium salt to form stable thioethers. These S-pyridinium derivatives exhibit a well-defined maximum at 308–316 nm region in the UV spectrum as a consequence of the bathochromic shift from the reagent maximum. Among the three functionalities of the sulfur amino acids potentially able to displace the halogen atom at the 2-position of the pyridinium

ring activated towards nucleophilic substitution, only sulfhydryl group reacts. This means that no multiple derivatives are formed. Conditions for application of this derivatization reaction scheme for analysis of cysteine and metabolically related compounds in physiological fluids by means of HPCE and HPLC will be discussed.

Determination of total plasma homocysteine

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In earlier years, interest in homocysteine in clinical chemistry was very much focussed on classical homocystinuria of homozygotes for cystathionine β-synthase deficiency. Such patients present with a Marfan-like phenotype and show early onset of arteriosclerosis as well as life-threatening thrombo-embolism among their most prominent symptoms. In classical homocystinuria highly elevated plasma concentrations of the homocysteine-dimer homocystine can easily be detected by methods for amino acid analysis as they are routinely used in selective screening for inborn errors of metabolism.

More recently, however, mild and moderate hyperhomocysteinaemia have also been reported to predispose to premature vascular diseases, including embolic strokes. Therefore, analytical approaches have attracted attention, which allow the determination of total plasma homocysteine, which is comprised of homocysteine, homocysteine-heterodimers with other thiol compounds (including homocysteine-protein adducts) and free homocysteine. A variety of analytical methods has been described so far, involving gas-chromatography techniques with mass-selective detection, high-performance liquid-chromatography with pre- or post-column-derivatization or electrochemichal detection, as well as an automated immunoassay. An overview will be provided and the possibilities and limitations of the different approaches will be discussed. Special emphasis will be put on the pre-analytical conditions for the determination of total plasma homocysteine, the different ways of sample preparation and the current role of methionine loading in detecting humans who carry an increased risk for vascular diseases.

Determination of low molecular weight thiols in small biopsy specimens from human tissues

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Accurate measurement of low molecular thiols in biological samples has long presented an analytical challenge. The methods available for measuring the various forms of the thiols has presented less of a challenge than the actual preparation of the biological samples. This has been underlined in various experiments which have shown that the sample processing may alter GSH and CySH values in samples taken from various tissues.

The principal problem in determining cellular thiols is loss due to oxidation during extraction and subsequent manipulation. We have developed a technique in which small tissue biopsies are rapidly frozen, followed by homogenisation in 5-sulfosalicylic acid prior to derivatization of the thiols. Recovery experiments have shown that the recovery of glutathione using this procedure is >95% and CySH is 70%.

By using NEM as a thiolchelator combined with a simple precolumn derivatization reaction with mBrB and HPLC separation of the bimane adducts we have been able to develop a sensitive and specific measurement of CySH γ-glu-CySH CyS-Gly and GSH as well as their disulphides.

This methodology has been successfully applied to small biopsy specimens (20–60 mg depending on the tissue) taken from human skeletal muscle, and liver, as well as gastric and duodenal mucosa. We have also followed the changes in concentration of low molecular weight thiols in skeletal muscle following surgical trauma.

The advantage of this HPLC analysis is that it enables rapid (25 minute turnabout time) simultaneous detection of several low molecular weight thiols. This will facilitate the study of low molecular thiols in various human tissues under pathophysiological conditions.

Homoserine in human and animal organisms

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The existing data on L-homoserine in animal and human organisms are controversial. In 1955–1964, L-homoserine was described in some of patients with neuroblastoma and cystinuria, in rats and mice treated with methionine. These scarce findings have not been subsequently confirmed. Recent literature contains no clear indications about L-homoserine.

Using purified E. coli homoserine kinase (HSK) we found in mouse liver extract and human urine a substrate which is phosphorylated to give a product resembling L-o-phosphohomoserine. Its amount in normal urine is very low (6 to 8 nmol/ml), but its higher concentration was found in 12 out of 17 examined patients with hepatitis B. Three patients with acute form of the disease contained 100 to 130 nmol per ml. The high level maintained during two months then declined parallel to the decrease of prothrombin and aspartate aminotransferase/alanine aminotransferase ratio, clinical tests in hepatitis B. Since HSK can phosphorylate some (artificial) analogues of L-homoserine, we performed direct determination of the urine substrate after its partial purification by chromatographic separation (using HSK assay as detection probe) in an automated analyser; we revealed a peak in the position of L-homoserine containing it in amount about 23 nmol.

Our data show that L-homoserine is formed at low level in mammalian organism and is in pathological circumstances, neuroblastoma, cystinuria (according to earlier findings), and hepatitis B (as shown in this study) produced in high amounts.

A possible origin of L-homoserine from L-methionine in mammals will be discussed.

The role of mitochondria in the metabolism of cysteine

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The main metabolite of cysteine in mammals is sulfate, which is produced through the cysteinesulfinate pathway, and the final step of the pathway is sulfite oxidase reaction in mitochondria. In 1968, Crawhall et al. reported that β-mercaptolactate-cysteine mixed disulfide was excreted in the urine of a patient with β-mercaptolactate-cysteine disulfiduria. In the same year we reported that the same mixed disulfide was excreted in normal human urine. These discoveries suggested us that cysteine was metabolised through β-mercaptopyruvate as an intermediate. In 1992, we proposed a pathway of cysteine metabolism in mitochondria, mercaptopyruvate pathway (MP pathway), (Ubuka et al, Amino Acids, 2, 143–155). We suggested that the physiological significance of this pathway is to supply non-oxidized divalent sulfur atoms to thiosulfate sulfurtransferase reaction and excess sulfur is metabolized to sulfate. Taurine is another main metabolite of cysteine in mammals, which is pro-

duced in cytoplasm through the oxidation of cysteine sulfur. Sulfate and taurine constitutes appr. 95% of sulfur excreted in the urine of rats and both cysteinesulfinate and MP pathways contribute to sulfate formation and the former to taurine. We have studied overall metabolism of cysteine by determining the excretion of urinary sulfate and taurine in rats fed with high- and low-protein diets. Sulfate/taurine ratio of rats fed a high protein diet was similar to that in rats fed a standard protein diet, but the ratio is high in rats fed a low protein diet, indicating that mitochondrial sulfate formation predominates than taurine formation when cysteine supply is low.

New findings of sulfur-containing amino acid metabolism: Is protein-bound methionine used to maintain hepatocyte antioxidant/antielectrophile status and cell viability?

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The antioxidant/antielectrophile capability of a cell largely depends on the cell's ability to restore GSH levels once the GSH has been depleted as a result of conjugating electrophiles or oxidation and GSSG efflux from the cell. However little is known about the cellular metabolic processes required for GSH resynthesis. Such knowledge could result in new strategies for treating chemotherapy resistant tumor cells by compromising their ability to resynthesize GSH or by protecting patients' tissues by increasing their GSH synthetic ability. We have shown that when hepatocyte GSH was depleted 90% with 1-bromoheptane in 30 min, the intracellular GSH levels recovered to above 50% within 3 hr when incubated in an amino acid free buffer. The recovery was markedly accelerated and was 100% when methionine, homocysteine or cysteine were added thereby indicating that the methionine transmethylation cycle and transsulfuration pathway for cysteine formation were not affected by the GSH depleting agent. This GSH replacement in the absence of amino acids was inhibited by propargylglycine (a γ-cystathionase inhibitor) or adenosine (a homocysteine hydrolase inhibitor). The lysosomal protease inhibitor, leupeptin, or endocytosis inhibitors also prevented GSH recovery. These inhibitors however did not affect GSH recovery if methionine or cysteine were added to the incubation mixture. These observations imply that once cell viability is threatened by GSH depletion, then damaged proteins are endocytosed for hydrolysis by lysosomal proteases so as to replenish the methionine pool for GSH biosynthesis. Furthermore, cell susceptibility to alkylating agents was increased by those protease or endocytosis inhibitors. The success of protease inhibitors as chemoprotective, antimetastatic or chemotherapeutic agents could thus be partly explained by their ability to compromise GSH resynthesis in tumor cells.

Comparative study of L-cysteine desulfuration in liver of vertebrates

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The desulfuration pathway of L-cysteine metabolism, important as a source of metabolically active reduced sulfur, was examined in tissues of such evolutionary different species as frog *Rana temporaria* and mouse. The activity of 3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2), cysteathionine γ-lyase (EC 4.4.1.1), rhodanese (thiosulfate sulfurtransferase, EC 2.8.1.1), and the level of sulfane sulfur compounds were determined in liver, kidney, brain and heart homogenates. The activity of all the investigated enzymes either in liver and kidney of the frog or the mouse, was significantly higher in comparison to the value determined in other tissues. For each of the investigated frog tissues, changes that were noted in the enzymatic activities and in the level of sulfane sulfur compounds were dependent both on the season of the year, and on the character of the tissue. Significant differences in the level of metabolically active reduced sulfur in frog liver homogenates in different seasons of the year indicate that in autumn L-cysteine is actively metabolised through the desulfuration pathway with the formation of sulfane sulfur compounds which in other seasons are utilized by rhodanese. Scanning microscope studies of the content of sulfur in liver cryostat sections from the mouse and frog in autumn showed levels which were almost two times higher in frog than in mouse liver. A high rhodanese activity in frog liver was confirmed histochemically. The size of granules resulting from the enzymatic test corresponded to the size of the mitochondria, what indicates their formation in these organelle. No cross-reactivity between frog rhodanese isolated from mitochondrial fraction of liver and beef rhodanese was demonstrated using either antiserum against beef enzyme or antiserum against frog enzyme. Moreover, the molecular weight of purified mitochondrial rhodanese from frog liver, determined by the method of HPLC was about 9 kDA, i. e. much below the value for the beef enzyme. This large difference in molecular weight and the lack of common antigenic determinants suggest the occurrence of different forms of this mitochondrial sulfurtransferase in beef and frog liver. A possible role of sulfane sulfur compounds and of rhodanese in frog liver is also taken into consideration.

Property of an active center of rat mercaptopyruvate sulfurtransferase

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Mercaptopyruvate sulfurtransferase (MST, EC 2.8.1.2) catalyses transsulfuration reaction from mercaptopyruvate to thiol compounds or cyanide. MST is widely distributed in rat tissues and localized in cytoplasm and mitochondria [1, 2]. Rat MST was purified to homogeneity [3]. cDNA of the enzyme was also cloned and the recombinant MST was overexpressed in *E. coli* [4]. In a comparative study of rat MST and rhodanese (EC 2.8.1.1), MST was found to be evolutionarily related to mitochondrial rhodanese [3, 4].

A catalytic site Cys247 in rhodanese is conserved in MST (Cys247), but adjacent residues, Arg248 and Lys249 in rhodanese are replaced with Gly248 and Ser249, respectively in MST. In bovine rhodanese, Lys249 and Arg186, located at the entrance of the pocket of an active center, serve as binding sites for thiosulfate [5]. Arg187 (corresponding to Arg186 in rhodanese) is conserved in MST. Arg196, found only in MST, is located near the pocket of an active center in an estimated tertiary structure of rat MST.

In rhodanese mutant obtained by site-directed mutagenesis, R248C (substitution of glycine for Arg248), K249S, and R248G & K249S (double replacements) resemble catalytic properties of MST. On the other hand, in MST mutants, C247S loses both MST and rhodanese activities, suggesting that Cys247 is a catalytic site. Catalytic property of S249K is converted to that of rhodanese. R187G and R196G show decrease in MST activity, suggesting that Arg187 and Arg196 are binding sites for mercaptopyruvate.

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Sulphur amino acid metabolism in two rat hepatoma cell lines

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It has been reported that some tumour cells isolated from patients are methionine dependent in culture [1]. This dependency may be exploited in cancer treatment [2]. We were interested in the biochemical defect that causes the methionine dependency.

Phil-1 is a well differentiated rat hepatoma cell line; HTC is an undifferentiated rat hepatoma cell line. Both cell lines were grown under conditions with and without menthionine to establish their methionine requirements, Phi-1 grew in all conditions but HTC cell could not grow when methionine was replaced with homocysteine (which is converted to methionine by methionine synthase).

We analysed the activity of methionine synthase in both cell types to determine whether a defect in the activity of this enzyme could be responsible for methionine dependence.

Cells were grown in media containing added vitamin B_{12} and folic acid (required for methionine synthase activity), and either 200 μM L-methionine (M-200), 100 μM L-methionine (M-100), 100 μM DL-homocysteine (H-100) or 100 μM methionine with 100 μM homocysteine (M/H-100).

Table 1

Media type	Methionine synthase activity Phi-1 cells	nmol methionine/ hr/mg protein ±sem HTC cells
M-200	4.1 ±0.4	1.8 ±1.6
M-100	21.9 ±1.8	4.2 ±1.2
H-100	47.3 ±9.8	3.3 ±0.8
M/H-100	23.6 ±4.2	4.4 ±0.6

Table 1 shows methionine synthase activity measured as previously described [3] for confluent cells after four days in the various media types, n = 3–5.

The results show that methionine synthase activity can be upregulated in the methionine independent cell line Phi-1 when methionine levels are decreased to 100 μM or 0 μM methionine. In contrast the methionine dependent HTC cells do not increase methionine synthase activity, and have a lower basal activity than Phi-1 cells.

The effect of these growth conditions upon other elements of the sulphur amino acid pathway are currently under investigation.

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Differences of methionine metabolism depending on melaninogenesis

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Methionine participates in pheomelanin synthesis through a transformation into cysteine which by condensation with DOPA forms 5-S-Cysteinil-DOPA which is a monomer unit of pheomelanin.

The excretion of tyrosine and methionine metabolites (p-HPPA, Homogentisin Acid and 5-S-Cysteinil-DOPA) with the urine and activity of tyrosine aminotransferase, cystationine-β-synthetase and glutathione reductase in liver and the skin and DOPA-chrome oxidoreductase in hair bulbs of white, black and yellow guinea pigs were studied.

Initiation of melaninogenesis was made by irradiation of animals with sun light and administration of them with L-tyrosine and L-methionine (1–3 mMol per kg) on normal ration.

The results of a study of the tyrosine aminotransferase, cystathionine-β-synthase and glutathione reductase activities in the liver and skin and DOPA-chrome oxidoreductase activity in the hair bulbs of albino animals, taking the excretion of p-HPPA, homogentisic acid, and 5-S-Cysteinyl-DOPA with the urine in these animals into account, just as under conditions of initiation of melaninogenesis by sunlight or loading with amino acids, suggest that the inhibition of the synthesis of melanin pigments in the skin of white guinea pigs is evidently limited at later stage of melaninogenesis in these animals. In addition, the metabolic processes of the initial stage of melaninogenesis in albino animal (if they occur) are evidently identical with the metabolic processes characteristic of pheomelanin synthesis. We should also conclude that there is a close interrelationship between the metabolism of sulfur-containing amino acids and the tyrosine metabolism; moreover, the metabolism of these amino acids is differentiated depending on the sate of melaninogenesis and on the type of melanin synthesized.

Evidence for apical localization of a novel, sodium-dependent glutathione transporter in mouse brain endothelial cells (MBES)

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Our previous work has shown that gluthatione (GSH) is transported across the blood brain barrier by a specific, carrier-mediated process. We have used MBEC-4, an immortalized mouse brain endothelial cell line, to establish the presence of Na^+-dependent and Na^+-independent GSH transport and have localised the Na^+-dependent transporter using domain-enriched plasma membrane vesicles. Partial, but significant Na^+-dependency of ^{35}S-GSH uptake (~40–70%) was observed in cultured MBEC and in oocytes injected with MBEC-poly(A)$^+$-RNA. Uptake in HepG2 and Cos-1 cells and in poly (A)$^+$RNA injected oocytes was NA^+-independent. Plasma membrane vesicles from MBEC were separated into three fractions (30%, 34% and 38% sucrose, w/w) by density gradient centrifugation. Sodium-dependent glucose transport, reported in the abluminal membrane, was associated with the 38% fraction (abluminal). Sodium-dependent GSH transport was present in the 30% fraction which was identified as the apical (luminal) membrane by localization of P-glycoprotein 170 by Western blot analysis. In MBEC depleted of GSH with BSO, a significant increase in intracellular GSH could be demonstrated only in the presence of Na^+. Localization of Na^+-dependent transport to the luminal membrane and its ability to drive up intracellular GSH may find application in delivery of GSH to the brain *in vivo.*

L-Cysteine; its central pressor action mediated by vasopressin secretion and autonomic nervous activation

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Neurotransmitters play essential roles in the central nervous system (CNS) but their number and kind are incompletely

known. The previous studies have shown the potential role of several amino acids in the CNS for the cardiovascular control [1, 2]. Central stimulation with L-cysteine (2 μmol, ic) as well as those with L-proline (2–10 μmol, ic) and L-arginine (2–10 μmol, ic) has induced a strong pressor action in the conscious rat. The pressor action of those amino acids can be mediated by autonomic nervous activation and/or vasopressin secretion. The present paper compared this pressor mechanism of L-cysteine with those of L-proline and L-arginine [3–5]. Blockade of the autonomic nervous system significantly attenuated the pressor effect with L-arginine (10 μmol, ic), but augmented that with L-proline (10 μmol, ic) and L-cysteine (2 μmol, ic). Additional inhibition with the vasopressin antagonist (iv), however, completely suppressed that augmentation with both L-proline and L-cysteine. Prior iv injection of vasopressin antagonist alone inhibited the pressor response to ic injection of L-proline but not L-cysteine. The results indicate that L-arginine stimulates the CNS for the autonomic nervous system, L-proline for vasopressin secretion, and L-cysteine for both the autonomic nervous system and vasopressin secretion. L-cysteine in addition to L-arginine and L-proline might play an important role as a neurotransmitter or neuromodulator in the CNS for the cardiovascular control.

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Differential chemoprotection against acetaminophen-induced hepatotoxicity by latentiated L-cysteines

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Novel thiazolidine prodrugs were prepared by the condensation of L-cysteine with aldose disaccharides. Using a disaccharide in prodrug construction allows for a terminal cyclic sugar moiety to be present on the prodrug, which may allow the delivery of the agent to specific receptors, such as the asialoglycoprotein receptor (ASGPR) of hepatocytes, that require specific structural motifs for recognition. Three L-cysteine prodrugs were synthesized with a pendant cyclic galactose moiety; two related glucose-bearing prodrugs were synthesized for comparison. The prodrugs were design to release L-cysteine, which is then available to support glutathione (GSH) biosynthesis and provide cytoprotection against a variety of toxic insults. Protection studies in Swiss-Webster mice used acetaminophen (APAP, 575 mg/kg), a well documented hepatotoxin which depletes GSH at overdose. Three prodrugs performed well against APAP-induced hepatotoxicity, although structure-activity relationships were not obvious. Co-administration of selected prodrugs with a 400 mg/kg dose of APAP prevented the short-term depletion in hepatic GSH and also reduced hepatotoxicity, as determined by histological damage and serum levels of alanine aminotransferase. A single dose of the prodrugs alone had no effect on hepatic drug metabolizing enzmyes [glutathione S-transferase (GST), NDA(P)H: quinone oxidoreductase (QOR), UDP-glucuronosyltransferase (UGT), and cytochrome P450] but, concordant with the reduction of hepatotoxicity, the latentiated forms prevented the significant elevation in QOR activity and mRNA and GST mRNA elicited by APAP itself. GST activity, UGT activity and mRNA, and cytochrome P450 concentration were all unaffected by APAP or the prodrugs.

Biological properties of 2-methyl-thiazolidine-2,4-dicarboxylic acid, the product of nonenzymatic condensation of L-cysteine with pyruvate

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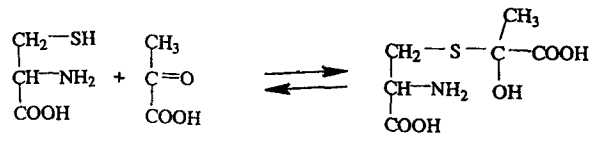
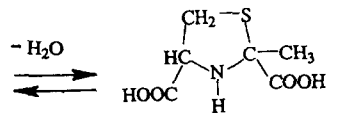
Pyruvate formed in the course of L-cysteine metabolism (both *in vivo* and *in vitro*) reacts nonenzymatically with the excess of L-cysteine to 2-methyl-thiazolidine-2,4-dicarboxylic acid (CP).



L-cysteine pyruvate semimercaptal



thia zolidine-2,4-dicarboxylic acid (CP)

This compound in a reverse reaction constitutes a source of cysteine, the amino acid limiting biosynthesis of glutathione (GSH) in cells. GSH plays an important role as a cellular protector against oxygen reactive species and toxic compounds.

We have observed that CP leads to the following effects in cells:

- elevation of non-protein sulfhydryl levels and sulfurtransferase activities in mouse liver,
- time and dose dependent increase of glutathione in mouse liver,
- selective modulation of glutathione level in Ehrlich Ascites tumour bearing mouse,
- increase of the intracellular level of non-protein sulfhydryl and strong stimulation of the proliferation of IL-2 dependent CTLL-2 cells,
- simultaneously increase the viability of CTLL-2 growing without IL-2 in medium,
- increase of GSH and cysteine and decrease of lipid peroxidation in liver and brain in mice chronic intoxicated with ethanol,
- preventing of mice liver *(in vivo)* and of HepG2 cells *(in vitro)* against the hepatotoxic dose of paracetamol.

Concluding 2-methyl-thiazolidine-2,4-dicarboxylic acid may serve as a nontoxic cysteine prodrug for cells and all these results suggest its protective properties.

Effect of cystathionine and cystathionine metabolites on the stimulus coupled superoxide generation and phosphorylation of tyrosine residues in human neutrophils

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Human peripheral blood polymorphonuclear leukocytes were pre-incubated with cystathionine and cystathionine

metabolites found in the urine of the patients with cystathioninuria. Among the cystathionine metabolites, cystathionine ketimine significantly enhanced the N-formyl-methionyl-leucyl-phenylalanine-induced superoxide generation, but cystathionine and cyclothionine did not enhance the superoxide generation. Cystathionine ketimine also enhanced superoxide generation induced by opsonized zymosan but not those induced by arachidonic acid and phorbol myristate acetate. Superoxide generation induced by cystathionine ketimine was inhibited by genistein, an inhibitor of tyrosine kinase, and was enhanced by 1-(5-isoquinoline-sulfonyl)-2-methyl-piperazine, an inhibitor of protein kinase C. The effect of cystathionine and cystathionine metabolites on the phosphorylation of tyrosine residues was also studied with human neutrophils. Among the cystathionine metabolites, cystathionine ketimine markedly increased phosphorylation of a 45 kDa protein with time and the phosphorylation depended on the concentration of cystathionine ketimine, while cystathionine and the reduced form of cystathionine ketimine (cyclothionine) did not increase the phosphorylation of the 45 kDa protein. The phosphorylation of the 45 kDa protein induced by cystathionine ketimine was inhibited by genistein and herbimycin A, inhibitors of tyrosine kinase, but was not inhibited by 1-(5-isoquinoline sulfonyl)-2-methyl-piperazine and staurosporine, inhibitors of protein kinase C.

Glutathione reductase activity of hemoglobin

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Infection of H_2O_2-generating bacteria to Japanase acatalasemia patients caused progressive oral gangrene (Takahara disease), and Takahara and his group suggested that severity of catalase deficiency in blood was an important factor to cause the disease. However, acatalasemic mice established by Feinstein et al. have no health problem. We were interested in studying H_2O_2-removal activities in erythrocytes and examined the removal rates by catalase, gluthathione peroxidase and hemoglobin in acatalasemic and normal mouse hemolysates. In this study, we examined glutathione reductase (GR) since H_2O_2-removal rates by glutathione peroxidase were depended upon glutathione contents in erythrocytes. Hemolysates was applied to a carboxymethyl-cellulose column. GR activity in the eluate was examined. The activity was eluted two different fractions. One fraction was GR activity, and another fraction was eluted with hemoglobin. The latter activity was proportional to hemoglobin content in the eluate, and the activity could use NADH as a substrate. Compared to the rates by GR in erythrocytes, rates by the activity separated from hemolysates were substantial. We want to discuss this activity since it was thought that GR was a sole enzyme to reduce oxidized glutathione in erythrocytes.

Regulation of transport, metabolism and biological functions of *S*–nitroso compounds in human erythrocytes by cysteine

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Nitric oxide (NO), *S*-nitrosocysteine (SNC) and *S*-nitrosoglutathione (GSNO) are putative endothelium-derived relaxing factors. *S*-nitrosoalbumin (SNALB) is the most abundant and most stable physiological circulating NO carrier in mammals. NO, SNC, GSNO, SNALB and other *S*-nitroso compounds are potent vasodilators and inhibitors of platelet aggregation. It is well established that human erythrocytes are responsible for the inactivation of NO via oxidation to nitrate by oxyhemoglobin. The importance of red blood cells for the metabolism of *S*-nitroso compounds are still poorly understood. In the present study we investigated the metabolism of NO, SNC, GSNO, SNALB and other *S*-nitroso compounds and NO donors in washed human red blood cells (WHRBCs) and human whole blood. Incubation of *S*-[^{15}N]-nitroso-L-cysteine (L-S[^{15}N]C) with WHRBCs resulted in time- and concentration-dependent saturable formation of cytosolic *S*-[^{15}N]-nitrosoglutathione (GS[^{15}N]O), [^{15}N]nitrate and L-cysteine in the cytosol. Cytosolic GS[^{15}N]O and [^{15}N]nitrate were structurally identified by mass spectrometry. D-S[^{15}N]C was also transported into and metabolized in the WHRBCs but at a considerably lower rate compared with L-S[^{15}N]C. Incubation of erythrocytes with lowered intracellular levels of GS[^{15}N]O by (mean ± SD, n = 3) 80 ±15% and 98 ±5%, respectively, while lysine (10 mM) inhibited by only 65 ±10%. Re-incubation of L-S[^{15}N]C-treated WHRBCs with the original plasma resulted in a concentration-dependent formation of plasma *S*-[^{15}N]-nitrosoalbumin and inhibition of ADP-induced platelet aggregation. Incubation of WHRBCs with GSNO and *S*-nitroso-*N*-acetyl-penicillamine, DETA NONOate, Angeli's salt or nitric oxide (NO) did not result in significant intracellular formation of GSNO. In human whole blood *in vitro* GSNO was quantitatively oxidized to nitrate (k_1 = 0.0345 min^{-1}, $t_{1/2}$ = 20 min) although erythrocytes do not possess a transport system for GSNO. GSNO metabolism in the erythrocytes was found to depend on extracellular cysteine concentration. Our results suggest that plasma cysteine is the key physiological thiol regulating transport, metabolism and biological functions of *S*-nitroso compounds in human erythrocytes via a specific transport system for SNC.

β-Amino aciduria induced by (Aminooxy)acetate

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Injection of (aminooxy)acetate (AOA), a potent inhibitor of amino-transferases, to rats increased the excretion of β-alanine (BALA), β-aminoisobutyric acid (BAIBA) and γ-aminobutyric acid (GABA). We developed a method of simultaneous determination of these β- and γ-amino acids in the urine and tissues. Amino acids in trichloroacetic acid extracts of tissues and in urine of normal and AOA-treated rats were converted to dabsyl derivatives and analyzed by reversed-phase HPLC. An injection of 15 mg of AOA per kg of body mass increased urinary excretions of BALA (27-fold), BAIBA (13-fold) and GABA (9-fold) in the 24-h urine after the injection. BALA in the liver increased 10 times at 2 h after the AOA injection, and that in the kidney, brain and blood plasma increased also significantly. BAIBA was not detected in these tissues, but it was detected at 2 h after AOA injection. GABA in the brain increased significantly in response to AOA injection. These results seem to be due to the inhibition of transaminases involved in the metabolism of these amino acids. Taurine and hypotaurine are also β-amino acids containing sulfonic and sulfinic group, respectively. Urinary excretion of taurine and hypotaurine increased by the administration of cysteine, and the excretion increased further by AOA injection. On the other hand, sulfate excretion decreased by the simultaneous injection of AOA with cysteine. The increase in taurine and hypotaurine excretion seem to be due mainly to the inhibition of transaminase(s) involved in the sulfate formation.

Gas chromatographic determination of hydrogen sulfide and acid labile sulfide in rat tissues

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In 1992, we proposed mercaptopyruvate pathway (MP pathway), in which hydrogen sulfide is formed. Hydrogen sulfide may also be formed by cystathionine-γ-lyase reaction. In order to study cysteine metabolism in mammalian tissues, we have developed a method to determine hydrogen sulfide and acid labile sulfide in rat tissues. A Hitachi gas chromatograph G-5000A with flame photometric detector (GC-FPD) equipped with a column of 5% polyphenyl ether 5rings on Uniport HP 80/100, 3 mm x 6 m was used. Tissue homogenate (10.0 ml) prepared with 5 vol of 0.9% sodium chloride solution at 0 °C was bubbled with N_2 gas with or without 5 ml of 50% phosphoric acid. The gas was introduced into 10 ml of 0.1N sodium hydroxide solution. An aliquot of this solution placed in a vial was acidified with 70% phosphoric acid, and the vial was incubated at 37 °C for 60 min. An aliquot of the head space gas was analyzed by GC-FPD. Calibration curve was prepared using known amount of Na_2S solution by mixing with 70% phosphoric acid in a vial. The square root of the peak area was proportional to the amount of Na_2S between 10 and 100 nmol per vial. The recovery of Na_2S as H_2S when Na_2S was added to rat liver homogenate was 103.2 ±6.5%. When liver and heart homogenates were acidified as above, 110 ±25.0 (n = 6) and 258.7 ±12.9 (n = 4) nmol/g of fresh tissue, respectively, of H_2S was detected. Free H_2S was not detected when the homogenate was not acidified.

Calcium dependency of excitatory sulfur-containing amino acid stimulated 3H-GABA release from rat olfactory bulb

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Beside glutamate (Glu), other amino acids as L-cysteine sulfinic acid (CSA) and L-homocysteinic acid (HCA) may have an excitatory role in the CNS acting on glutamate receptors (GluR). We have previously shown that the release of 3H-GABA may be triggered by Glu, CSA and HCA through NMDA and non-NMDA receptors (Garcia et al, 1995; Jaffe and Garcia, 1997) in the external plexiform layer of the olfactory bulb (EPL). In this preparation mitral cell dendrites of the olfactory bulb establish reciprocal synapses with GABAergic granule cell dendrites at the level of the EPL, where the excitatory input is through GluR. It is well known that the exocytotic release mechanism of neurotransmitters depends on the increase of cytosolic Ca^{2+}, which can be achieved by Ca^{2+} release from internal stores, or by Ca^{2+} entry through the voltage dependent Ca^{2+} channels (VDCC) and through Ca^{2+} permeable GluR, both NMDA or AMPA/Quisquilate type. Here we have studied the relative importance of these possible Ca^{2+} sources for the 3H-GABA release mechanism from granule cell dendrites of the EPL induced by CSA and HCA. We have used a $0Ca^{2+}$/10 mM Mg condition and Cd^{2+}, to block or reduce Ca^{2+} entry, and an inhibitor of Ca^{2+}-Calmodulin dependent processes, trifluoperazine to affect intracellular Ca^{2+} mobilization. With the $0Ca^{2+}$ condition the SAA induced 3H-GABA release, was completely blocked while with 50 uM Cd^{2+} the SAA stimulated release was not affected. Trifluoperazine significantly inhibited the stimulated GABA release. These results could indicate that intracellular Ca^{2+} buffering mechanisms and Ca^{2+} entry through Glu receptors are involved in SAAs stimulated GABA release in this preparation. On the contrary, Ca^{2+} entry through VDCC appears not to be important for the SAAs stimulated GABA release. However further experiments with other VDCC blockers are necessary to confirm this conclusion.

Regional expression and histological localization of cysteine sulfinate decarboxylase mRNA in the rat kidney

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Taurine, a putative osmolyte in the kidney, was previously localized in various segments of the nephron, while taurine-transporter (TauT)-mRNA showed a more restricted distribution. Thus, a local synthesis of taurine could explain its presence in some renal cells. Cysteine sulfinate decarboxylase (CSD) and cysteine dioxygenase (CDO) belong to the cysteine sulfinate pathway leading to taurine from cysteine. While a CSD activity was previously measured in whole kidney extract, no information about CSD-mRNA regional expression and histological localization is available yet. Western blot and Northern blot were performed in the four main dissected rat renal zones using respectively an antiserum against recombinant CSD and a [^{32}P]dCTP-labelled CSD-cDNA probe. SCD-mRNA, CDO-mRNA and TauT-mRNA contents were comparatively determined through RT-PCR. *In situ* hybridization was carried out using [^{35}S]CTP-labelled CSD-RNA probe. A single protein (53 kDa) and a single mRNA (2.5 kb) were detected, which appeared both more enriched in the outer stripe of the outer medulla (OS). CSD-mRNA, CDO-mRNA, TauT-mRNA were similarly enriched in the OS. *In situ* hybridization of CSD-mRNA showed a strong labelling in the proximal straight tubules of cortical medullary rays and OS. So, taurine synthesis through the cysteine sulfinate pathway occurs primarily in a restricted portion of the nephron, the proximal straight tubule. The localization of taurine along the nephron differs from these of TauT-mRNA and CSD-mRNA. This suggests that other metabolic pathways and/or taurine-transporters remain to be identified and localized in the kidney.

Methylation of peptides derived from two GRG-containing proteins by S-adenosylmethionine: Protein arginine N-methyltransferase

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S-Adenosylmethionie: protein arginine N-methyltransferase (protein methylase I; EC 2.1.1.23) methylates the guanidino nitrogen of arginine residues in protein utilizing S-adenosyl-L-methionine as the methyl donor. This enzymatic methylation of polypeptides yields N^G-monomethyl, N^G,N^G-dimethyl and N^G,N'^G-dimethylarginine with release of S-adenosyl-L-homocysteine. It has been observed that these methylated arginine residues are present in Gly and Arg rich motifs. In order to investigate the GRG-containing peptides serve as the methyl acceptors *in vitro* for the N-methyltransferase, we have synthesized several oligopeptides of different chain length and sequences which are identical or homologous to the GRG-containing region of the Stimulator of HIV-1 TAR RNA-binding protein(SRB) and fibronectin. These results showed that the heptapeptide, AGGRGKG (Res. 16–22 in SRB) methylated by rat liver protein arginine N-methyltransferase with K_m value of 50 μM. The same peptide in which both NH_2- and COOH-termini were blocked by acetyl and amide groups, respectively, was poor substrate than the unblocked pep-

tide. The longer oligopeptide (Res. 10–28 in SRB) exhibited much higher substrate activity with decreased Km value (8.3 μM). Amino acids analysis by HPLC confirmed that the methylated product was N^G-monomethylarginine and N^GN^G-dimethylarginine (asymmetric), which are known enzymatic products by nuclear protein/histone-specific N-methyltransferase. The synthetic heptapeptide, GRGDSPK, which is derived from the cell attachment sequence of fibronectin showed negligible substrate capability. However, when the N-terminal Gly of that peptide was extended with extra Glys, the methyl acceptibilities increased in proportion to the number of Gly added. Interestingly, while the unmethylated peptides blocked β-hexosaminidase(Hex)-induced DNA synthesis, the methylated counterpart did not block Hex-response. These results indicate that there are another potential substrate for protein arginine N-methyltransferase which might regulate the function of these proteins *in vivo*.

Low vitamin B12 does not obviously compromise transmethylation in adults on a free diet

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The lower reference for plasma vitamin B12 is not well defined and is commonly set against haematology criteria, typically about 150 ng/L. There is, however, evidence for biochemical perturbation, as assessed by plasma concentration of remethylation intermediates, which suggests that a lower limit of about 220 ng/L would be more appropriate. We have attempted to improve the definition further by relating creatine, the major product of transmethylation, to vitamin B12 in a cohort of patients referred for neurological screening, but otherwise unselected in terms of symptoms, age, sex or diet.

We have found nor correlation between plasma creatine or vitamin B12 concentrations (range 101–2000 ng/L), suggesting that in these patients vitamin B12 is not a determinant of transmethylation flux. One possible explanation for this is the ready availability of methionine. It is possible to derive a theoretical intake of methionine at which the requirement for remethylation is zero, the daily intake of protein needed to achieve this being easily attainable on a free "western" diet.

Although plasma concentrations of methionine, homocysteine and creatine may not accurately reflect intracellular metabolism, we tentatively conclude from this preliminary data vitamin B12 is not critical in maintaining essential 1-carbon (transmethylation) reactions in the presence of an adequate dietary supple of methionine.

Free Radicals and Amino Acid Oxidation

Radiation damage to proteins and amino acids

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Amino acids, peptides and proteins are preferred targets of reactive oxygen species produced as a result of ionizing radiation. The initial reactions are quite well established and involve hydrogen abstraction, one-electron transfer and addition depending on the nature of the reactive species. However, subsequent product-forming mechanisms are complex and much less understood. Here, we will present examples on how peptide sequence and conformation around an amino acid can affect reaction pathways of initially produced reactive intermediates.

The reaction of hydroxyl radicals with methionine yields methionine hydroxysulfuranyl radicals. Evidence for proton transfer between the latter and an N-terminal amino group in Thr-$(Pro)_n$-Met (n = 0–4) has been obtained resulting in cleavage of the Thr side chain into acetaldehyde. Competitively, the methionine hydroxysulfuranyl radical can convert into intramolecularly sulfur-caboxylate bonded intermediates. Reaction efficiencies depend on the distance between the reacting groups and peptide conformation and flexibility, supported by molecular dynamics simulations. In another example, we will show how peptide sequence promotes the diastereoselective oxidation of methionine by reactive oxygen species. The latter pathway is especially important with regard to the diastereoselective enzymatic repair of methionine sulfoxide by methionine sulfoxide reductase, demonstrated here for the protein calmodulin.

(Supported by the NIH.)

Modified thiolamines: Improvements in radiation protection?

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The need for protection against the toxic and mutagenic effects of ionizing radiation comes from many different directions: occupational exposure, nuclear accidents, environmental sources, and protection of normal tissue during the therapeutic irradiation of cancer. Sulfhydryl-containing compounds, including cysteamine, cysteine, and WR-1065, have long been known to possess radiaprotective properties, but their therapeutic utility is limited by side effects at radioprotective doses and limited oral activity. To avoid these drawbacks, thiazolidine prodrugs of the parent drugs were prepared. The prodrugs were designed to liberate the parent thiolamine either enzymatically or nonenzymatically, which may allow for the fine-tuning of biological effects. Toxicity of the novel agents in Chinese hamster V79 cells was investigated, as was protection against radiation-induced lethality and DNA single-strand breaks. Representatives of various classes of agents will be compared and contrasted.

Mechanisms of thiolamine chemoprevention

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Aminofostine (WR-2721, Ethyol®, S-2[3-aminopropylamino]-ethylphosphorothioic acid) and its thiol and disulfide forms represents a class of thiolamine compounds that can confer protection against cell killing (cytoprotection), mutagenic

and carcinogenic processes (chemoprevention), and spontaneous metastasis formation. Cytoprotection results from the reduction of radiation or drug induced damage to cells via free radical scavenging, hydrogen atom donation (chemical repair), and auto-oxidative mechanisms. The anti-mutagenic effects result from the polyamine like structure and function of the disulfide form of amifostine that allows it to stabilize chromatin and enhance the fidelity of DNA repair. The anti-metastatic effect is thought to be due to thiol and disulfide involvement in modifying redox dependent processes that lead to changes in: transcription factor activation (i. e. NFκB), gene expression (i. e. Mn-SOD), and enzyme activity (i. e. topoisomerase-IIα). These properties are also exhibited by the thiols N-acetyl-cysteine (NAC) and oltipraz, but at much higher concentrations. The relative net positive charges of the thiol and disulfide forms of amifostine (i. e. +2 and +4, respectively) are in contrast to the net negative charges of NAC and oltipraz. It is proposed that amifostine's enhanced effectiveness at relatively low concentrations as compared to NAC and oltipraz in modifying transcription factor activation and gene expression is due to its enhanced ability to localize within the microenvironment of nuclear and mitochondrial DNA.

(Supported by National Institutes of Health, National Cancer Institute, RO1 grant CA37435.)

Effects of thiazolidine prodrugs on mutation in A_L cells

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Genetic diseases like cancer are caused by mutations so that *in vitro* studies of mutagenesis can illuminate mechanisms and cancer causing potency of radiations. It follows, also, that prevention of mutation could reduce the incidence of cancer. But the success of such studies depends on the capacity accurately to quantify and study all of the kinds of mutations involved in carcinogenesis, from small intragenic ones to loss or rearrangements of tens of millions of base pairs. Experiments will be described using various chemicals, heavy metals, and radiations of different LET demonstrating that A_L human x hamster hybrid cell *CD59* assay allows the sensitive and rapid quantitation of all of these kinds of mutants and the simple molecular analysis of the kinds of mutations induced. The role of oxidative damage in radiation mutagenesis and alteration of mutagenesis by radioprotectors such as WR-1065 [N-(2-mercaptoethyl)-1,3 propanediamine and the thiazolidine prodrug ribose-cysteine RibCys will be described.

(Supported by grants from NASA, NIH-NCI & the Waldren/Vannais Lab Discretionary Fund.)

The toxicity and post radiation antimutagenicity of WR 1065 varies in related murine L5178 Y lymphoblastic cell lines

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The L5178Y (LY) cell lines are heterozygous at the thymidine kinase (TK locus) and include LA-S1, which is deficient in DNA double-strand break (dsb) repair, and LY-SR1 and LY-R16, both of which are proficient in dsb repair relative to LY-S1 cells. LY-R16 cells were found to be much more sensitive to the cytotoxic effects of WR 1065 than where the other two cell lines, and addition of catalase to the medium reduced the toxicity and the occurrence of apoptosis. Over a year's time, LY-R cells became more resistant to the cytotoxic effect of WR 1065 and H_2O_2, and the level of catalase had increased from previous values. The results indicate that the toxicity of WR 1065 in LY-R cells is caused by its metabolism to H_2O_2 that is subsequently converted to reactive oxygen species. WR 1065 provided equal protection to the LY-S1 and LY-SR1 cell lines against the cytotoxic and mutagenic effects of ionizing radiation when present 30 min before and during the exposure. However, a 3-hour post radiation treatment with WR 1065 reduced the frequency of radiation-induced $TK^{-/-}$ mutants in LY-S1 cells to a much greater extent than in LY-SR1 and LY-R16 cells. The post radiation treatment did not significantly affect cell survival, dsb rejoining, or cell cycle distribution. It is possible that post radiation WR 1065 increases the fidelity of dsb rejoining.

New strategies in radioprotection based on unconventional use of common pharmacologics

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Unconventional treatments are currently being explored for use in protecting against both early and late-arising pathologies induced by ionizing radiation. The ultimate aim of this effort is to develop nontoxic, effective radioprotectants that afford to the prophylaxed individual an elevated resistance to tissue injury and subsequent pathology. The treatments under review encompass such diverse pharmacologic classes as: novel cytokines, cytokine mimics, organ-specific stimulants and repair enhancers, polyfunctional radical trapping agents and nutritional antioxidants. Of the several treatments currently under study, two show particular promise and are being actively pursued. The first involves the use of 5-androstenediol (AED), a steroid with broad spectrum, radioprotective attributes. This agent protects against simple tissue injury as well as against septic complications that often follow such injury. It has low toxicity, can be given through injection or orally, and can substantially enhance resistance to potentially lethal injury by increasing the LD50/30 radiation dose level by >25%. The second agent is buthionine sr-sulfoximine (BSO). BSO is a well known radiosensitizer under acute irradiation. However, following chronic oral administration, BSO appears to lower cancer risk associated with radiation exposure by selectively inhibiting the abnormal expression of several oncogenes (*ras* family oncogenes) and tumor suppresser genes (p53) during early phases of evolving cancer. Initial successes in protecting irradiated animals against both early and late-arising pathologies using these new and unconventional agents will be described and discussed relative to a new evolving paradigm for preventive treatments.

Quantification of free 3-nitrotyrosine in human plasma by gas chromatography-tandem mass spectrometry: A novel method for the assessment of oxidative stress *in vivo* in humans

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3-nitrotyrosine has been suggested as a novel marker of reactive nitrogen species mediated oxidative stress. To date, no reliable data exist on the concentration of free 3-nitrotyrosine in plasma of healthy humans. For the accurate and precise quantification of free 3-nitrotyrosine in human plasma at the basal state

we developed and fully-validated a gas chromatographic-tandem mass spectrometric method. Various careful precautions have been taken in order to avoid artefactual formation of 3-nitrotyrosine from tyrosine and nitrite both present in human plasma at concentrations of 70 μM and 3 μM, respectively, during sample treatment. Endogenous plasma 3-nitrotyrosine and externally added 3-nitro-L-[$^2H_3$]tyrosine for use as internal standard were isolated by high-performance liquid chromatography (HPLC) of 200-μl aliquots of plasma ultrafiltrate (20 kDa cut-off), extracted from a single HPLC fraction by solid-phase extraction, derivatised to their *N*-pentafluoropropionyl *n*-propyl ester trimethylsilyl ether derivatives and quantified by negative-ion chemical ionization in the selected-reaction monitoring mode. Overall recovery was determined as 50 ±5% using 3-nitro-L-[$^{14}C_9$]tyrosine. The limit of detection of the method was 4 amol of 3-nitrotyrosine. 3-nitrotyrosine added to human plasma in the range 1–2 nM was quantitated at an accuracy of >80% and at a precision of >94%. In plasma of six healthy humans a mean concentration of 2.9 ±0.6 nM was determined by the method. This is the lowest concentration reported so far for plasma free 3-nitrotyrosine. Endogenous 3-nitrotyrosine was identified in human plasma by generating a daughter mass spectrum. This method should be useful to investigate the reliability of plasma free 3-nitrotyrosine as a measure of reactive nitrogen species associated oxidative stress in vivo in humans.

Radical scavenger activity of 5-S-cysteinyldopa-enkephalin, a precursor of pheo-opiomelanins

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In previous works we demonstrated that tyrosine-containing opioid peptides (i. e. enkephalins, exorphins) are oxidized by tyrosinase giving rise to soluble pigments, the opiomelanins. Recently we obtained results indicating that, in the presence of cysteine, the dopaquinone resulting from enkephalin oxidation undergoes nucleophylic addition with the sulfhydryl group leading to the formation of adducts, the cysteinyldopaenkephalins. In order to define a possible functional role of these adducts, we have indagated on the radical scavenger activity of the 5-S-cysteinyl isomer of tyrosinase-oxidized Leu-enkephalin (5-CDEnK).

The data obtained demonstrated that 5-CDEnk can (in a dose-dependent manner) scavenge the superoxide anion. The compound showed a high activity even at low concentration. 5-CDEnk was a scavenger much more efficient than cysteinyldopa and Leu-enk.

5-CDEnk also scavenged hydroxyl radical. The order of effectiveness of the hydroxyl scavenger tested was: 5-CDEnk >DMSO >Leu-enk >>mannitol.

5-CDEnk was also shown as an efficient inhibitor of lipid peroxidation; it decreased the rate of linoleic acid peroxidation ABAP-induced. Also peroxyl radical was efficiently scavenged by 5-CDEnk.

It is well known that in certain physiological or pathological conditions there is an overproduction of reactive oxygen species, including superoxide anion, hydroxyl and peroxyl radicals, which are dangerous to the cell. It is also ascertained that the first step of the overall oxidative phenomeny linked to the oxidative stress is the entry in the cell of cysteine or glutathione. The results here reported lead us to think that cysteinyldopaenkephalins could be formed in vivo as response of the cell to oxidative stress conditions, i. e. they might be effective endogenous antioxidant that protect lipids and proteins against oxidative damage.

Hydroxyl radical-induced DNA-protein cross-linking under gamma-irradiation: Cross-links of thymidine with lysine and arginine in calf thymus nucleohistone

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Hydroxyl radical-induced formation of DNA-protein cross-links involving thymidine (dThd) and basic amino acids such as lysine (Lys) and arginine (Arg), that are major constituents of histone and facilitate its binding to DNA in chromatin, was studied to get chemical insight into the structures and the mechanism. The structures of dThd-Lys and dThd-Arg cross-links as isolated by HPLC from the model γ-radiolysis systems in N_2O-saturated aqueous solution were identified, using a fast atom bombardment-liquid chromatography-mass spectrometry (FAB-LC-MS). Characterization by a gas chromatography-mass spectrometry (GC-MS) was also performed for trimethylsilylated HCl-hydrolysates of the cross-links. Compared with a cross-link between thymine and Lys formed in a model γ-radiolysis system, considerable degradation occurred by the HCl-treatment and trimethylsilylation. A mechanism by which 5-hydroxy-5,6-dihydrothymidin-6-yl radical [dThd-5-OH C(6)•] undergoes recombination with C(4)-centred radical of Lys [Lys C(4)•] accounted for the observed LC-MS and GC-MS of each cross-link. Trimethylsilylated HCl-hydrolysates of calf thymus nucleohistone after γ-irradiation in N_2O-saturated aqueous solution were further analyzed by a GC-MS with a selected-ion monitoring technique (GC-MS/SIM), by reference to several fragment ions characteristic of the dThd-Lys and dThd-Arg cross-links. The GC-MS/SIM analysis demonstrated that both the dThd-Lys and dThd-Arg cross-links are formed in the nucleohistone under generation of hydroxyl radicals.

Oxidation of methionine and cysteine residues in actin by reaction with chloramine T

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In general, methionine oxidation to the sulfoxide derivative may inactivate proteins. In vivo oxidation of Met in specific proteins has been associated with physiological and pathological conditions, such as ageing, cataract, inflammatory disease, arteriosclerosis, neurological disorders, rheumatoid arthritis and emphysema [Oliver et al (1990) Proc Natl Acad Sci USA, 87: 5144–5147; Stadtman ER (1995) In: Esser K, Martin GM (eds) Molecular aspect of ageing, Wiley, London, pp 129–142]. However, Met residue oxidation in proteins does not necessarily cause either structural changes or loss of biological activity. A mild oxidising reagent for the selective oxidation of methionine is chloramine-T (CT), which, at pH 7.0–8.5, oxidises only Met and Cys.

Determining the number of both total and free thiol groups for actin monomer by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (ETNB), we found that increasing CT concentrations, up to a 10-fold molar excess to actin, gradually oxidised the only exposed thiol group of native G-actin (Cys374). When the protein was treated with a CT molar excess of 20 or higher, Cys10 was also oxidised. Comparison of CT-oxidised and native actin by Trp intrinsic fluorescence gave no indication of significant conformational changes for CT concentrations ranging from equimolarity to 10-fold molar excess. Differently, at higher ratios, a remarkably reduction of Trp fluorescence and a red shift in the mission wavelength maximum were observed, both indicating protein unfolding. CNBr cleavage pattern of oxidised G-actin indicated that oxidation of both Met44 and Met47, and probably some others not yet identified, occurred. Polymerization kinetics was studied by following the increase in the 90°

light scattering intensity at 546 nm. At increasing CT concentrations, actin polymerization was progressively slackened and the steady state level of F-actin concentration was considerably decreased; at ratios higher than 7, inhibition of polymerisation was complete. When actin was subjected to CT oxidation after blocking the Cys374 SH group with DTNB, polymerization inhibition was still observed, indicating that modified Met residues take some part in the intermonomer binding during polymerization, in addition to the Cys374 thiol group. The CT inhibitory effect on actin assembly was confirmed by two additional independent assays of polymerization, i. e. high-speed sedimentation assay and a high-shear viscometry assay.

Structural changes and conformational stability in actin resulting from Cys-374 oxidation

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Cell rounding and plasma membrane blebbing as well as shortening of microfilaments, which aggregate side-to-side into short bundles, suggests that cytoskeletal elements of the cell cortex could represent one of the cellular targets of oxidative stress [Hinshaw et al (1991) Arch Biochem Biophys 288: 311–316]. In cultured cells, actin thiols are one of the targets for the oxidative stress [Mirabelli et al (1988) Arch Biochem Biophys 264: 261–269; Omann et al (1994) Arch Biochem Biophys 308: 407–412]. In addition, H_2O_2 and diamide alters actin dynamics in solution [DalleDonne et al (1995) Biophys J 69: 2710–2719; Milzani et al (1997) Arch Biochem Biophys 339/2: 267–274]. Recently, Keck (1996) [Keck (1996) Anal Biochem 236/1: 56–62] showed that only exposed methionine residues were susceptible to oxidation by t-butyl hydroperoxide (t-BH) in native recombinant interferon gamma and recombinant tissue-type plasminogen activator. On the other hand, the t-BH treatment of human red blood cells causes damage in cytoskeletal protein thiols [Caprari et al (1995) Chem Biol Interact 94: 243–258; Lii and Hung (1997) Biochem Biophys Acta 1336: 147–156].

The susceptibility of monomeric actin (12 μM) to methionine and cysteine oxidation when treated with t-BH was investigated. CNBr cleavage pattern is unaffected by the oxidative chemical treatment at the t-BH concentrations (1–20 mM) used in our experiments. This indicates that oxidation of methionine residues did not occur. In fact, one of the five cysteine residues of the actin molecule, Cys-374, is the site of the oxidative modification. Perturbations in the intrinsic tryptophan fluorescence and the decreased susceptibility of oxidised actin to limited proteolysis by α-chymotrypsin and subtilisin suggest some alterations of protein conformation in helix 74–92 in subdomain 1, as well as in the central segment of surface loop 39–51 in subdomain 2. $\Delta G(H_2O)$, the free energy of unfolding at infinite denaturant dilution, derived from urea denaturation curves, differs by 0.657, 2.437 and 2.757 kcal/mol for the native actin and actin oxidised by 1,10 and 20 mM t-BH, respectively, indicating a lower conformational stability for the oxidised actin.

Methionine and S-adenosylmethionine (SAM): Sources of radiation-induced DNA-methylation

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Methionine is one of the radiosenitive amino acids in the nucleohistones and in some polypeptides. SAM is the coenzyme of many methyltransferase and plays an important role in cell biochemistry. It is well known that an imbalance of transmethylation results in over-methylation of some substances and undermethylation of others within cancer cells.

Aqueous solutions of L-(methyl-^{14}C)methionine, L-^{35}S-methionine, and S-adenosyl-L-(methyl-^{14}C)methionine with different concentrations were purified from volatile autoradiolytic degradation products by repeated freeze-drying. Aliquotes were then gamma-irradiated with a dose rate of 5 Gy/h for doses up to 3 Gy, and measured by liquid scintillation counting. The resulting dose effect curve for L-(methyl-^{14}C)methionine is higher by a factor of about two than the dose effect curve of ^{35}S-methionine, due to the fact that in the case of (methyl-^{14}C)methionine, volatile products formed from $^{14}CH_3$ and $^{14}CH_3S$ radicals are measured; whereas in the case of ^{35}S-methionine only volatile products resulting from $CH_3{}^{35}S$ radicals are counted. The cleavage rate for methyl radicals from SAM is smaller than that of methionine.

To test methylation and thiomethylation of DNA (methyl-^{14}C)methionine and ^{35}S-methionine were irradiated together with isolated DNA in deoxygated aqueous solutions. The separation of DNA and methionine was performed by gel filtration on Sephadex G25 columns, and the radioactivity of DNA measured in a scintillation counter.

The present date for the radiation-induced DNA alkylation at a dose of 5 Gy are comparable to the extent of DNA alkylation in mice after a single treatment with an alkylating agent, ENU (N-ethyl-N-nitrosourea), which produces tumors in 43% of the animals, found by other authors.

Furthermore, the single polynucleotides pA, pC, and pG were irradiated together with (methyl-^{13}C)methionine in aqueous solution and separated on Sephadex G50 columns. The mass spectrometric analysis of the polynucleotides showed an increase of the ^{13}C-content in the sequence pG >pC <pA.

The radiation-induced methylation and thiomethylation of DNA could open a new pathway to the understanding of the molecular reactions of carcinogenesis. The results underline the requirement for irradiation of other biological substances, for example the methionine-containing tumor suppressor gene p53.

The origin of chirality: New aspects by radiation-induced isotope fractionation during decarboxylation and deamination of L-leucine

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The role of isotope effects in radiation-induced selection processes is of special interest in connection with the origin of chirality.

The radiation-induced decarboxylation of L-phenylalanine-1-^{13}C and of L-leucine-1-^{13}C show sigmoid dose effect relations for the cleaved CO_2 typically for the radiation damage in organic solids.

Recent findings concerning the decarboxylation and deamination demonstrate that there is not only a carbon isotope effect during decarboxylation L-leucine-1-^{13}C but also a nitrogen isotope effect during deamination. For this purpose several samples of about 2 grams of the amino acid were irradiated with a ^{60}Co-source under vacuum with doses of 17 to 500 Gy. Since decarboxylation and deamination take place simultaneously the reaction products CO_2 and NH_3 had to be separated and the ammonia converted into molecular nitrogen for stable isotope ratio measurements.

The experiments show that deamination is favoured to decarboxylation. Both reactions lead to an enrichment of the light isotopes ^{12}C and ^{14}N, respectively, in the corresponding gases. The decarboxylation of D-leucine was shown to be more effective than that of L-leucine earlier. If this were valid for the deamination, too, there were two different reactions cooperatively favouring the degradation of the D-form. Deamination and de-

carboxylation independent from each other give rise to the loss of asymmetric centres of the amino acids.

During prebiotic evolution, the radiation on the early Earth could have led in the same way to a predominance of the other L-amino acids involved.

Effect of proline on the production of singlet oxygen

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Molecular oxygen in electronic singlet state is a very powerful oxidant. Its damaging action in a variety of biological processes has been well recognised. Here we report the singlet oxygen quenching action of proline. Singlet oxygen ($^1O_2$) was produced photochemically by irradiating a solution of sensitiser and detected by following the formation of stable nitroxide radical yielded in the reaction of $^1O_2$ with the sterically hindered amine (2,2,6,6-tetramethylpiperidine, TEMP). Illumination of a sensitiser, toluidine blue led to a time dependent increase in singlet oxygen production as detected by the formation of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) by EPR spectrometry. Interestingly, the production of TEMPO was completely abolished by the presence of proline at concentration as low as 20 mM. These results show that proline is a very effective singlet oxygen quencher. Other singlet oxygen generating photosensitizer like hematopophyrin and fluorescein also produced identical results with proline. Since proline is one of the important solutes which accumulate in many organisms when they are exposed to environmental stresses, it is likely that proline accumulation is related to the protection of these organisms against singlet oxygen production during stress conditions. A possible mechanism of singlet oxygen quenching by proline is discussed.

Tryptophan degradation along the kynurenine pathway, protective and toxic implications during immune activation

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Interferon gamma (IFN-γ) produced by activated T-cells enhances free radical generation and induces a number of proteins including the enzyme indoleamine 2,3-dioxygenase (IDO) in astrocytes and macrophages. IDO is the first enzyme of the kynurenine pathway, leading to the synthesis of quinolinic acid (a known neurotoxin) as well as NAD. DNA damaging agents such as H_2O_2 reduce NAD levels in astroglia due to increased poly(ADP-ribose)polymerase (PAPRP) activity.

This study investigates the hypothesis that IDO induction in the human astroglioma cell line, HTB-138, results in increased *de novo* synthesis of NAD^+.

IDO activity increased significantly in astroglia following IFN-γ treatment. IFN-γ increased NAD^+ levels in astroglia to 112 ±5% of control following PARP inhibition. This effect was completely abolished by inhibiting IDO with 6-chloro-D-tryptophan. After 30 minutes exposure to 100–800 μM H_2O_2 NAD^+ levels decreased progressively to 47 ±2.8% of control at 800 μM H_2O_2. This loss in NAD^+ was significantly moderated in cells pretreated with IFN-γ. Lactate dehydrogenase activity was significantly elevated in control but not IFN-γ pretreated cells following exposure to moderate amounts of H_2O_2.

These results indicate that *de novo* synthesis of NAD^+ is increased in IFN-γ treated astroglia in which IDO is induced. This appears to contribute to the maintenance of intracellular NAD^+ levels and cell viability under conditions of an increased free radical burden, but may lead to the synthesis of potential neurotoxin, quinolinic acid.

Taurine dichloramine as an intermediate in sulfoacetic aldehyde formation

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Neutrophiles (PMNs) during the phagocytic process release reactive oxygen species (ROS). At the presence of the hydrogen peroxide the hypochlorite (HOCl) and taurine chloramine are the products of myeloperoxidase (MPO) action in phagolysosomes. The formation of the taurine chloramine is favoured due to the (30 mM) concentration of taurine in medium. At present the knowledge about the function and the products of the further decomposition of this compound is poor. However, it has been reported [Cunningham C, Tipton KF, Dixon HBF (1998) Biochem J 330: 939–945] that the sulfoacetic aldehyde is formed in the fagocytizing PMNs and its formation is taurine monochloramine mediated.

In fagolysosome when the pH is decreased to 5 the taurine dichloramine is formed from monochloramine. In the presence of the hydrogen peroxide; di-, monochloramine and taurine could undergo the oxidative deamination process.

In our report we examined the sulfoacetic aldehyde formation in the systems containing H_2O_2, taurine mono- and dichloramine within the pH range 5.3–7.4. Chloramines were obtained nonenzymatically or were generated with MPO/H_2O_2/Cl^- system. The presence of sulfoacetic aldehyde was confirmed in all tested systems, although its formation is accelerated in those containing taurine dichloramine and hydrogen peroxide at pH 5.3. It was also shown that the unchlorinated taurine is the source of aldehyde at the presence of H_2O_2 but only in acidic solutions. In examined systems the conversions taurine derivatives to aldehyde raises to 10%. Presented kinetics reveal that oxidative deamination in these systems is the slow process (hours/days) but could lead to μM-mM levels of aldehyde in the medium. The production of sulfoacetic aldehyde in the MPO system reaches the comparable level as for nonenzymatic ones and its increase correlates with the decrease of dichloramine concentrations in the samples. It seems that MPO plays the double role in taurine oxidative deamination. In the absence of Cl^- or at the excess of hydrogen peroxide the quick deamination is favoured probably via MPO/H_2O_2 complex. In the full system containing Cl^- the enzyme catalyses the monochloramine formation which via dichloramine is slowly oxidized to the aldehyde.

Sulfoacetic aldehyde formation was confirmed with use of the ^{1}HNMR spectra of separated 2,4-dinitrophenylhydrazone and its presence was monitored with HPLC method of its cyanine derivative. Additionally its antimicrobial activity as compared to that of the taurine chloramine is presented.

Content of total glutathione in the cerebrospinal fluid of patients with complete stroke

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Tripeptide glutathione represents one of the main component of cell antioxidative defense, as well as a clue factor of cell surviving.

Contents of total glutathione and malondialdehyde (MDA) were measured in the cerebrospinal fluid (CSF) of patients (n = 19) with completed stroke, confirmed by CT-scan. Samples were collected 48 hours after the ischemic episode. Control group included patients (n = 11) suspected on lumbar disc disease, who underwent diagnostic lumbar radiculography.

Total glutathione content was decreased about 33% in the

CSF of stroke patients, compared to control values (control 0.397 ±0.154 nmol/ml CSF). At the other hand, MDA content, as a measured of membrane lipid peroxidation, was increased about 89% compared to controls.

Elevated content of total glutathione could be result of enhanced level of oxidized form of this tripeptide, as a consequence of oxidative stress and consumption of GSH in process hydroperoxide detoxification mediated by glutathione peroxidase.

Gamma-camera lymphoscintigraphy and determination of an antigen, specific for the ovarian carcinoma in patients, suffering from ovarian tumors

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Patients with carcinoma of the ovaries, twenty in total, were investigated at ages between 25 and 60. All the patients were subject to indirect lymphoscintigraphy of the abdominal lymphatic nodules in order to reveal metastases. The colloids were labelled with Technetium 99 and injected into both dorsal surfaces of the feet. The registration of the labelled lympathic chains was accomplished in a γ-camera Sigma-4100 Ohio on the second and third hours after injection.

The changes in the abdominal lymphatic nodules were characterized by a moderate decrease of the phagocytic activity of the homolateral iliacus and inguinalis lymphatic nodules. The discrepancies were visualized by asymmetry and decreased storage of the radiocolloid in the lymphatic nodules, disrupted lymphatic chain and absence of the radiocolloid in some lymphatic nodules.

Our results indicate that lymphoscintigraphy can be a method to record changes in the phagocytic activity of the lymphatic nodules, localized homolaterally to the ovarian carcinoma.

Polyamines

Polyamine and neoplastic growth

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The research into the relationship between polyamines and cancer was developed first as the evaluation of polyamine levels in physiological fluids of healthy and cancer-bearing patients, then in successive steps in the actual search of polyamine analogues as effective adjuvant therapeutic agents for advanced carcinoid tumors. Currently, attention is being given in our laboratory to polyamines as building blocks for other type of biomolecules by the action of transglutaminases. This expansion of studies was made possible by the observation that transglutaminases and the product of their action (gamma-glutamyl-polyamines) are involved in cancer cell growth and differentiation [1]. Since the first report by D. H. Russell about polyamine and cancer [2], several polyamine analogues have been found to affect polyamine pool homeostasis and inhibit tumor cell growth [3]. The cytotoxicity of these molecules appears as a result of their ability to selectively activate the cell death pathway [4]. Our last data about the involvement of polyamines and their transamidated products in cancer suggest that these aliphatic molecules play a role in the cascade of molecular changes which influence cancer cell differentiation [5]. The basic principles underlying cellular mechanisms modulated by polyamines and their analogues are becoming more clear and ongoing laboratory and clinical observations will direct an expansion of this emerging novel approach to biological response modification, chemoprevention and chemotherapy.

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Molecular mechanism of polyamine stimulation of the synthesis of oligopeptide-binding protein

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We have suggested that the degree of polyamine stimulation of oligopeptide-binding protein (OppA) synthesis is dependent on the secondary structure and position of the Shine-Dalgarno (SD) sequence of OppA mRNA [Igarashi K, Saisho T, Yuguchi M, Kashiwagi K (1997) J Biol Chem 272: 4058–4064]. To study the structural change of OppA mRNA induced by polyamines and polyamine stimulation of initiation complex formation, four different 130 mer OppA mRNAs containing the initiation region were synthesized *in vitro*. The structural change of these 130 mer OppA mRNAs induced by polyamines was examined by measuring their sensitivity to RNase T_1, which is specific for single-stranded RNA, and to RNase V_1, which recognizes double-stranded or stacked RNA. In parallel, the effect of spermidine on mRNA-dependent fMet-tRNA binding to ribosomes was examined. Our results indicate that the secondary structure of the SD sequence and initiation codon AUG is important for the efficiency of initiation complex formation, and that spermidine relaxes the structure of the both SD sequence and the initiation codon AUG. The existence of a GC-rich double-stranded region close to the SD sequence is important for spermidine stimulation of fMet-tRNA binding to ribosomes. Spermidine apparently binds to this GC-rich stem, and causes a structural change of the SD sequence and the initiation codon, facilitating an interaction with 16S rRNA of 30S ribosomal subunits.

A role for polyamines in the regulation of apoptosis

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Apoptosis is an altruistic form of cell death associated with characteristic changes in morphology and ultimately fragmenta-

tion of the cell into apoptotic bodies without an ensuing inflammatory response [Kerr JFR, Wyllie AH, Currie AH (1972) Br J Cancer 26: 239–257]. A common feature of apoptosis is the fragmentation of DNA into multiples of 180–200 bp fragments due to the activation of an endogenous endonuclease. These fragments can be detected on agarose gel electrophoresis as a characteristic DNA "ladder" or by an ELISA method. The human promyelogenous leukaemic cell line, HL-60, can readily be induced to undergo apoptosis in response to anticancer drugs such as etoposide. Using 10 μM etoposide we have shown that apoptosis can be detected as early as 4 h after exposure to etoposide using electron microscopy and by 8 h using agarose gel electrophoresis. DNA fragmentation was also detected by the ELISA method from approximately 4 h with maximum fragmentation rates being reached at around 12 h [Lindsay GS, Wallace HM (1999) Biochem J 337: 83–87]. We also found a decrease in the total polyamine content in cells undergoing apoptosis as well as increases in polyamine oxidase (PAO), spermidine/spermine BN^1-acetyltransferase (SSAT) and polyamine export. In view of these changes in polyamine metabolism associated with the apoptotic process the aim of this study was to determine whether modulation of polyamine content either up or down would regulate apoptosis induced by etoposide. The addition of exogenous polyamines decreased DNA fragmentation HL-60 cells treated with etoposide following a 6 h incubation. Spermine was the most effective polyamine although acetyl polyamine derivatives were also active in decreasing the amount of DNA fragmentation. The polyamines did not however prevent apoptosis occurring rather they delayed the onset of the process. Decreasing polyamine content by pretreatment with DFMO also decreased the amount of etoposide-induced apoptosis. While these results may initially seem contradictory, preliminary evidence suggests that it may be changes in the activity of ornithine decarboxyalse (ODC) which are important in the regulation of apoptosis as opposed to changes in the polyamine content of the cell *per se*.

Transglutaminase-catalyzed spermidine incorporation into fibronectin and laminin reduces the *in vitro* metastatic behaviour of B15-F10 melanoma cells

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Metastasizing tumor cells disseminate invading the surrounding tissue and colonise distant sites [1]. Invasion can be facilitated by proteins which stimulate tumor cell attachment to host cellular or extracellular matrix components, but may be counterbalanced by factors which can impair their action. Polyamines and transglutaminases (TGs) are potential tools for the control of the metastatic potential of tumor cells [2]. TGs are enzymes catalyzing an acyl transfer where the carboxamide group of a peptide-bound glutamine is the acyl donor. Polyamines may act as acyl acceptors, leading to the formation of mono- or bis-(γ-glutamyl)derivatives. An inverse correlation between TG activity and the metastatic behaviour of cancer cell has been established, suggesting an involvement of TG in cancer cell motility and/or attachment to extracellular matrix [3]. We recently showed that the increase of TG activity reduced the invasiveness of B16-F10 melanoma cells [4]. Since the invasion of malignant cells involves basement membrane proteolysis, we investigated whether the covalent modification of basement membrane proteins by TG and polyamines could interfere with tumor adhesive and invasive ability. Fibronectin and laminin were incubated with TG and spermidine, to allow covalent incorporation of the polyamine. The "polyaminated" proteins were used for adhesion experiments and as barriers in an *in vitro* invasion assay. The results showed that the adhesiveness of tumor cells onto substrates coated with "polyaminated" proteins decreased by 20–25%, and the migration through the modified protein was significatively lower than the control (68% reduction for fibronectin, 48% for laminin). Our findings suggest that the TG-catalyzed covalent modification of extracellular matrix proteins by polyamines may impair tumor cell attachment to the basement membrane, but also may impede the proteolytic cleavage of extracellular matrix by metalloproteinases.

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The enzymatic oxidation products of spermine induce cytotoxicity on human colon adenocarcinoma cells

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In order to increase knowledge of the role of polyamines and their oxidation products for possible applications in cancer therapy, we have previously investigated cytotoxicity induced by purified bovine serum amine oxidase (BSAO) in Chinese hamster ovary (CHO) cells, under normal and hyperthermic conditions. BSAO caused considerable cytotoxicity in the presence of exogenous spermine. Cytotoxicity was increased by 42 °C hypterthermia. Exogenous catalase and aldehyde dehydrogenase protected against the cytotoxic effect at both 37 °C and 42 °C, showing that two species were responsible for cytotoxicity: hydrogen peroxide and aldehydes.

In order to verify the effects induced by the oxidation products of spermine in human cancer cells, we examined cytotoxicity in colon adenocarcinoma cells, both sensitive (LoVo WT) and drug resistant (LoVo DX) cells. Cytotoxicity was evaluated by plating efficiency test on cells after incubation in PBS-1%BSA containing BSAO (16 μg/mL) and spermine (0.1–6 μM) at 37 °C. BSAO and spermine alone were not toxic at these concentrations. Cytotoxicity was dependent on the concentration of spermine and on the incubation time. Cell survival experiments clearly showed that LoVo DX cells were more sensitive to the above treatment than LoVo WT cells. This finding was confirmed by scanning ad transmission electron microscopy observations. After treatment with BSAO and spermine, resistant cells showed morphological and ultrastructural modifications more pronounced than sensitive cells. In fact, LoVo DX cells appeared roundish with numerous surface blebs and large areas deprived of microvilli. Furthermore, almost all mitochondria of resistant cells showed condensed matrix and dilated cristae.

These results suggest that BSAO could have clinical potential in cancer treatment and in combination with hyperthermia may prove to be effective against drug-resistant tumors.

Polyamine transport in *Escherichia coli* and *Saccharomyces cerevisiae*

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PotE protein in *Escherichia coli* can catalyze both uptake and excretion of putrescine. The uptake of putrescine is dependent on the membrane potential and the excretion is based on the putrescine-ornithine antiporter activity. Amino acids involved in both activities were identified using mutated PotE. It was found

that Cys62, Trp201, Trp292 and Tyr425 were involved in both activities. Mutations of Tyr78, Tyr90 and Trp422 predominantly decreased the uptake activity, whereas mutations of Lys301 and Tyr308 mainly decreased the excretion activity. All these amino acid residues were found to be located in the transmembrane helixes or in the cytoplasmic region of PotE which has 12 transmembrane segments.

Properties of a membrane protein which has putative 12 transmembrane segments encoded by *YLL028w* were examined using *Saccharomyces cerevisiae* cells transformed with the gene. The transformed cells became resistant to polyamine toxicity, and the resistance was overcome by bafilomycin A_1, an inhibitor of vacuolar H^+-ATPase. Although spermine uptake activity of the transformed cells was almost the same as that of wild type cells, the uptake activity of vacuolar membrane vesicles from the transformed cells was higher than that from wild type cells. When the *YLL028w* gene was disrupted by inserting the *HIS3* gene, the cells became sensitive to polyamines, and spermine uptake activity of the vacuolar membrane vesicles decreased significantly. The accumulated spermine in *YLL028w* gene-disrupted cells decreased greatly compared with that in wild type cells. The results indicate that a membrane protein encoded by *YLL028w (TPO1)* is a polyamine transport protein on the vacuolar membrane.

Role of interferon regulatory factor-1 in ornithine decarboxylase gene expression during monocytic activation and differentiation

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Ornithine decarboxylase (ODC), the rate-limiting enzyme in the biosynthesis of polyamines, is thought to be crucial in cellular growth, proliferation and differentiation. In a previous study we observed that the induction of ODC gene expression, followed by polyamine accumulation, is an important step for macrophage activation, as demonstrated by a conspicuous decrease in superoxide anion (O2-)release using selective ODC inhibitors. We also hypothesized a role for the transcription factor IRF-1 in the regulation of the ODC gene during IFN-g-induced macrophage activation. IRF-1 is a member of the Interferon Regulatory Factors family, which includes over 10 members. This factor, originally characterized as a regulator of IFN-a, and-b, and other IFN-inducible genes, is encoded by a delayed early gene induced in response to IFN-g and to a variety of macrophage-activating factors. Recent investigations have demonstrated that this factor has a role in cell proliferation, differentiation and apoptosis. In the present study we investigate the correlation between IRF-1 activation and OLC gene expression in U937 human promonocytic cells during PMA-induced differentiation. The differentiation state of cells was evaluated by FACS analysis of CD11a, CD11c and CD14 expression, morphological analysis and O2-production. We provide evidence that the activation of IRF-1 in monocyte-macrophage differentiation of human U937 monoblastic cells induced by PMA tightly correlates with an increase in the expression of the ODC gene.

[This work was supported by grants from Associazione Italiana Ricerca sul Cancro (AIRC).]

Thiols and polyamines in the cytoprotective effect of taurine on carbon tetrachloride-induced hepatotoxicity

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Taurine (2-aminoethanesulfonic acid), a sulfur amino acid, is present in all mammalian tissues. It is not incorporated into protein and in fact the most abundant free amino acid in many tissues. Taurine is taken in via the diet in carnivores and omnivores and is also synthesized form cysteine or methionine present in the diet. The depletion of liver taurine in rats increases their susceptibility to the hepatotoxicity of carbon tetrachloride (CCl_4). Taurine also protects against the cytotoxicity of hydrazine and 1,4-naphthoquinone in isolated rat hepatocytes. The mechanism by which taurine protects hepatocytes injury induced by CCl_4 is not fully understood. In a previous study, we reported that cellular polyamines play an important role in this mechanism. The relationship between cellular glutathione (GSH), protein SH levels, and lactate dehydrogenase (LDH), with respect to the effect of polyamine on the cytoprotective ability of taurine in CCl_4-induced toxicity in isolated rat hepatocytes, was examined. CCl_4 induced a LDH release and decreased cellular thiols and polyamine levels. Treating with taurine reversed these depletions. The effect of CCl_4 was also reversed by the addition of exogenous polyamines. Pretreating with α-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase, which is a key enzyme in polyamine biosynthesis and therefore used to deplete cellular polyamine, prevented the protective effect of taurine. Adding diethyl maleate, a cellular glutathione-depleting agent, reduced the effect of exogenous polyamines. The role of polyamine in the cytoprotetive effect of taurine in CCl_4-induced toxicity may therefore be by preventing, among others, GSH and protein-SH depletions.

Effect of polyamines on vascular cells

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Several studies showed that polyamines play an important role in vascular cell growth and function. Other studies showed that oxidized polyamines exert a cytotoxic effect on endothelial cells at high concentration [1], although more recent studies showed that the cytotoxic effect of polyamine can be only partially due to an oxidative metabolic pathway, but another mechanism, even unclear, is related to this pharmacologic effect [2,3]. The objective of this study was to examine the effect of the polyamines, putrescine and spermidine, on aortic endothelial cells (AEC) and aortic smooth muscle cells (ASMC). In the presence of 10% fetal calf serum in the growth medium, spermidine (1–40 micromolar) exhibited a dose dependent cell death promoting effect on vascular cells, whereas putrescine was uneffective at the same concentrations. It is noteworthy that the range of concentrations used in the described experiments was close to those found in some *in vivo* conditions. In the absence of fetal calf serum neither polyamine caused cell death. FACS analysis and DNA ladder analysis showed that cell death was due to apoptosis. Heat treatment of FCS and ultrafiltration through a 10 Kda cutoff filter markedly reduced the effect of spermidine on ASMC and AEC as well as the treatment of both cell types with inhibitors of polyamine oxidase and transglutaminase, i. e. two catabolic pathways of polaymines, inhibited almost completely the cytotoxic activity of spermidine both on AEC and ASMC. Moreover, the overexpression of transglutaminase type II increased the sensitivity to spermidine in both cell types. These results show that both AEC and ASMC undergo apoptosis via both a polyamine oxidase and a transglutaminase-dependent reaction. The occurrence of this effect requires a heat sensitive factor present in FCS which may be the serum polyamine oxidase. The difference of potency of spermidine on two vascular cell types and the occurrence of such effect at concentration close to the physiological ones suggest a modulatory role for this polyamine

and suggests a novel pathogenetic mechanism underlying vascular diseases like diabetes, arterial injury and atherosclerosis [4,5,6].

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Plant polyamine binding proteins: receptor or part of membrane transport systems?

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By virtue of their cationic nature at physiological pH values, polyamines bind to anionic cell constituents such as nucleic acids, phospholipids and negatively charged protein residues of membranes both affecting their structure and function [1]. One possible mechanism by which polyamines might act as growth substances involves their binding to specific regulatory proteins. In both animal and plant systems it has been postulated that these compounds may play a role in the post-translational modifications of proteins as well as in the modulation of many enzyme activities. Despite the large amount of information on the covalent interactions between polyamines and proteins [2], little is known about the non-covalent binding of polyamines to natural membrane proteins, although this may represent one of the fist steps in their action at cellular level. In previous works the main characteristics of specific spermidine binding to plasma membrane vesicles isolated from etiolated zucchini [3] and to total solubilized protein from zucchini plasma membrane vesicles [4] were pointed out. Recently, a 60 kDa polypeptide doublet, showing specific spermidine-binding activity, was identified in microsomal fractions of maize coleoptiles. This doublet was both N-terminus and partially internal sequenced. Protein databases were screened for possible protein homologies. Rabbit polyclonal antibodies were raised and will be used in the future for an immunocytochemical and subcellular localization of these spermidine-binding proteins in maize cells. In this talk we'll tray, even by virtue of the recent knowledge obtained in plant, animal and microorganism fields, to address the question given in the title.

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Transglutaminase activation and polyaminated polymeric protein formation: evidences for a new class of uremic toxins

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Blood coagulation factor XIII (FXIII) is a transglutaminase (TGase) which catalyzes the covalent incorporation of amines, including polyamines (PA), into proteins. Polyamination reactions are physiologically important and may modify the structural and/or the catalytic properties of protein and peptides. Recently, we demonstrated that the *in vitro* cross-linking between plasma proteins and PA results in the formation of polyaminated polymeric proteins (PPP) that might share many of the biological properties of free PA. In particular, we provide evidence for a role of PPP in the hypoproliferation of bone marrow erythroid cells CFU-E possibly due to either the interference with the response to erythropoietin or the impairing of the cell cycle and proliferative signalling.

Several studies have demonstrated elevated levels of free PA in uremia; in the meantime, we first show that PPP accumulate in the uremic plasma. In fact, a new class of high molecular weight (HMW) toxins, in which spermidine (SPD) is the main PA substrate of the reaction with plasma proteins, can be observed in the blood of at least a part of the chronic renal failure patients and particularly in the hemodialysis (HD) ones. The possibility that FXIII is activated in the blood of these subjects has focused our attention. This heterogeneous group of PPP could be involved in the inhibition of erythropoiesis thus contributing to anemia of HD patients; moreover, like free PA, they could interfere with the cell cycle and trigger apoptosis.

The most common dialysis techniques, such as HD, appear poorly effective in removing both free PA and PPP. Preliminary evidence has been provided on the efficacy of a last generation of high-flux dialyzers (cut off ≥70 KDa) in eliminating PPP from the uremic plasma.

Polyamines are unevenly distributed within the rat and rabbit kidney

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Measurement of aliphatic polyamines in the kidney has been generally performed on the whole organ, however, it is often missed that the kidney is composed of a variety of cells. Thus, it remains unknown how polyamines are distributed within the kidney. The aim of this study was to establish the distribution pattern of the main polyamines within the kidneys of male and female rats and rabbits. It is shown that polyamines were unevenly distributed along the cortico-papillary axis of both rats and rabbits and that each polyamine exhibited its own distinct distribution pattern. Putrescine levels increased gradually from the cortex to the papillary tip in rabbits, whereas, in rats, fluctuations in putrescine level were more marked. In the six zones of the rabbit kidney, spermidine and spermine content were dramatically higher in females than in males. This difference was less marked in rats. Spermidine content was higher in the cortex than in the medulla, regardless of sex or species. Spermine content and distribution showed sex and species differences. These data revealed the heterogeneous distribution of polyamines with-

in the kidney. Therefore, in experimental animals, separation of the renal zones is essential to detect local variations in polyamine content of the kidney. In addition, measurement of polyamine content in each renal zone will provide new informations to understand their physiological roles.

Polyamine effects on pyruvate dehydrogenase (PDH) activity in energized heart mitochondria

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In previous papers it has been demonstrated that spermine is able to stimulate PDH activity both in purified enzyme and in permeabilized mitochondria isolated from adipose tissue, liver and heart. At present any report refer to the action of spermine on PDH activity in intact mitochondria. In particular the polyamines can be transported into the matrix by means of a specific uniporter operating at high membrane potential. The aim of this work is to ascertain if in energized heart mitochondria, in the absence of exogenous MG^{2+} and Ca^{2+} and in presence of EGTA, the polyamines are able to reach the inner compartment where PDH is located and to stimulate it.

The obtained result show that the polyamines induce a stimulatory effect on PDH activity, in particular way with spermine. The maximum effect is reached at 0.3–0.5 mM concentrations. Higher concentrations exhibit a gradual lesser effect. 1.5–2 mM spermine, instead, provokes a strong inhibition. Furthermore it has also been observed a transient decrease, followed by subsequent increase, in the phosphorylation of the alpha-subunit of the enzyme complex, by enhancing polyamine concentration. This effect is much more marked in the presence of spermine.

The present results clearly demonstrate that the polyamines, by entering the matrix of heart mitochondria are able to reach the PDH compartment and to regulate its activity at the level of the phosphorylation state of the alpha-subunit. The observed effects are also independent from both Ca^{2+} and Mg^{2+}.

Some approaches to the chemical regulation of polyamine synthesis *in vitro*

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Eight novel chemically modified pyridoxal analogues were evaluated for their ability to regulate the polyamine biosynthesis in cell-free system of tissues with high mitotic index such as regenerating liver and hepatoma G-27. All of them were derivatives of 2-methyl-5-oxo-1,4-dihydroindene-[1,2-B]-pyridine-3-carbonic acid and were used in final concentration 0.1 mM in the reaction mixture. The derivatives were differed in radicals in positions 3 and 4 of pyridinix cycle. 2-Difluorometoxyphenyl and 2-nitrophenyl were located in 4 positions. Ester substituents were in 3 positions.

Ornithine decarboxylase (ODC) activity and polyamine (PA) levels were determined. The effect of compounds was expressed as a ratio of ODC activities and PA concentrations in control and tested samples. Almost all of investigated substances demonstrated activation both ODC activity and PA synthesis except agent 769: methylic ether of 2-methyl-4-(2-difluorometoxyphenyl)-5-oxo-1,4-dihydroindene-[1,2-B]-pyridine-3-carbonic acid. It strongly inhibited PA formation in both test-systems. The activation of PA synthesis in hepatoma G-27 tissue were less effective than in regenerating one. Agent 1694: n-dodecylic ether of 2-methyl-4-(2-nitrophenyl)-5-oxo-1,4-dihydroindene-[1,2-B]-pyridine-3-carbonic acid, activated ODC and the biosynthesis of PA in cell-free system of regenerating tissue and inhibited ODC activity and the rate of PA formation in hepatoma G-27 tissue.

The data obtained indicate the different sensitivity of PA biosynthesis to chemical action in test-systems with controlled (regenerating liver) and uncontrolled (epatoma) rate of cell proliferation. These compounds may be useful for investigation of pecularities of PA metabolism, to regulate cellular PA levels and could serve as the basis for the control of both PA metabolism and RNA and DNA synthesis. Moreover, they clearly show the possibilities to work out some approaches to the chemical regulation of cell proliferation by chemical analogues as potential antitumor agents.

Polyamines effect on structural integrity of isolated lysosomes

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Direct influence of diamines and polyamines on structural integrity of isolated rat liver lysosomes were investigated. The lysis of these organelles was initiated by joint action of physical and chemical factors of intracellular medium such as osmotic pressure ($\pi = 0.75$), pH 7 and temperature (t = 50 °C). Lysosome suspension was incubated in 0.7 M sucrose solution with 1 mM ADTA at 50 °C and pH 7 together with tested agent. The effect of compounds was expressed as percentage of initial absorption (A_{520}) and initial lysosome lysis rate (ΔA_{520}/min).

All investigated compounds demonstrated protective properties with different extent which increased in line: cadaverine, putrescine, spermidine and spermine. So they at 10 mM concentration in incubating medium decreased lysosome lysis on 13, 14, 84 and 97% accordingly. Polyamines stabilized isolated lysosomes more effective than diamines. Dose-dependent effect was revealed.

The data indicate that diamines and polyamines may stabilizede isolated lysosomes by forming solvate complexes with phospholipids and other acid components of their membranes. Consequently polyamines would regulate the activity of cell process in which lysosomes are involved, in particular cell proliferation.

Modification of Amino Acids

Overview for modification of amino acids

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Brief overview of current status of modifications on amino acids will be presented. The modifications occurring at post-translational level on several amino acids side chains have gained much interest in recent years in term of their biological functions associated with specific side chain modifications. These modifications are highly economical in terms of cellular energy requirement, since a group transfer reaction (equivalent to one mole of ATP) could often results alterations in their structural and functional activities. Brief comment will be presented in respect to emerging new side chain modification on proteins.

Characterization and functional significance of $^{\alpha}$N-methyl methionine formation in the small subunit of rubisco

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Rubisco is post-translationally modified at multiple sites in both the large (LS) and small (SS) subunits during import and assembly. Since Rubisco is the most abundant protein in the world, the modified amino acids represented by this post-translational processing, $^{\alpha}$N-actylproline, $^{\alpha}$N-methylmethionine, and $^{\epsilon}$N-trimethylsine, are the most common modified amino acids. Methylation of the $^{\alpha}$-amino group of Met-1 of SS occurs after proteolytic removal of the N-terminal transit sequence. Recombinant processed forms of the SS expressed in *E. coli* and [^{3}H-*methyl*] AdoMet were utilized as substrates in assays designed to detect and characterize Rubisco SS $^{\alpha}$N-methyltransferase (Rubisco SSMT) activity in spinach chloroplast lysates. Kinetic analyses suggest an apparent Km of 3.8 μM for AdoMet and 0.5 ßμM for recombinant SS. However, in these assays Rubisco SSMT activity failed to exhibit continued linearity with increased enzyme concentration. Incubation of recombinant SS with increased amounts of chloroplast lysate for 20 minutes prior to addition of AdoMet resulted in a 70% decrease in the incorporation of radiolabel. However, methylation of the SS prior to incubation resulted in apparent stability. Since N-terminal protein processing can have a substantial influence on protein stability, the influence of the SS penultimate and N-terminal amino acid residues on Rubisco SSMT activity, as well as *in vitro* stability, were investigated by generating substitutions at these positions using site-directed mutagenesis. Our hypothesis is that Rubisco SSMT activity operates with a specificity established by the penultimate and N-terminal amino acid residues of SS, and that in competition with chloroplast-localized amino peptidase(s), these enzymatic processes serve as determinants which regulate the *in vivo* stability of SS in the chloroplast.

Aspartyl esterification can reduce the peroxisomal proliferation, induced by dehydroepiandrosterone

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The purpose of this study was to determine whether the esterification of DHEA with aspartate (DHEA-aspartate) could reduce the peroxisomal proliferation, induced by DHEA itself, without loss of its antiosteoporotic activity. Female Sprague-Dawley rats were ovariectomized, thereafter, DHEA and DHEA-aspartate were administered, ip. at 0.34 mmol/kg BW 3 times a week for 8 weeks. To assess the effects of DHEA and DHEA-aspartate on bone, we carried out the bone morphometric analysis of trabecular bones in proximal tibia and biochemical assays in serum and liver. The adverse effects of DHEA and DHEA-aspartate on liver were assayed by changes in liver weight, the activities of peroxisomal enzymes, the levels of TBARS and carbonylated protein and peroxisomal morphology. DHEA-aspartate treatment on ovariectomized rats significantly increased trabecular bone area in tibia as much as DHEA treatment. Calcium level, osteocalcin concentration and alkaline phosphatase activity were not significantly different between DHEA-aspartate and DHEA treatments. DHEA-aspartate treatment significantly reduced the liver weight and the ratio of liver to body weight and palmitoyl-coA oxidase activity in liver compared with DHEA treatment and also showed nearly normal morphology of hepatic peroxisome, while DHEA treatment increased the number and size of peroxisome. DHEA-aspartate reduced carbonylated protein content but not in TBARS in liver compared with ovariectomized control. From these results, we found that DHEA-aspartate has the antiosteoporotic effect in ovariectomized rats with a marked reduction of hepatomegaly and peroxisomal proliferation compared with DHEA.

Carboxyl methylesterification of prenylated cysteine residue in G-protein family

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In eukaryote several signal transduction proteins are known to have a highly conserved C-terminal sequence of a CAAX motif, in which C, A and X represent cysteine, aliphatic amino acid and any amino acid, respectively. These proteins are Ras superfamily proteins (such as γ-subunit of heterotrimeric G protein, α-subunit of cGMP phosphodiesterase and yeast mating factor), and undergo three sequential posttranslational modifications at the CAAX motif, namely i) prenylation of cysteinyl residue, ii) proteolytic cleavage a AAX to generate C-terminal prenyl cysteine, and finally iii) carboxyl methylesterification of prenylated cysteine, catalyzed by the membrane-bound protein isoprenyl cysteine O-methyltransferase (EC 2.1.1.100). We have purified the cysteine methyltransferase from bovine brain membrane 130-fold with a yield of 1.8% by applying DE-52 chromatography, Superdex-75 FPLC, non-denaturing PAGE and SDS-PAGE. The molecular weight of the enzyme was determined to be about 30-kDa, based on the Superdex-75 elution profile as well as a photoaffinity labelling of the enzyme with S-adenosyl-L-[*methyl*-^{3}H]methionine. The partially purified enzyme activity was stimulated 30 ~40 fold by 2 mM guanosine 5'-O-(3-thiotriphosphate), while ATP did not show any effect at the same concentration. A synthetic substrate/inhibitor, N-acetyl farnesyl-cysteine which served as a substrate for the membrane-bound enzyme inhibited the purified enzyme activity in a concentration dependent manner with a complete inactivation at 2 mM. These results suggest further investigation on this anomalous reaction with the membrane-bound enzyme.

Oxidation of 3-(3,4-dihydroxy phenyl)-L-alanine (levodopa) and 3-(3,4-dihydroxy phenyl)-2-methyl-L-alanine(methyl dopa) by manganese(III) in pyrophosphate media: Kinetic and mechanistic study

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Manganese(III) has been stabilised in weakly acidic solution by means of pyrophosphate and the nature of the complex was elucidated spectrophotometrically. Stiochiometry of manganese(III)-oxidation of the substrate, levodopa and methyl dopa in pyrophosphate medium was established in the pH range 2–4. The reaction shows a distinct variation in kinetic order with respect to [Mn(III)], a first order dependence in the lower pH range, decreasing to fractional order above pH 3. While other common features include first order dependence on [dopa], positive fractional order dependence on $[H^+]$ and inverse first order dependence on [Mn(II)] in the entire pH range were studied. Effect of varying ionic strength and solvent compositions were studied. Added ions such as SO_4^{2-}, ClO_4^- alter the reaction rate probably due to the change in the formal redox potential of Mn(III)-Mn(II) couple because of the changes in coordination environment of the oxidising species. Evidence for the transient existence of the free radical intermediate is given. Cyclic voltammetric sensing of levodopa and methyl dopa at various pH has indicated that dopaquinones are not the likely oxidation products under the kinetic conditions. Activation parameters have been evaluated using the Arrhenius and Erying plots. Mechanism consistent with the kinetic data have been proposed and discussed. These studies are expected throw some light on the levodopa metabolism.

Derivatives of glutamic acid as new surfactants

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Starting from glutamic acid, different types of surfactants have been synthesised by using original modular strategies. Monosubstituted zwitterionic amides of glutamic acid obtained with excellent yields show good surface activity. The grafting of a second hydrophobic side-chain leads to bicatenar cationic surfactants or to disubstituted nonionic cyclic compounds. In order to reduce the hydrophobic character of the bicatenar surfactants, a second synthetic method has been developed, allowing the introduction of a polar sugar group into these molecules. The surfactant properties of several of the products have been determined by physico-chemical methods such as surface tension measurements and compression isotherm studies by means of a Langmuir balance.

Simple and efficient synthesis of silylated monomers on the basis of amino acids for the preparation of grafted silica

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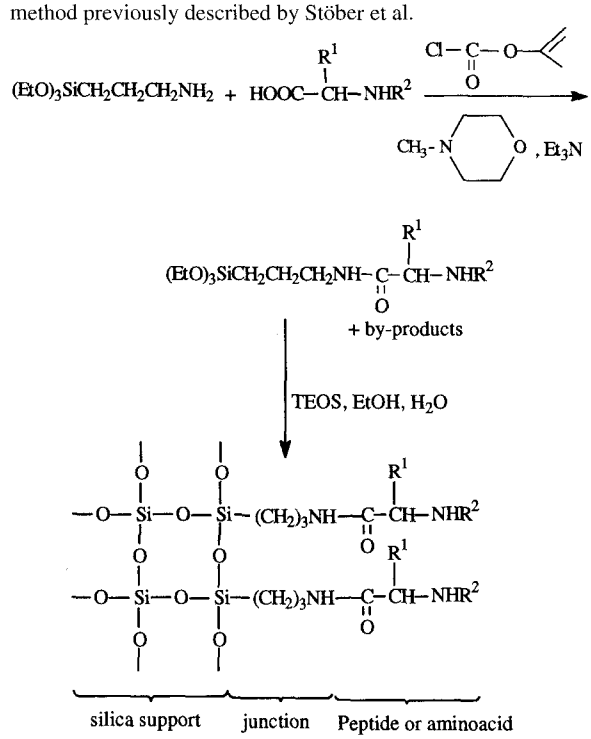
The production of grafted silica has largely expanded during the last fifteen years due to the multitude of potential applications for this kind of new materials: chemistry, biology, non-linear optics, environment, . . . Our objective is not only to prepare but also to characterise modified silica, which can selectively complex bivalent or trivalent metallic ions such as Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+} or Cr^{3+}. For this purpose, we propose grafted silica of the structure *I*. Reaction of aminopropyltriethoxysilane with amino acid or peptides like carnosine (β-alanine-histidine) used as chelating moiety allows to obtain silylated monomers which are copolymerised with tetraethoxysilane (TEOS) using a method previously described by Stöber et al.



Structure I. Synthesis of sylilated monomer and structure of the modified silicia

This simple method is applicable to different structures of amino acids and peptides. Several preparations of modified silica have been realised, and their characterisation (RMN ^{13}C, ^{29}Si, size determination . . .) is under study.

Bis-*p*-cresol derivative of allysine for analysis of allysine residue of elastin and collagen

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The first step in normal cross-linking in elastin and collagen is the formation of α-aminoadipic-δ-semialdehyde, allysine, through oxidative deamination of specific peptidyl lysine by the enzyme lysyl oxidase (LO; EC 1.4.3.13). The precise determination of allysine in elastin and collagen is thought to be important for elucidating several physiological phenomena, since the nature and extent of cross-links in elastin and collagen plays an important role in connective tissue turnover. For this reason, we synthesized a bis-*p*-cresol derivative of allysine in elastin, by *p*-cresol. This derivatization was carried out under condition of acid hydrolysis (6N HCl, 110 °C, 48 h). A bis-*p*-cresol derivative of allysine was isolated from bovine ligamentum nuchae elastin hydrolysates obtained in 6N HCl containing 5% (w/v) *p*-cresol. This derivative was characterized by ultraviolet spectroscopy, mass spectrometry and NMR spectroscopy, and identified as 2-amino-6,6-bis(2-hydroxy-5-methylphenyl)hexanoic acid. A rapid, sensitive high-performance liquid chromatographic method was developed for the determination of allysine as bis-*p*-cresol derivative.

Nutrition

Dispensable amino acid metabolism in burn patients: Mass spectrometry and positron emission tomograph (PET) studies

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Burn injury causes not only hypermetabolism and protein catabolism, but also a disturbance in the relationship between indispensable (IDAA) and dispensable (DAA) amino acids, which contributes to protein catabolism and altered amino acid (AA) requirements. Studies have revealed injury-induced alterations in plasma AA concentrations and nitrogen balance (NB), which may serve as a basis for improving AA compositions for nutritional support. However, alterations in AA concentration could be caused by changes in either its production, or utilization, or the combinations of the above changes in either direction for NB. We hypothesise that exploring the altered kinetics of individual AA and protein turnover leads to better understanding the metabolism of AA's which will be a more solid basis for assessing AA requirements.

AA solution containing both IDAA's and DAA S has been found to result in better NM in surgical patients that containing IDAA only, indicating the indispensability of certain "DAA" after trauma. By suing multiple stable isotope labelled AA tracers, we have explored the metabolic kinetics of individual IDAA, DAA, and their relationships (rates of DAA *de novo* synthesis, release from and incorporation into proteins, oxidation and conversion to other DAA and/or metabolites) in burn patients. The studies revealed significant alterations in the metabolic fate of certain AA's: tyrosine and phenylalanine, cysteine and methionine, proline and the urea cycle intermediates, arginine, citrulline and ornithine, and γ-glutamyl cycle activities after burn injury. Hence, exogenous supply of certain *"DAA's"* may be *indispensable* for patients. The kinetic findings may further help improve nutritional support. The stable isotope models and the potential applications of PET in assessing protein metabolism will also be discussed.

Is arginine (Arg) essential in human nutrition?

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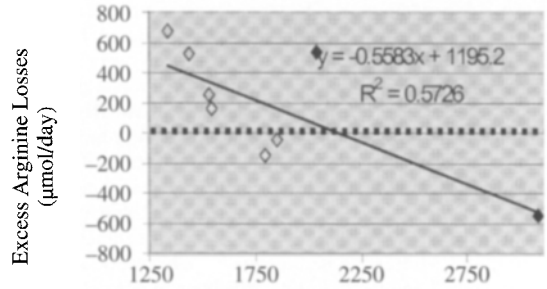
In adults, Arg is generally considered a dispensable amino acid despite a low rate of *de novo* synthesis. In newborns, studies done by Batshaw et al (1976) and more recently by Ball and colleagues support the concept that Arg may be an indispensable amino acid during the neonatal period.

Method: In order to estimate net de novo synthesis, *in vivo,* in neonates fed parenterally exclusively, I evaluated Arg metabolism measuring plasma amino acids fluxes and whole-body rate or Arg oxidation base on a single pool model. Protein turnover was measured using the leucine: α-ketiosocaproic acid model.

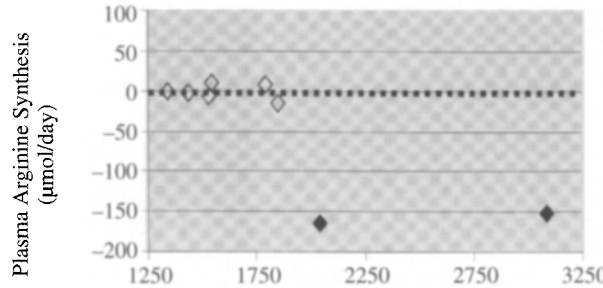
Experiments: After being on TPN formula that provided a minimum of 2.5 gm of protein/kg/day and a minimum of 120 kcal/kg/day for 4–5 days, a known primed, constant infusion of [5-^{13}C]Arg and [5,5,5-$^{2}H_2$]leucine was given for five hours. Blood and breath samples were taken once before and twice, at 30 minute intervals, at the end of the infusion. Measured expired $^{13}CO_2$ was corrected for under-recovery of ^{13}C-label from infused [5-^{13}C]ornithine. Isotopic abundance of amino and keto acids was measured by GC/MS as methyl ester-TFA and t-BDMS derivatives, respectively.

Results: Under these conditions, plasma Arg flux was measured at 209 ±13 μmol/kg/h. Daily excess losses via Arg oxidation was measured at 175 ±385 μmol/kg with a daily Arg intake averaging 1827 ±523 μmol/kg. A significant correlation exists between excess losses and dietary intake as shown in Fig 1. The rate of appearance of free plasma arginine from net *de novo* synthesis was measured at -40 ±69 μmol/kg/h and shown a poor correlation between these two (Fig. 2). The small excess in daily Arg oxidation and the low rate of appearance of free Arg in plasma support the concept that neonates have a very limited rate of Arg de novo synthesis. Leucine appearance from protein breakdown was 172 ±60 μmol/kg/h, which represents 11.4 ±4.0 g/kg/day of protein.



(◇ Normal Parenteral Nutrition, ♦ Arg-Supplemented Diet)

Fig. 1. Eccess Arg losses



(◇ Normal Parenteral Nutrition, ♦ Arg-Supplemented Diet)

Fig. 2. Plasma Arg synthesis

Conclusion: Net rate of endogenous Arg synthesis is present in neonates receiving TPN but is relatively small in comparison to the whole-body turnover of the amino acid. These results are consistant with a limiting capacity for neonates to have a net rate or arginine synthesis supporting the recommendation that Arg is indispensable in newborns.

Amino acid metabolism in the gut

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The small intestine is not only responsible for digestion and absorption of proteins but plays a prominent role in the catabolism of dietary amino acids. The fractional rate of protein synthesis in the gut is considerably higher than in other tissues. Gut and liver together account for one forth of the whole-body pro-

tein synthesis. The pioneering work of Windmueller and coworkers on intestinal glutamine utilization and studies performed during recent years demonstrate that enteral amino acid nutrition is crucial for gut integrity and that 30 to 50% of nutritional amino acids (e. g. leucine, phenylalanine, lysine, glycine, glutamate) are taken up by intestinal tissues. In part these amino acids are used as precursors for the synthesis of glutathione, nucleotides, proteins and other products as well as a fuel for enterocytes. In addition, it appears that the molecular form in which dietary amino acids are supplied (free amino acids or proteins) and the amino acid composition of proteins determine the amino acid availability for splanchnic protein synthesis. The gut modulates the entry of dietary amino acids into portal circulation, and thus, may affect amino acid requirement in health and disease.

A so far not well understood area is the amino acid metabolism of the human intestinal microflora and its interaction with the host. It was shown by us and others that in non-ruminant animals and man lysine and threonine produced by microbes contribute to the homeostasis of those amino acids and to small intestinal protein synthesis. However, since the site of absorption within the intestinal tract as well as the source from which microbial amino acids are absorbed (microbial peptides, free amino acids, or proteins) are not known little quantitative data are available.

Peptides in clinical nutrition

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Particular clinical conditions result in certain amino acid (AA) deficiencies, antagonisms or imbalances causing specific changes in AA metabolism and requirements. Consequently, several AA are now classified as conditionally indispensable: cystine and tyrosine (infancy, impaired liver function), glutamine (hypercatabolic and hypermetabolic situations), and taurine (infancy, TPN, uremia). Thus, these AA should be essential parts of any nutritional efforts in the given situations. However, several unfavorable physical/chemical properties of the free AA mentioned hamper their use as substrates in aqueous preparations for artificial nutrition. Tyrosine and cystine are poorly soluble (0.4 and 0.1 g/L). Cysteine rapidly oxidises to cystine (risk of precipitation); at slightly alkaline pH, H_2S is formed. Glutamine quantitatively decomposes during heat sterilization and storage to yield pyroglutamic acid and ammonia. Taurine might be not transported via plasma membranes due to a very high i.c./e.c. transmembrane gradient.

In order to overcome this problem, we successfully synthesized dipeptides with C-terminal glutamine, cystine, tyrosine, and taurine residues. Their high purity, excellent solubility in water and stability during processing and storage approved these new peptides as suitable substrates for clinical nutrition. Numerous in vitrostudies and experimental investigations provide strong evidence that synthetic dipeptides are rapidly cleaved after parenteral/enteral administration and that the liberated AA are used to support i.c. and e.c. AA pools. Several prospective, randomised, controlled, double-blind clinical studies in metabolically stressed patients (e. g. elective operation, polytrauma, sepsis, bone marrow transplantation) demonstrated that the parenteral administration of glutamine-containing dipeptides beneficially influence the outcome of the patients (diminished length of hospital stay, lower mortality and morbidity).

It is, thus, to conclude that synthetic dipeptides containing indispensable AA should be an integrated part of artificial nutrition in various diseased state.

Use of fasting plasma profile equivalents (FPPE) of the essential amino acids in ranking protein quality. I. Predicting the drop in serum urea levels in Sprague-Dawley rats fed a supplemented diet

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An earlier study showed that serum urea levels in Sprague-Dawley rats were lowered 44% when three supplementary essential amino acids (EAA) were added to Purina Laboratory Chow. The quantities of lysine (7.17 grams), tryptophan (1.32 grams) and threonine (1.49 grams) added to 427 grams of Chow (100 grams of protein) were derived from the fasting rat plasma. The sum of the excess FPPE values was 42% lower for the supplemented ration.

Use of fasting plasma profile equivalents (FPPE) of the essential amino acids in ranking protein quality. II. Pilot studies showing 25 to 28% reduction in 24-hour urinary urea after feeding essential amino acid supplemented rations to a human subject

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A blend of four essential amino acids: Tryptophan (80 mg), Methionine (90 mg), Valine (103 mg) and Lysine hydrochloride (128 mg) was calculated to be a universal supplement for 60 commonly consumed proteins based on an average human fasting plasma profile. Consumption of quantitatively and qualitatively similar meals led to 24 to 28% reductions in 24-hour urinary urea when two unit doses were taken immediately after each meal.

Dietary THR reduces plasma PHE levels in patients with hyperphenylalaninemia

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Background: In order to achieve normal intellectual development, the plasma PHE levels of PKU patients must be lowered by special diets which mainly contain free amino acids without PHE. In a recent animal study, we found a reverse correlation between the PHE and the THR concentrations in both plasma and brain. Therefore, it was the aim of this study to investigate further whether the supplementation of THR to a given diet might also decrease the plasma PHE levels in patients with hyperphenylalaninemia.

Patients: In a crossover design 12 patients with hyperphenylalaninemia were randomly assigned to one of two supplementation groups (THR or placebo). After a feeding period of our weeks and a six weeks' wash-out period the supplementations were changed crosswise to the respective second treatment. Blood was obtained at the start and the end of each supplementation period. THR was supplemented in a dosage of 50 mg/kg/day. A mixture of maltodextrines was used as placebo supplement.

Methods: The plasma amino acid measurements were performed by using ion exchange chromatography. Statistical analysis of plasma THR and PHE levels was performed by using the t-test with special attention to crossover effects according to Lembacher (two-period crossover analysis).

Results: Increasing THR intake lead to increased plasma THR levels ($p < 0.001$). Due to the inverse correlation between plasma THR and PHE levels ($r = -0.546$; $p = 0.0155$) increased THR intake resulted in reduced plasma PHE levels ($p = 0.0234$) (Fig. 1).

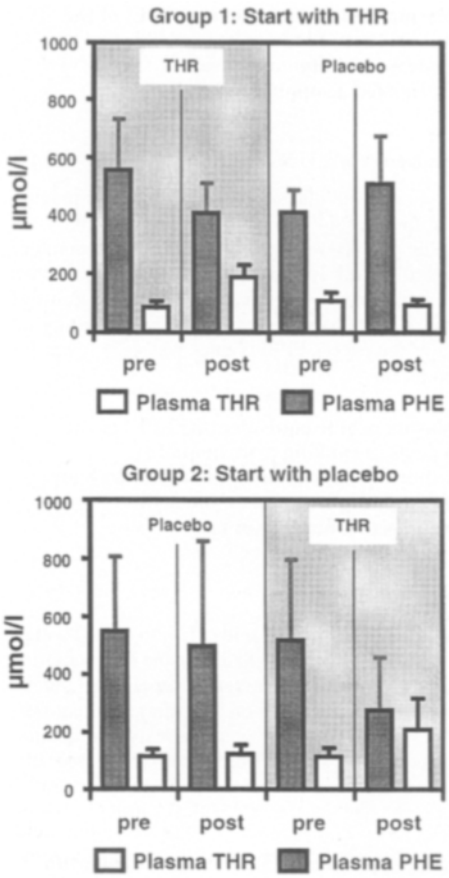

Fig. 1

Discussion: The data support the hypothesized reduction of plasma PHE levels by dietary THR. Thus, THR supplementation might represent a new method for dietary treatment of PKU. However, as the study does not allow conclusions concerning the underlying mechanism, further investigations are necessary to identify the causal relationship for the effect of dietary THR on plasma PHE levels.

Effect of amino acids from millet grains on cholesterol metabolism *in vivo* and in cultured hepatic cells

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Millets have been used for human consumption in Africa, Asia and Central America. Recently, in Japan the consumption of proso, foxtail and Japanese barnyard millets is increasing because it is thought that millets may have a health benefit. However, no study on the physiological functionality of millets has been done except for our works.

We showed that level of plasma high-density lipoprotein (HDL)-cholesterol elevates when animals were fed protein from proso millet. The mechanism by which acts on plasma HDL elevation is not, however, clear. Therefore, this paper examined the effect of amino acids or peptides from these millets on apolipoprotein A-1 (apo A-1) secretion in cultured hepatic cells. Exp 1: After rats were administered protein solution (5 g/100 ml saline), blood from portal vein was collected and plasma amino acid composition was examined. Plasma levels of Leu, Met, Phe, Trp and Ala were high in rats fed millet protein, whereas those of Ile, Val, Tyr and Pro were lower than these levels of animals fed casein. Exp 2: To investigate effect of amino acid on apo A-1 secretion, primary culture of hepatic cells were done. The hepatic cells from rats fed millet secreted more apo A-1 than those from animals given casein. These results may suggest that amino acid composition of millets would affect on apo A-1 synthesis or secretion although further study needs. The effects of peptides from these millets will be also examined.

Amino acids as possible precursor of volatile fatty acids during fermentation process in the manufacture of fish sauce

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This study aimed to investigate the role of amino acids as precursor of volatile acids in the manufacture of fish sauce. The mechanism by which volatile acids are formed from amino acids during the manufacture of fish sauce has not been fully elucidated. In our previous study, we found that bacteria and lipid were possible precursors of straight carbon chain volatile acids during fermentation process of fish sauce [1]. It was hypothesized that oxidative deamination of protein amino acids would give branched-chain and not straight-chain acids which are predominant in fish sauces. It was further proposed that isoleucine could be degraded into one molecule of each of acetic and propionic acids by a coenzyme A reaction [2, 3]. In this study, free amino acids in different concentrations either in singles or in mixtures were added to fish mixtures before incubation. Incubation was carried out at 31 ± 1° C for a specified period and the liquid was collected for quantitative and qualitative analyses of volatile fatty acids. There seemed to have an increase of acetic and propionic acids in the leucine and iso-leucine added fish mixtures but formation of branched chain volatile fatty acids have yet to be confirmed. This will be discussed further.

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Amino acids composition of low protein diet for chronic renal failure

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Low protein diet (LPD) is commonly used in the treatment of patients with chronic renal failure and can effectively suppress the decline of glomerular filtration rate. In this diet, valid composition of amino acids (AA) should be maintained to prevent protein malnutrition, however, there were few data available evaluating AA composition of LPD. In order to assess the quality of LPD, dietary composition and serum concentrations of amino acids were studied in 15 patients with chronic renal failure on LPD. Dietary intake was calculated from precisely weighed food records during 2 days just before blood sampling. Validity of dietary AA composition was judged from AA score (AA-S) proposed by FAO/WHO.

Actual daily intake of protein in studied patients was 0.70

± 0.10 g/kg, energy 32.9 ± 3.5 kcal/kg, essential AA 13.0 ± 5.5 g, total nitrogen 5.4 ± 1.6 g. Mean AA-S was 94.1 ± 4.4, and the scores of 3 patients were 100 (group I) and those of 12 patients were below 100 (group II). There was no significant difference in protein intake between two groups. The limiting AA in the group II were threonine in 9, valine in 1, lysine in 1 and leucine in 1. The percentage ratio of essential AA/protein intake was significantly higher in group I than in group II (43.9 ± 8.9 vs 33.8 ± 4.0%, $p < 0.01$), and of animal/vegetable protein was significantly higher in group I than group II (53.2 ± 5.6 vs 37.9 ± 10.4%, $p < 0.05$). There wer no significant relationship between each amino acid intake and their serum concentrations.

In conclusion, to maintain AA-S at 100 in LPD, over 60% of dietary protein should be composed of animal protein.

Pre- and post-operative aminoacidemia in breast cancer

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Conflicting aminoacidemia alterations have been described during breast cancer and were not validated as specific to the mammary tumor. The aim of this work was firstly to establish the specific modifications of plasma free amino acid (AA) concentrations during breast cancer and secondly to determine evolution of aminoacidemia after tumor excision.

The study included 18 healthy volunteers without breast cancer, and 19 breast cancer patients, submitted to a clinical mammary examination to stage tumor. A single plasma sample was collected in control women, whereas in patients blood was drawn the day before (D0), and 5 days (D5), 1 month (M1) and 6 months (M6) after tumor ablation. Plasma AA concentrations were measured by ion exchange chromatography. Results are expressed as mean ± SEM. Student t-test, test H and ANOVA were used for statistical analysis.

Most of the tumors were of small size (in cm: 2.14 ± 0.92 × 2.08 ± 1.03) and with a good prognosis (65% stage I). Plasma levels (µmol/l) of serine (Ser), glutamate (Glu) and ornithine (Orn) were higher ($p < 0.05$) in patients at D0 (respectively 124 ± 3, 68 ± 7 and 113 ± 13) than in control women (respectively 110 ± 6, 48 ± 5 and 77 ± 7). Tumor excision induced a normalization in aminoacidemia (in µmol/l at D5: Ser: 114 ± 4, at M1: Glu: 55 ± 6 and at M6: Orn: 82 ± 6, NS *versus* values of healthy subjects).

We confirm alterations of Glu and Orn plasma concentrations during breast cancer, previously described by others and we prove that they are specific to the tumor since the concentrations of these two AA return to their basal levels after tumor surgical removal. In addition, Glu, Orn and Ser plasma concentrations in breast cancer are very sensitive since their alterations occur in patients with small tumor. Consequently these modifications of aminoacidemia could be used in the early screening of breast cancer process and recurrence. However, further studies are required to fully evaluate this possibility.

Involvement of substance P (SP) in the regulation of amino acid absorption across the small intestine

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There is increasing evidence that Substance P is abundant in the gut nerves and primary afferents. It has been reported that fibers containing SP-like immunoreactivity terminate on enteric ganglia, and binding sites specific for SP have been found in the intestinal mucosa and submucosal ganglia. Several studies suggested that SP may act directly on mucosal epithelial cells and indirectly through submucosal nerves to alter water and electrolyte secretion in the small and large intestine. However, the contribution of SP to the regulatory mechanisms governing amino acid absorption remains unknown. The present study describes the actions of SP on active amino acid absorption across the rat small intestine. Alanine absorption was measured using the single-pass perfusion technique. Continuous intravenous infusion of SP (100 pmol/kg-min) reduced alanine absorption by 38%. Intraluminal perfusion of 0.1 µM SP inhibited alanine absorption by 34%. *In vitro* experiments, SP decreased alanine uptake by jejunal strips in a dose-dependent manner. Chemical ablation of the extrinsic innervation and myenteric plexus by pretreatment with Benzylalkonium Chloride (BAC) abolished this inhibitory effect of SP. Neonatal treatment with capsaicin and subdiaphragmatic vagotomy produced a similar effect. On the other hand, SP reduced alanine uptake by mucosal scrapings which are essentially devoid of neural innervation by 13%. Our results suggest that SP interferes with alanine absorption through an inhibitory mechanism which is modulated by the neural network.

Gastrectomized patients are in a state of chronic protein malnutrition. Analyses of 23 amino acids

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Malnutrition is one of the major postoperative complications of radical subtotal or total gastrectomy for gastric cancer. To evaluate a nutritional status and abnormality in protein metabolism in these patients serum concentrations of 23 amino acids were measured by HPLC in forty patients who underwent either subtotal ($n = 20$) or total ($n = 20$) gastrectomy more than 6 months prior to this analysis. Serum concentrations of total amino acids (AA) and non essential AAs (NEAA) were not different between gastrectomized patients and healthy controls ($n = 50$). However, concentrations of essential AAs and EAA/NEAA and branched-chain AA (BCAA)/total AA ratios were significantly lower in patient groups than in normal controls. Each EAA was decreased and concentrations of glutamate and citrulline were increased in both patient groups compared with controls. The major differences between patients with subtotal and total gastrectomies included an increased ornithine and a decreased arginine concentration in patients with subtotal gastrectomy. These changes suggest that malabsorption of protein from the intestinal tract causes persistent proteolysis in the skeletal muscle for long periods of time after surgery in these patients and that changes in ornithine and citrulline may reflect more severe alterations in those with total gastrectomy.

The role of amino acids in flavour

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The predominant flavour of some food products develops during processing or home preparation. Nonenzymatic browning (NEB) or Mailard browning is responsible for the most pleasant flavours enjoyed by consumers. Generally speaking, NEB is a reaction between carbonyls and amines. The carbonyls in foods are most often reducing sugars, while the amines come from either amino acids or proteins. The major end products of NEB are melanoidins and other nonvolatile (and insignificant to flavour) compounds. However, a number of intermediates and minor products of the reaction are formed which are significant to flavour. Typically, some minor products make the largest contribution to flavour. Many factors influence the rate of flavour pro-

duction and particular flavour pathway predominating in the reaction. The influence of temperature on flavour formation may be understood better by recalling that each particular pathway of flavour formation has its own activation energy. The overall flavour components and their concentrations are strongly affected by the type of amines and carbonyls source, in addition to the reaction temperature.

Since aspartic acid and fructose are common components in plenty of food varieties, they are selected as model system reactants in the present work. Three separate samples of uniformally ground mixture of aspartic acid and fructose (ratio 1:3, respectively), were subjected to heating in closed system at three different temperatures (60, 100 and 120 c) for 24 hours. The flavour concentrate of each sample was isolated by conventional method. The components of the corresponding flavour concentrate was estimated by GLC. Forty-nine components were separated out of which thirty-four components could be identified. These components are related to different classes of organic compounds such as aldehydes, furans and pyrazines, and are recorded by different concentration for the different reaction temperature as shown collectively in Table 1.

Table 1

Class	Concentration		
	60 C	100 C	120 C
Aldehydes	16.1	5.6	2.6
Furans	5.1	50.4	40.7
Mono & dialkyl pyrazines	4.7	2.8	5.1
Polyalkyl pyrazines	0.6	60.9	82.2

Detailed discussion, tabulated data and figures are available.

Changes of free amino acids in Edam cheese made with freeze-shocked mesophilic lactic acid bacteria

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Accumulation of free amino acids has been studied for 6 weeks on series of trials Edam cheese was made with freeze-shocked mesophilic lactic acid bacteria at –10, –20° C for 24 and 96 hrs. The free amino acids content increase significantly with ripening the cheese trials, however, more noticeable increases were found to be associated with the freeze-shocked trials. The total free amino acids ranged from 40.36–71.36 mg/100 g cheese just after salting at 3 weeks and gradually increased to reach ranged of 92.34–177.77 and 262.50–478.76 mg/100 g cheese after 3 and 6 weeks ripening for all trials, respectively. Twelve individual free amino acids showed significant development from trials in long term ripening. Whilst the six weeks cheese content more Lys, Thr, Ser, Glu, Pro, Ala, Val, Met, Iso, Leu, Tyr, Phe and less His, Asp, Gly, Met and Tyr. An acceleration of the rate of most amino acids accumulation was observed in all trials towards the 6th week of ripening; this has been attributed to the adjunct modified mesophilic lactic acid bacteria.

Modelling amino acid nutrition in ruminants

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From trials using postruminal infusion of amino acids (AA), it is now evident that AA from microbes, and from feed that escapes ruminal fermentation, are not sufficient to meet requirements of ruminants. Modelling AA nutrition in ruminants is of interest for the new scientific knowledges required, and for practical benefits to improve milk protein yield and to decrease nitrogen wastage. In order to be used in practice, the models must be efficient and easy to use.

Some problems related to building such models will be discussed. The first is related to the way to express values: absolute, relative to the sum of essential AA, or to the sum of total AA. Other problems are related to the choice of AA composition of microbial (bacteria and protozoa, free or attached bacteria), bypass (methods of estimation), endogenous fractions, and their relative true digestibility.

The last problem is the estimation of requirements. Different approaches can be used: factorial approach, determination by the break-point of blood concentrations, by the maximum of organ extraction rate. These approaches were discussed in the case of lactation in comparison with methods using milk protein response curve to increasing postruminal supply of one AA coupled with measurement or estimation of AA intestinal flow. Two opposed combinations of these elements used by the CPM and the French AADI system were compared. Suggestions to improve practical AA nutrition of ruminants were given in conclusion.

Amino acid metabolism by rumen microorganisms

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Digestion of feed in the digestive tract of ruminants is dominated by microbial fermentation in the rumen, which is a large sac anterior to the abomasum, or gastric stomach. This evolutionary development gives ruminants the ability to digest fibrous feeds and to use the products of fermentation efficiently. However, it also means that dietary protein is broken down extensively before it reaches the abomasum. Rumen microbes break down dietary protein only partly to provide amino acids for protein synthesis: their main purpose is to provide energy for microbial growth in an environment where the availability of energy limits microbial growth for much of the daily cycle. Thus, much dietary amino acids-N appears as NH_3, which is lost from the rumen by diffusion across the rumen wall. The nutritional challenges posed by ruminal microbial amino acid metabolism are therefore (i) to decrease dietary amino acid breakdown and (ii) to maximise the efficiency of amino acid incorporation into microbial protein. Ciliate protozoa and anaerobic bacteria comprise the great majority of the microbial biomass in the rumen. The bacteria are principally responsible for the processes of proteolysis, peptide hydrolysis and amino acid deamination, but the protozoa have an important role in determining the flow of microbial protein from the rumen, and additionally they have several interesting features of their amino acid metabolism. An important group of deaminative bacteria comprises species which are non-saccharolytic; they include various *Clostridium, Eubacterium, Peptostreptococcus* and others species atypical of the most numerous ruminal bacterial species. Their numbers and activity can be controlled by a number of different feed additives. The protozoa undertake a variety of metabolic modifications of amino acids, including lysine synthesis from bacterial diaminopimelic acid and pipecolic acid formation from lysine. A biosynthetic glutamate dehydrogenase has recently been cloned from *Entodinium caudatum,* indicating that protozoa may carry out more *de novo* synthesis of amino acids than had previously been suspected. The main impact of protozoa, however, is that they ingest and digest rumen bacteria, causing the overall yield of microbial protein to be decreased. The suppression of ciliate protozoa is therefore an important priority for amino acid nutrition in ruminants.

Tissue requirements: Amino acids for muscle growth

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Assessing amino acid requirements for muscle growth is difficult owing to many factors, such as breed, sex, nutrition or the endocrine status which interfers in the process of muscle protein synthesis. Moreover, it is difficult to discriminate the specific requirements for maintenance and growth. However, there are some biological indicators providing valuable informations. Although plasma or intracellular amino acid concentrations are poor indicators, they underline that methionine concentration is low, as compared to the other amino acids. More accurate approaches are arterio-venous differences through and uptakes by a muscle mass. Uptake reflects net amino acid absorption and excretion through the sarcolemma and amino acid metabolism inside muscle cells. Most studies reported in the literature show that uptake of methionine and lysine are the lowest among the essential amino acids, especially when the data are reported to the amino acid concentration in muscle protein. By contrast, branched chain amino acids show higher uptakes probably because these molecules are involved in muscle energy metabolism. During growth, endocrine status improves amino acid absorption, especially of neutral amino acids by the insulin sensitive receptors. Anabolic hormones such as insulin, T3, T4 and IGF-I increase the contribution of protein synthesis in protein turnover and also the intracellular and interorgan recycling of amino acids. In conclusion, it could be emphasized that among essential amino acids, branched chain amino acids are important for energy metabolism while methionine and lysine are limiting factors for protein synthesis.

Protecting amino acids from ruminal breakdown: Technology and efficacy

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Considerable progress has been made in understanding amino acid (AA) requirements and the development of models for predicting passage of digestible AA to the small intestine (SI) of ruminants. This has greatly contributed to improving the precision of diet formulation for ruminants. Across a variety of diets lysine (Lys) and methionine (Met) are the most limiting AA for lean tissue growth of cattle and milk protein production of lactating cows. Dose-response relationships indicate that the optimum contribution of Lys and Met is 7.3–7.6% and 2.4–2.6% of total digestible AA respectively, to maximize content and yield of milk protein. However concentrations of Lys, Met, or both in digestible ruminally undegraded feed protein are generally lower than the optimal. Because concentrations of Lys (~8.0% of total AA) and Met (~2.5%) in ruminally synthesized microbial protein are close to optimal, the challenge in ration formulation is to achieve the ideal amounts of both Lys and Met in digestible protein using conventional feed proteins. It is generally accepted that free amino acids are rapidly deaminated in the rumen, therefore effort has been made to develop technologies for supplying Lys and Met in forms that are not degraded in the rumen without substantially compromising their digestibility in the SI. The physical-chemical properties of Lys are such that application of most technologies are currently limited to Met. The technologies most worthy of note are: (1) combination pH-sensitive polymer/fatty acid coating, (2) fat or fatty acid and mineral matrices or coatings, and (3) liquid sources of Met analog (DL, 2-hydroxy-4-methylthiobutanoic acid; HMB). Technology #1 relies on the physico chemical differences between the rumen and abomasum and provides a product with ruminal stability exceeding 90% after a 24-h incubation, intestinal availability values that approximate 90% and thus, biological availability greater than 80%. Consistent responses to the feeding of pH-sensitive protected Met (or Met and Lys) when cows are fed Met (or Met and Lys) deficient diets are significant increases in content and yield of milk protein and significant increases in plasma levels of Met (or of Met and Lys). In contrast to technology #1 in which postruminal delivery is independent of digestive enzyme function, technology #2 relies on identifying a combination of process and materials that provides a coating or matrix giving a reasonable degree protection against degradation, provided by the relatively inert characteristics of saturated fat in the rumen, plus intestinal digestibility. When fed to cows consuming Met-deficient diets, the mean response was that milk yield of the cows was raised by 1.2 l/h/d (+3.1%), milk fat percentage was increased by 1.2 g/kg and protein content was increased by 0.4 g/kg. Technology #3 relies on the fact that HMB can, as a result of being associated with the liquid outflow from the rumen, partially escape degradation and is absorbed post ruminally. When a supplement of 30 g/h/d of HMB was supplied to the rumen, fractional degradation of the HMB was 0.196 resulting in a ruminal escape of 40%. Of the original dose of HMB, 60% was ruminally degraded, 27.6% was absorbed prior to the duodenum and 12.4% entered the duodenum. The technologies demonstrate the value of supplementing Met-deficient diets with sources of methionine. Presently these technologies do not offer a completely cost effective solution to supplying amino acids to ruminants but the target levels of supply have been identified and solutions to the present challenges will be found.

Tissue requirements: Amino acids for gut function

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The conventional view of the ruminant gastrointestinal tract (GIT) is that of "the great provider". Indeed, its ability to supply nutrients for whole body metabolism and anabolic gain from dietary sources which cannot be digested by non-ruminants is perceived as a great benefit to the animal and to mankind. Yet recent studies have started to indicate that the nutrient requirements of the GIT (for both protein and energy) represent a major competition for these absorbed nutrients, to the extent that the GIT may be the single most influential factor in the poor utilisation of nutrients and poor productivity in present-day ruminant production systems. Amino acid (AA) use by GIT tissues and secretions can represent 40 to 60% of whole body AA flux in forage-fed animals, with the majority (80%) of these AA being extracted from circulating arterial blood, i. e. rather from digesta-derived AA during absorptive metabolism. Further, the losses of metabolic N associated with this turnover of GIT protein (as oxidised AA and the non-resorption of endogenous secretions) equate to >30% of the daily supply of absorbed AA. Part of the reason for the GIT's excessive need for AA is the inherent anatomy of tract, but factors which alter the environmental conditions within the lumen of the GIT can also have a significant influence on GIT protein turnover and hence the partition of absorbed nutrients towards more productive purposes. Recent studies indicate that both subclinical nematode infection and the use of antimicrobial agents alter the rates of GIT protein turnover (and associated losses of metabolic nitrogen), with these changes exerting a strong influence on the anabolic capacity of the animal.

Partial purification and properties of glutamate-phenylpyruvate aminotransferase from rumen bacterium *Prevotella albensis*

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Phenylalanine (Phe) is one of the aromatic amino acids and is thought to be essential for ruminant animals. The synthesis of Phe through the aminotransferase (AT) of phenylpyruvic acid (PPY) by mixed rumen bacteria and protozoa has recently been demonstrated by Amin and Onodera (1997), and in widely studied aerobic microorganisms as *E. coli* (Powell and Morrison, 1978), *Pseudomonas putida* (Ziehr and Kula, 1985) and *Pseudomonas fluorescens* (Evans et al., 1987). The AT activity exists in the rumen has already been demonstrated, where Phe AT activity by mixed bacteria and protozoa has also been reported (Tsubota and Hoshino, 1969, and Bathia et al., 1980), but the enzymes are not yet purified and there have been no report concerning the glutamate-phenylpyruvate AT (GPAase) (EC 2.6.1.64) of rumen bacteria. This study was conducted to partially purify and investigate some characteristics of GPAase from rumen bacterium *Prevotella albensis*.

Prevotella albensis were grown overnight at 39° C in media 2 (Hobson, 1969). The cells were collected by centrifugation at 27,000 × g for 15 min at 4° C, disrupted by sonication and the supernatant was collected by centrifugation at 37,000 × g for 45 min at 2° C and used as crude enzyme. The enzyme was partially purified by ammonium sulphate precipitation (80%) and column chromatography with Phenyl-superose (eluted with a linear concentration gradient (0.02 M to 0.2 M) Na-phosphate buffer (Na-PB) (pH: 6.2), DEAE-toyopearl 650 M (eluted with a linear concentration gradient (0.05 M to 0.5 M) NaCl in 0.01 M Na-PB (pH: 6.2), and Sephacryl S-100 HR (eluted with 0.01 M Na-PB (pH: 6.2). All elution contained 0.1 mM PLP & 0.01% 2-mercaptoethanol (2-ME). Protein was monitored by the measurement of absorbance at 280 nm during purification. The protein concentration of the enzyme was measured by the method of Bradford (1976). The reaction mixture for AT assay was composed of 0.1 M Na-PB (pH 6.2), 0.5 mM pyridoxal phosphate (PLP), 10 mM ammonium chloride, 15 mM L-glutamic acid sodium salt, 5 mM PPY sodium salt, 2.6 units glutamate dehydrogenase (GDH), 2 mM NADH and transaminase enzyme extract in a total volume of 200 µl. The reaction was initiated by the addition of the enzyme to the mixture and incubated at 50° C for 20 min and measured by the change in absorption at 340 nm by a micro plate reader. The molecular size of the GPAase was determined by native (omitting SDS and 2-ME) and SDS-PAGE according to the method of Laemmli (1970). GPAase was run through a native gel at 4° C and was identified by means of coupled reaction catalysed by GDH and diaphorase, leading to the reduction of INT to a coloured formazan (Bergmeyer, 1987). The band was cut off, treated and run through SDS-PAGE.

GPAase from a cell free extract of a rumen bacterium *Prevotella albensis* was partially purified 320-folds. The GPAase activity was assayed at different pH values from 4.0 to 10.5 under standard condition. The optimum pH of GPAase was 6.2. The enzyme was also stored at different pH values as mentioned above for 15 min at 4° C, and the remaining activities was assayed. The GPAase was reactive over a wide range from pH 4.5 to 9.5. The activity of GPAase was also measured at various temperatures from 25–70° C at pH 6.2 under standard condition. The maximum reaction velocity was observed at a temperature of 50° C. At higher temperatures over 60° C, the activity declines. Thermal stability of GPAase was also investigated by determining the residual activity after keeping the enzyme at various temperatures as mentioned above for 15 min in 0.1 M Na-PB (pH 6.2) with 50 µM PLP. The GPAase was stable below 60° C. The molecular weight of the GPAase was estimated to be 3.9 kDa. The GPAase activity was completely inhibited by the use of an AT inhibitor, aminooxyacetic acid.

The quality of dietary protein affects brain protein synthesis rate in aged rats

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The purpose of this study was to determine whether the quality of dietary protein affected the rate of brain protein synthesis in aged rats. Experiments were conducted on three groups of aged rats (30 wk) given the diets containing 20 g casein, 20 g gluten or 20 g gelatin/100 g for 10 d. The fractional rates of protein synthesis in brain, liver and kidney declined with a decrease of dietary quality of protein. In brain, liver and kidney, the RNA activity [g protein synthesized/(g RNA • d)] was significantly correlated with the fractional rate of protein synthesis. The RNA concentration (mg RNA/g protein) was not related to the fractional rate of protein synthesis in any organ. The results suggest that the rate of protein synthesis in the brain declines with the decrease of dietary quality of protein in aged rats, and that RNA activity is at least partly related to the fractional rate of brain protein synthesis.

In children and rats, oral rehydration solutions with glycine and glycyl-glycine result in hyperammonemia

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Diarrhea causes dehydration, electrolytes losses and malnutrition. Therefore the treatment of diarrhea includes the prompt administration of oral rehydration solutions and feeding. The purpose of this study was to compare the urinary production of ammonia and orotic acid in children with diarrhea and in normal rats that received two oral rehydration solutions. These solutions were identical in terms of their concentration of electrolytes but one of them contained glycine and glycyl-glycine as promoters of water absorption. Orotic acid was used as an indicator of hyperammonemia. The results showed that in the children with diarrhea (89 infants) those assigned to the SRO containing glycine (45 infants) excreted substantially more nitrogen and urea and 31 and 44% more ammonia and orotic acid than those (44 infants) assigned to the solution without glycine. This higher production of metabolites derived from amino acid catabolism was observed both at the first day and the second day of the study. In normal rats fed diets containing the same and 2 and 3 times the amount of glycine and glycyl-glycine consumed by the children, a higher production of ammonia and orotic aciduria was also observed. In this animals, the concentration of urinary ammonia and orotic acid was proportional to the concentration of glycine in the diet. These results indicate that the inclusion of glycine in oral rehydration solutions produces hyperammonemia in children and rats.

Total urinary nitrogen is a better predictor of amino acid catabolism than urinary urea in malnutrition and trauma

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The protein requirement of hospitalized patients, is determined from their excretion of urinary urea. This estimation is appropriated in healthy subjects since approximately 80% of the nitrogen they produce is excreted in the urine in the form of urea. However, urea production depends upon nutritional status, and the capacity of producing urea can be a limiting factor in cases of a very high rate of protein catabolism. In these cases, urinary urea may be less than 80% of the total urinary nitrogen and as a consequence the protein requirement may be underestimated. Accordingly, in this study, we measured the proportion of the total urinary nitrogen excreted in the form of urea in a) Humans fasting for 6 days, b) children with acute diarrhea, c) rats with fractures, burns or surgery and d) rats with different degrees of energy and protein deficiency. In all cases, total nitrogen was determined in the collected urine using a colorimetric method developed in our laboratory, which is useful for nitrogen balance studies in hospitalized patients (Nutr. Rep. International 38: 1129). The results showed that in the majority of the studied cases, urea represented considerably less than 80% of total urinary nitrogen excreted. Therefore we conclude that in order to estimate protein requirements from urinary nitrogen losses, total urinary nitrogen is better than urinary urea nitrogen.

Plasma amino acid ratios in hemodialysis patients on a protein supplemented diet

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Plasma amino acid (AA) concentrations are a reliable marker of renal function and a criteria for assessment of protein-energy malnutrition (PEM). The ratios between some of AA are considered much more informative compared to the plasma levels of the individual amino acids.

The aim of this investigation is to analyze the changes in plasma AA ratios in hemodialysis patients on a protein supplemented diet. 28 patients with signs of PEM were included in the study (16 F and 12 M; mean age 43.64 – 24 to 67 years and average duration of dialysis treatment 105.8 months – from 5 to 207 months) for a period of 6 months. The oral supplement "Nutridial" (62% protein content mathematically modeled mixture of soy concentrate, full powdered cow's milk and oats flour) was added to the daily ration in quantity of 0.3 g protein/kg. BW/day. Plasma AA spectre is ascertained pre-, 3 months, 6 months and one month after the end of the supplementation. AA ratios related to renal function – Val/Gly, Ser/Gly, Tyr/Phe, Trp/LNAA and to malnutrition EAA/NEAA, Ala/BCAA and Gly/BCAA are calculated.

A decrease in Ser/Gly ratio /from 0.55 to 0.42; $p > 0.01$) and an increase in Trp/LNAA ratio (from 0.04 to 0.07; $p > 0.001$) in the course of supplementation is registered. In all other ratios we do not calculate significant changes, which we consider a sign of stabilized nutritional status as a result of protein supplementation.

Changes in amino acid composition of pork and chicken meat exposed to various heat treatment

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The changes in amino acid (AA) content in two kinds of meat – pork and chicken (products of "AVES"plc., Bourgas, and "KAMTCHIA"plc., Shoumen) have been studied under various heat treatments – grilling, frying at 180° C and boiling at 100° C. We registered losses of sulfur containing AA (S-AA) and Tryptophan (TRP) during all kinds of heat treatment. Significant differences in AA spectres between frying and grilling were not found. In relation to the kind of meat, higher losses of TRP are ascertained for pork (65,6%) compared to white chicken meat (46,2%). This difference is even higher for S-AA.

These findings are explained by the oxidizing processes, as a result of which free radicals and hydroperoxides are released, probably causing decomposition of TRP and S-AA.

Different amino acids and proteins as precursors of some characteristic aroma

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With the interest of producing inexpensive replacement for expensive natural flavour, it was essential to study the flavour precursors and their sensory and chemical characteristics produced during heating.

Several model systems containing different amino acids, proteins and carbohydrates were used for preparation of meat, coffee and cookies flavours. The model system of hydrolyzed soybean protein, L-proline and ribose was used to prepare the roasted meat flavour. The isolated aroma was subjected to gas chromatography-mass spectrometric (GC-MS) analysis. Different chemical classes such as sulfides, disulfides, sulfur heterocyclic compounds, thiophenes and furans were identified. The odour profile of the mixture was described as roasted meat flavour, which might be due to the presence of the well representation of some contributor components for roasted meat aroma. Four model mixtures of different amino acids and starch were used for the preparation of coffee flavour. The volatile components identified in the aroma of each model mixture were grouped in different chemical classes, furans, furanones, carboxylic compounds, pyrroles, sulfur containing compounds, pyrazines, pyridines, and oxyzoles. The odour sensory test of the four model mixtures revealed that the mixture containing therionine, cysteine and starch had the best results, it was described as excellent roasted coffee aroma with high intensity. This may be attributed to the presence of the most potent odourants of roasted coffee, 2-furfural mercaptane, 2-acetyl pyrazine and furaneol.

The analysis of wheat germ (low priced protein) showed that it comprises different amino acids, which can be considered as precursors for cookies aroma. In view of this finding, the model mixture of wheat germ and fructose was used to prepare the cookies flavour, the odour profile of the heated mixture was described as excellent sponge cakes odour. This was confirmed by the presence of the volatile components, which are responsible for crackers and toasted note such as 2-acetyl pyridine and 2-ethyl-3-methyl pyrazine.

Study in order to document conventional food, specify its nutritional value

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In the Near East, burghul has been known for many centuries as a conventional food made from wheat. It is generally produced on small scale in the villages after the wheat harvest.

This study was conducted to evaluate the effect of various processing operation on the main constituents of wheat and on the properties of the final product, in order to find out the best

processing procedures that could be practised under local conditions for the production of high quality burghul.

Burghul was made from 4 different wheat varieties: Two samples of soft wheat (SW) and two samples of hard winter wheat (HW) by traditional method (involving cooking, sun drying, steeping, stone grinding, redrying, removal of husks and sieving to obtain a fraction with a particle size greater than 0.5 mm) and by similar processes, in the laboratory the chemical composition, amino acid and mineral (Fe, Cu, Mn, Zn, Mg, Ca) contents of wheat before and after cooking were determined. The obtained results indicated that all the investigated wheat varieties can successfully be used for burghul making. The ash and mineral contents were lower in burghul than in the initial wheat. Protein and amino acid content was little affected. The decrease was greater for burghul made from soft wheat than for the burghul made from hard wheat, the statistical analyses of sensory evaluation of different burghuls showed that there was insignificant difference in some sensory characters. The source of wheat used for processing is also responsible for the observed variations. However, burghul produced from hard wheat was better in colour and appearance than produced from soft wheat.

Rumen protozoa can produce tyrosine from phenylalanine in an anaerobic condition

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Tyrosine (Tyr) is a semi-essential amino acid and is normally produced from phenylalanine (Phe) in the mammalian liver in presence of oxygen. Reports on the formation of Tyr from Phe in anaerobic condition by rumen microorganisms are contradictory. Scott et al. (1964) found no Tyr or its precursor from L-[U-^{14}C]Phe by mixed rumen microorganisms which was supported by Allison (1965) with mixed rumen bacteria. Amin and Onodera (1997) observed an increase in some compound at the same retention time as Tyr and *P*–hydroxyphenylacetic acid (HPA) by HPLC analytical method (Amin et al., 1995) during the study of Phe metabolism, but they could not identify the compound because of the limitation of the HPLC method. Recently we developed a quantitative HPLC determination method of aromatic amino acids and their eleven related compounds with which Tyr and HPA could be separated (Khan et al., 1998). We found that 15.2 and 4.6% of Phe was converted to Tyr after 12 h incubation by mixed rumen bacteria (B) and protozoa (P), respectively (Khan et al., 1999). The present study was conducted with L-[U-^{14}C]Phe to confirm the formation of Tyr from Phe by the rumen microorganisms.

Suspensions of B and P were prepared from goat rumen content as described by Onodera et al. (1992). Microbial suspensions (4.0 ml) was incubated for 12 h after adding 10 µCi (0.2 ml) of L-[U-^{14}C]Phe (Sp. Act. 460 mCi/mmol) and 0.1 ml of L-Phenylalanine so that the final concentration of Phe is 0.1 mM. Aliquots of sample from CO_2fraction, ether fraction, water soluble fraction and hydrolysates were taken into the scintillation vial containing 10 ml of scintillation fluid to count the radio activity. Rest of the hydrolysates and water fractions were desalted by "Amberlite" CG-120 (H form) resin and used for thin layer chromatography (TLC) and counting radio activity.

After 12 h incubation radioactivity increased (10.5 and 4.9% in B and P, respectively) in CO_2 fraction, indicating the decarboxylation of Phe by rumen microorganisms. Large amount of radioactivity (76.0 and 38.2% in B and P, respectively) was found in ether fractions, which indicates an abundant production of fatty acids by the degradation of Phe. Desalted supernatant fluid of B and P contained 5.2 and 38.1% of the total radioactivity. About 6% of added L-[U-^{14}C]Phe (12.1% of the degraded) was converted to Tyr by rumen protozoa and most of it was observed in the supernatant. Very little radioactive Tyr (0.81% of the added radio activity) was present in the supernatant of B. Only 4.2 and 1.2% of the radioactivity was detected in the desalted hydrosylates of B and P, respectively. The net degradation of L-[U-^{14}C]Phe by B and P was about 80.0 and 52%, respectively. The results of the present study strongly support our previous results, though a big difference was observed in B and the reasons are necessary to find out. Finally the present study confirmed that rumen protozoa can produce Tyr from Phe in an anaerobic condition.

"Red Bull" energy drink significantly improves concentration, reaction time, memory, mood, aerobic and anaerobic endurance

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The energy drink "Red Bull" is widely used by students who report an improved subjective "lift" and work endurance. These properties were examined in 2 studies comprising 12 and 14 completing subjects (age 18–35 years), with treatments including Red Bull 250 ml (taurine, glucuronolactone, caffeine, sucrose, glucose, vitamins B2, B6, B12, niacin, panthenol), "dummy" energy drinks based on carbonated water and flavourings, water, or no drink control in a repeated measures, latin-square attempted double-blind design. They were assessed both pre-drink and from 30 minutes post-drink. Study 1 assessed heart rate, blood pressure, reaction time and subjective alertness; with aerobic endurance assessed post-drink by measuring endurance on an exercise bicycle whilst maintaining 65–75% maximum heart rate. Study 2 assessed number cancellation (concentration) and memory; with max. speed on an exercise bicycle giving post-drink anaerobic endurance.

Red Bull (study 1) significantly reduced choice reaction time in comparison to other treatments and increased subjective alertness; whilst heart rate and blood pressure increases were non-significant. Aerobic endurance capacity was significantly increased in comparison to other treatments. In study 2 significant increases in number cancellation and immediate memory recall were also seen after Red Bull, with anaerobic endurance significantly enhanced against other treatments.

Subjective improvements with caffeine alone are not always matched by performance [Rush et al (1993), Beh Pharmacol 4: 562–572], whilst Lettko and Meuer [Ann NY Acad Sci (1990) 585: 513–515] found vitamin B improved psychological state, and Owens and Benton [Npsychbiol (1994) 30: 106–113] reported faster reaction time with glucose. The combination of active ingredients may therefore be responsible for the wide range of improvements.

On the effect of intake of an "energy drink" on cognitive functions of car drivers

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In a randomized placebo-controlled double-blind study in 100 volunteers the effect of consumption of a taurine-enriched caffeine-containing "energy drink" on cognitive functions was measured by use of the well established d2 test. The volunteers were recruited at a highway rest station and randomly assigned to the placebo or verum group. The tests of cognitive functions were performed after detailed instruction of each volunteer shortly before and after a two hours car drive following consumption of the drink.

As a result of the study it turned out that the enhancement of total cognitive performance from test 1 (prior to ingestion of the drink) to test 2 (2 hours after ingestion) was 23.9% higher in the verum group as compared to the placebo group. Similarly, the decline of the relative rate of errors from test 1 to test 2 was

53.9% higher in the verum group as compared to the placebo group.

The results of this pilot study show that intake of taurine-enriched and caffeine containing "energy-drinks" can ameliorate cognitive performance over a time period of 2 hours. This is very relevant in connection with the fact that more than 50% of the deaths on highways are caused by insufficient cognitive performance of the drivers. A follow-up investigation with a larger number of volunteers for statistical verification of the results of this pilot study is recommended.

D-Amino Acids and Racemisation

D-Aspartic acid in osteoarthritic and normal knee cartilage

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Aspartic acid (Asp) is one of the fastest racemizing amino acids, such that significant levels of D-Asp may accumulate in slow turnover or non-metabolized proteins during the life span of a human. Previous reports show that proteoglycans of cartilage have relatively long half-lives (slow turnover). This fact coupled with aging and hyperthermia associated with joint inflammation of osteoarthritis led us to hypothesize that racemized D-Asp may correlate with osteoarthritic damaged cartilage in human knee joints. We have refined existing HPLC techniques to determine and quantify D- and L-Asp in hydrolysates from normal cartilage compared to osteoarthritic cartilage obtained from knee joint replacement surgery. Analysis of over fifty osteoarthritic cartilage samples indicates that there are significant levels of D-Asp in the diseased cartilage (20–80 nmole/g; 2–4% of the total aspartate). Normal knee cartilage, as control, is much more difficult to obtain. Analysis of a small number of normal cartilage samples shows similar levels of D-Asp are present (20–80 nmole/g; 2–4% of total Asp) indicating no difference between the normal and the osteoarthritic damaged cartilage. Further analysis of more normal samples and osteoarthritic samples is now underway and will be reported. This research is supported by a grant from NIH-NIGMS MBRS GM454555.

Role of free D-alanine in the intracellular isosmotic regulation of invertebrates

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Marine and freshwater crustaceans and several bivalve molluscs accumulate large amounts of free D–alanine (3–50 μmol/g wet wt.) in their muscle and other tissues. During seawater acclimation of freshwater crayfish *Procambarus clarkii* and several crab species, D-, L-alanine increased largely with the other several non-essential amino acids. These data indicate that D-alanine is one of the major osmolytes responsible for the tissue isomotic regulation of these species. Alanine racemase was found to catalyze the interconversion between D- and L-alanine in these crustaceans.

Only D-, L-alanine and inorganic ions increased significantly in muscle of Japanese mitten crab *Eriocheir japanicus* during the maturation in the river from July to October and during downstream spawning migration to estuaries in October. In the crabs captured in the sea, glycine and D-, L-alanine were largely elevated in place of inorganic ions declined. These data also suggest that D-, L-alanine in the tissues of this strong hyper-osmoregulator play an important role in the adjustment of salinity tolerance prior to and during downstream migration toward the sea. In contrast to the migration in the natural environment, glycine and L-proline as well as D-, L-alanine also increased significantly in the muscle of the freshwater mitten crab during artificial seawater acclimation.

These data also support our previous statements that D-alanine is the most compatible solute with less perturbing effects on the enzyme functions in the cell, even as compared with L-alanine and glycine which are widely used as the best osmolytes in various invertebrates for their intracellular isosmotic regulation.

Racemases in invertebrates

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Alanine racemase of crayfish *(Procambarus clarkii)* is suggested to take part in acclimation of the animal to increase in osmotic pressure of environmental water by producing D–alanine, which is not readily metabolizable. This enzyme has been purified from the muscle of clay fish 134000-fold to homogeneity as indicated on SDS-PAGE. The molecular mass determined by the electrophoresis was 58 kDa, while gel filtration of the native enzyme showed a molecular mass of 60 kDa, indicating that the native enzyme was a monomer. The enzyme lost its activity by treatment with hydroxylamine and required pyridoxal 5'-phosphate for reactivation. The K_m and V_{max} for L-alanine were 126 mM and 1560 μmol/min • mg protein, respectively. The pH optimum was 9.0 and the optimum temperature was in the range of 30–40° C.

The red blood shell, *Scapharca broughtonii,* has been found to contain substantial amounts of D-aspartate in some tissues, where its concentrations were comparable to those of L–aspartate, while D–enantiomers of other amino acids were present in only minute amounts. Examinations of tissue extracts revealed the presence of a racemase activity which was highly specific to aspartate and not active toward glutamate as well as neutral and basic amino acids. The concentrations of D–aspartate and L–aspartate were almost equal after a lengthy incubation of the extract with either of D-asparatete or L-aspartate at limited concentrations. The activity of crude enzyme preparations was severely decreased by hydroxylamine treatment and was markedly recovered by subsequent treatment with pyridoxal 5'-phosphate.

Uptake of D-aspartate into some selected tissues of rat

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Since the occurrence of D-amino acids such as D-Asp and D-Ser in mammals including humans has been reported, much attention has been focussed on the search for their origins.

We investigated the disposition of ^{14}C-D-Asp and -D-Ser in rats by means of whole body autoradiography following intravenous administration [1]. The radioactivity after dosing of ^{14}C-D-Asp was accumulated into the pineal and pituitary glands, where a large amount of endogenous D-Asp is present. When cold D-Asp was loaded, we observed a significant increase and a slight decrease of D-Asp concentration in these glands by 24 hrs [2]. Considering these results, it seems likely that a part of the endogenous D-Asp in these glands was originated from the circulated D-Asp incorporated from the exogenous sources such as food or gut flora.

In the case of ^{14}C-D-Ser, high accumulation of radioactivity was appeared in pancreas and corticomedullary area in kidney [3], suggesting a relation to renal tubular necrosis of rats by D-Ser administration [4]. The slow urinary excretion of D-Ser in rats as compared to dogs was also suggestive of the toxicity of D-Ser in rats [5].

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D-Amino acid substitutions in peptide immunosuppressors

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For several years our research group has worked on peptide immunosuppressors and their analogues including: cycloamanides, cyclolinopeptide A, PRP-peptide immunomodulator from ovine colostrum, tuftsin, thymopentin and lactoferrin. Numerous D-substituted analogues of the peptide immunosuppressors synthesized and tested by us retain the immunosuppressive activity of the native peptides. These investigations show that immune response reactions are generally less sensitive to the configurational changes of the amino acid residues than e. g. the enzymatic reactions. Some unpublished data and general conclusions will be given.

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Invertebrate neuropeptides containing D-amino acids

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In invertebrates, molluscs and arthropods have been regarded as sources of D-amino acid-containing neuropeptides. Until now, achatin-I, fulicin, fulyal, *Mytilus*–FFRFamide and crustacean cardioactive peptide-related peptide-III (CCAP-RP-III) have been isolated from snails and clam. The molluscan neuropeptides are short peptides (4–12 amino acid residues) and contain D-amino acids at position 2. In arthropods, crustacean hyperglycemic hormones (CHHs) only from Astacidea has been identified as the neuropeptides containing D-amino acids. The hormones consist of 72 amino acid residues and contain D-amino acids at position 3. Achatin-I, fulicin and fulyal from *Achatina fulica* showed excitatory and/or modulatory actions on several muscles and neurons, though their L isomers were devoid of activity. On the other hand, both FFRFamide and its L isomer from *Mytilus edulis* have the same excitatory effects on the anterior byssus retractor muscle. Moreover, CCAP-RP-III from *Helix pomatia* exhibited no remarkable activities on any of the muscles assayed; instead, its L isomer possessed various excitatory effects. In CHHs, the influence of isomerization in their physiological effects is different by the species. In the case of *Procambarus clarkii* (crayfish), both isomers of CHHs exhibited a hyperglycemic activity, while D isomer showed a more potent inhibition of ecdysteroidgenesis by Y-organ than L isomer. To investigate the biosythesis of such peptides, molecular biological studies have been performed on the neuropeptides from *A. fulica* and *P. clarkii*. Cloning of cDNAs encoding the precursor proteins of achatin-I, fulicin/fulyal and CHH suggests that D-amino acid-containing neuropeptides from molluscs and crustaceans are produced by posttranslational conversion of L- to D-amino acids in a peptide of ribosomal synthesis.

The effects of D-isomers of aspartic acid and glutamic acid on blood pressure and nociception

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Naturally occurring L-isomers of excitatory amino acids, aspartic acid and glutamic acid, play important roles in central cardiovascular regulation and nociception, but the effects of D-isomers of these amino acids are not well known. In the first part of this study the effects of intracerebroventricular (icv) injection of D- and L-isomers of aspartic acid and glutamic acid on mean arterial pressure (MAP) in conscious rats were compared. Direct pulsatile blood pressure monitoring was performed in male Sprague-Dawley rats after femoral artery cannulation via a pressure transducer connected to a polygraph. Icv injections of L-isomers of both aspartic acid (0.1–0.5 μmol) and glutamic acid (0.1–05 μmol) produced significant increases in MAP. D-aspartic acid (0.1 μmol) and D-glutamic acid (0.05 μmol) also produced increases in MAP. D-aspartic acid was as potent as L-aspartic acid, but D-glutamic acid was about 10 times more potent than L-glutamic acid in increasing MAP. In the second part of the study the effects of both D- and L-isomers of glutamic acid and aspartic acid on nociception were assessed by the tail-flick test in mice. Intraperitoneal injections of D- and L-glutamic acid (60–125–250 mg/kg) and D-aspartic acid [Onat et al (1995) Pharmacol Biochem Behav 51: 715–719] (115–230 mg/kg) induced significant increases in the tail flick latencies (TFL) compared to physiological saline, whereas L-aspartic acid (55–115–230 mg/kg) was ineffective. D-isomer of glutamic acid

was more potent than its L-isomer and D-glutamic acid was more potent than D-aspartic acid in increasing TFL.

Putative β-D-secretase in Alzheimer's disease

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The β-amyloid (β-AP) is a peptide consisting of 39–42 amino acids derived from a large protein precursor, the β-amyloid protein precursor (β-APP) by means of a hydrolytical enzyme, the β-secretase. After the β-AP has been generated, it becomes insoluble and aggregates to form amyloid plaques. It has been reported that at the N-terminal position of this peptide D-aspartic acid (D-Asp) is present and confers insolubility to the peptide due to the stereochemical instability. We hypothesized that a specific protease called by us β-D-secretase exists in human brain, which specifically cleaves the bond between L-Met and D-Asp at the position between 596–597 of β-APP. The cleavage generates the β-AP (the position 597 of the β-APP is corresponding to the position 1 (D-Asp) of the β-AP).

Using the following synthetic peptides: L-Met-D-Asp-L-Ala (position 596–598 of the β-APP), L-Lys-L-Met-D-Asp-L-Ala-L-Glu (position 595–599) and L-Asp-L-Lys-L-Met-D-Asp-L-Ala-L-Glu-L-Phe (position 594–600), we demonstrated the presence of such a hydrolase, and conclude that this enzyme occurs naturally in human brain. It is a membranal protein and in Alzheimer's brain it is present in significantly larger amount than normal brain.

D-Amino acids in hormonal neuropeptides of ribosomal biosynthesis in the Mexican crayfish, *Procambarus bouvieri*

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Neuropeptide hormones of the sinus gland of crustaceans (mainly lobsters and crayfishes) have been observed to be present frequently in two or more isomorphs. In 1994, Soyez et al. (J Biol Chem 269: 18295–18298) and Yasuda et al. (Gen Comp Endocrinol 95: 387–398) studied the isomorphs of the Crustacean Hyperglycemic Hormone (CHH) in the lobster *Homarus americanus* and in the crayfish *Procambarus clarkii,* respectively. When we studied the sinus gland neuropeptide hormones from the Mexican crayfish *Procambarus bouvieri,* we found that the CHH was present as two isomorphs, CHH-I and CHH-II, in a 3:1 proportion, separated by their different hydrophobicity in a μBondapak-Phenyl HPLC column. However, their amino acid composition, sequence, molecular mass p*I*, CD, and disulfide bonds were exactly the same. When both isomorphs were digested with trypsin and the peptide map was analyzed in an Ultrasphere-C18 column, only one peptide behaved differently in the two isomorphs and it corresponded to the N-terminal octapeptide, but their composition and sequence was the same: pEVFDQACK. In collaboration with Dr. Soyez, the two isomorphs of CHH were treated in an ELISA test with two antibodies against synthetic octapeptides, containing either L-Phe or D-Phe in the third position. The most hydrophilic hormone (CHH-I) reacted with the anti-L-Phe antibody and the less hydrophilic hormone (CHH-II) reacted with the anti-D-Phe antibody. We have now found the same situation in the two isomorphs of the Molt-Inhibiting Hormone (MIH-I and MIH-II) that have a L-Phe and a D-Phe, respectively, in the third position of their sequence.

[Granted by Consejo Nacional de Ciencia y Tecnología (CONACYT) and the Mexico-United States Foundation for Science, to A. Huberman.]

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Formation of four isomers at Asp-151 residue of aged human αA-crystallin by natural aging

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Proteins have been considered to consist exclusively of L-amino acids in living tissues. However, our previous studies showed that the Asp-151 residue in αA-crystallin inverts to the D-isomer in human, bovine, and horse eye lens during the aging. The high site-specific racemization of the Asp-151 residues in these αA-crystallins suggests that the Asp residues are stereochemically labile in the protein. Especially in human αA-crystallin, the D/L ratio of Asp-151 residue was higher than 1.0. Since racemization is defined as a reversible first order reaction, when D/L ratio reaches 1.0, racemization is in equilibrium. Therefore, a D/L ratio higher than 1.0 is not considered to be due to racemization, but rather results from stereoconfiguration inversion. Our report was the first observation that inversion occurred in the configuration of amino acids in vivo during the natural aging process. On the other hand, we also found that these enantiomers were simultaneously isomerized to form β-Asp (isoaspartate) residue. We measured that ratio of four isomers of Asp-151, which are normal L-α-Asp, biologically uncommon L-β-Asp, D-α-Asp and D-β-Asp in αA-crystallins obtained from human lenses of 0, 30, 60- and 80 year ranges. Biologically uncommon isomers increased with age and the amount of D-β-Asp was more than that of normal L-α-Asp in the αA-crystallin of the human lenses of 80's. This modification of the Asp residue likely affects the three-dimensional packing array of the lens protein. We proposed that D-amino acid could be a molecular indicator of aging.

Active site of tryptophanase for D-tryptophan degradation

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Chiral homogeneity has intrigued scientists since Pasteur's discovery of the optical activity of amino acids. The origin of optical activity has been discussed on the basis of abiotic chemical or physical process, but there has been still no general consensus on the question. This suggests that origins of homochirality involve other factors in addition to the process of chemical evolution. It is enzymes that maintain present exclusive use of L-amino acids in biological world. L-dominant biological world would not have been built without the stereospecificity of enzyme, which can be related to chiral homogeneity in early metabolism. If a mechanism for chiral homogeneity had been assembled through abiotic process in primitive environment, it might have been incorporated into early polypeptides, whose descendants may also be traced to present day enzymes. Today's high stereoselectivity should reflect historical consequence of enzyme evolution. In this context, the stereospecificity of enzyme is considered to hold the key to solve the riddle on the origin of homochirality. However, there is few discussion on the origin of homochirality from a viewpoint of enzymology. The reason is that the mechanism of the stereospecificity has remained unclear. Generally speaking, the substrate specificity of enzyme is ample in variation from narrow to broad

one. However, the stereospecificity for optical and geometrical isomers is very strict. If we have enzyme active to both L- and D-type of an amino acid, it is useful to study the mechanism of the stereoselectivity. This study provides that tryptophanase stereospecific to L type of tryptophan becomes active to D-tryptophan through probably reversible steric conformational changes when tryptophanase is exposed to highly concentrated diammoniumhydrogen phosphate solution. The enzyme reaction that can react with both antipodes at the same time has been never reported. This reaction depended on both diammoniumhydrogen phosphate and pyrodoxal 5'-phosphate. The activity was maximal at 3.1 M of diammoniumhydrogen phsophate concentrations. Inhibitors analogous to tryptophan such as indolepyruvate were used to characterize the active site of tryptophanase. Additionally, γ-rays was irradiated on tryptophanase to inactivate activity for D-tryptophan. Active site of γ-irradiated tryptophanase was also analyzed by an inhibitor, D-tryptophan. Inhibition reactions on tryptophanase and γ-tryptophanase were studied in terms of kinetics. Diammoniumhydrogen phosphate evoked another D-tryptophan-binding site remote from active site of L-tryptophan for the advent of activity for D-tryptophan. This shows that saline environment around enzyme is one of important factors to maintain the stereospecificity of enzyme.

Effect of D-amino acids on mitochondrial permeability transition in rat liver

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The physiologic role of D-amino acids (D-aa) in mammals has not been yet well established. Different studies focused on the distribution of D-aa and the enzyme D-amino acid oxidase (DAO) in several tissues. It has been suggested in mammals the detoxificant role of DAO catalyzing the oxidation of D-aa accumulated from cell wall of microorganisms (D'Aniello, A., 1993). Also, D-aa has been implicated to affect certain mitochondrial functions, as follows: membrane potential, intramitochondrial pH as well as respiratory chain (López-Ramírez, 1997). The opening of a protein-pore in the internal mitochondrial membrane is known as mitochondrial permeability transition (MPT); this event is characterized by mitochondrial swelling, membrane depolarization and uncoupling of respiratory chain and oxidative phosphorylation, among others, due to a variety of chemical compounds or pathologic events (Lemasters, J. J., 1997). In this work the main goal was to study the effect of certain D-amino acids on respiratory chain and the mitochondrial permeability transition. Male Wistar rats weighing between 250–300 g were used and liver mitochondria were isolated according to Saavedra-Molina (1997). The D-aa were: D-serine, D-methionine and D-thyrosine in a range of concentration from 0.01 μM to 1 mM. MPT was induced by the addition of 40 μM Ca^{2+} plus 3 mM phosphate-K^+ in a KCl-based incubation medium (pH 7.0, 7.4, 7.8). At pH 7.4 D-tir (0.01 μM) induced the highest mitochondrial swelling (53%) followed by D-met (20%) and D-ser (0%). Whereas, at pH 7.8 D-met (84%) and D-ser (55%) started mitochondrial swelling with 0.01 μM concentration; D-tir induced mitochondrial swelling more than the others D-aa at the same concentration (100%). These results concluded that the D-aa studied induced MPT under oxidative stress conditions in liver mitochondria under alcaline pH conditions more than acid pH.

[The authors appreciate the technical assistance of Mónica Clemente-Guerrero and the partial financial support of CONACYT (29609-N) and CIC-UMSNH 1999 grants.]

Molecular Regulation of Nitrogen Metabolism in Filamentous Fungi

GATA factors and nitrogen metabolism in filamentous fungi

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In filamentous fungi the utilization of most nitrogen sources other than ammonium and glutamine is dependent on a positive acting regulatory factor of the GATA protein family, which is required for the expression of many structural genes involved in nitrogen metabolism – e. g. AREA in *Aspergillus nidulans,* NIT-2 in *Neurospora crassa* or NRE in *Penicillium chrysogenum.* In the yeast *Saccharomyces cerevisiae* nitrogen metabolism is controlled by four GATA factors – Gln3p and Nil1p by activating, Dal80p and Gzf3p by repressing gene expression.

Recently, a second GATA factor involved in nitrogen metabolism has been identified in *Penicillium chrysogenum* (NREB) and subsequently also in *A. nidulans* (AREB) and *N. crassa* (NGF1). Despite a low overall amino acid sequence identity of approximately 30%, these GATA factors from filamentous fungi share several features with yeast Dal80p and Gzf3p. All proteins contain an N-terminal GATA type zinc finger motif – displaying 86% amino acid sequence identity – and a putative leucine zipper motif at the C-terminus. The putative DNA binding domain of NREB, expressed as a fusion protein in *Escherichia coli,* binds to GATA sites of its own 5'-upstream region as well as in the promoter of the nitrate assimilation gene cluster *in vitro.* Northern blot analysis revealed the presence of two *nreB* transcripts which differ in length whereby the level of transcription is subject to nitrogen metabolite repression. Consistent with a negative role in the regulation of nitrogen metabolism, overexpression of *nreB* in *Penicillium* leads to repression of nitrate assimilatory genes. RFLP analysis and inverse PCR have demonstrated that the *Aspergillus nreB* homologue *areB* is affected by rare mutations which lead to suppression of *areA* loss of function mutations. These data demonstrate a greater mutuality in nitrogen regulation between yeast and filamentous fungi.

Regulation of sulfur amino acid biosynthesis in *Aspergillus nidulans*: Physiology and genetics

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Biosynthesis and metabolism of sulfur amino acids in *A. nidulans* shown in Fig. 1 represents the richest repertoire of metabolic options described so far for sulfur metabolism. There are two pathways of cysteine synthesis: one involves steps 1 and 2 (the main pathway) and the alternative involves steps 5, 6 and 7 (Fig. 1); the latter functions when the main one is

impaired. Due to this fact, mutations affecting steps 1 and 2 suppress mutations in methionine synthesis (steps 3 and 4) since homocysteine formed in reaction 6 serves both for cysteine and methionine synthesis. Sulfate assimilation and a few other enzymes are repressed in the presence of a favourable sulfur source such as methionine in growth medium – phenomenon known as sulfur metabolite repression (SMR). Four apparently negative regulatory genes: *sconA, sconB, sconC* and *sconD* have been identified, mutations in which relieve sulfur metabolism from the SMR. The SCONB protein contains WD motifs characteristic to a family of regulatory proteins and the newly identified F-box by which it probably interacts with the SCONC protein, a homologue of the yeast SKP1 protein, crucial for many cell functions. A positive acting sulfur regulatory gene, *metR,* codes for a bZIP transcription factor. Mutations in this gene are epistatic to *scon* mutations which implies a cascade regulation. Folate enzymes were found to be induced by homocysteine and repressed by methionine indicating a regulatory interrelation between sulfur and folate enzyme biosynthesis. Genes regulating folate enzymes have been identified.

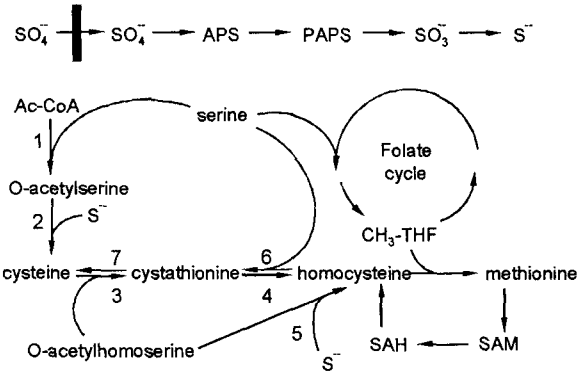

Fig. 1. An outline of sulfur metabolism in *Aspergillus nidulans*

Crosstalk between amino acid metabolism and fruiting body formation in *Aspergillus nidulans*

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Amino acid starvation results in two responses in the filamentous fungus *Aspergillus nidulans*. It induces increased transcription of genes regulated by the general control network and an arrest of sexual development at a defined checkpoint of fruit body formation. Mutant strains carrying a *cpcB* deletion express increased mRNA levels of general control regulated genes independently of the amino acid starvation signal and exhibit the same developmental arrest. The *cpcB* gene encodes a WD protein and a U24 small nucleolar RNA. The mutant phenotypes of impaired fruit body development and of increased transcription of the general control network can be suppressed by expressing either cDNAs of the *cpcB* wild-type gene or its mammalian homolog *RACK1*. The U24 snoRNA is not required for suppression. These data suggest an evolutionary conserved function for this WD protein in eukaryotic development and demonstrate a link between amino acid metabolism and sexual development in *A. nidulans*.

The fungus specific lysine biosynthesis pathway: Biotechnical importance, regulation and localisation

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To date most of the biosynthetic pathways leading to for humans essential amino acids have been found to be identical in fungi, plants and bacteria. An interesting exception is the biosynthesis of lysine via L-α-aminoadipic acid (L-α-AAA). This pathway which is named according to one of its intermediates aminoadipate pathway is found exclusively in higher fungi. In addition, L-α-AAA is a branch point of both lysine and penicillin biosynthesis in penicillin producing fungi such as *Aspergillus nidulans* or *Penicillium chrysogenum*. Previous investigations implied that the supply of L-α-AAA could be rate-limiting for the penicillin production. Hence, cloning of genes and overexpression of proteins of the lysine biosynthetic pathway might help to increase L-α-AAA levels and thus penicillin titers. Hence, we started to characterise the pathway both biochemically and at the molecular level. One of the genes, *lysF* of *A. nidulans,* was cloned and shown to encode homoaconitase. Using antibodies against *A. nidulans* homoaconitase together with the use of a GFP-LYSF fusion protein the cellular localisation of homoaconitase was studied. Furthermore, recent data on the regulation of lysine biosynthesis genes using *lacZ* gene fusions and Northern blot analyses emphasised their meaning for the production of fungal β-lactam antibiotics.

Nitrate utilisation in *Aspergillus nidulans*: An old model features new fashions

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Much attention has been paid lately to ecological, agricultural and human health problems related to nitrate contamination in hypertrophic waste water and intensively fertilised soil. The pioneering work by Cove and co-workers (reviewed in Cove, 1979) established *Aspergillus nidulans* as the major model organism for studying the nitrate assimilation pathway in eucaryotes.

In *A. nidulans,* two positively acting regulatory genes – *nirA,* a GAL4-type Zn^{++}-cluster protein mediating induction, and *areA,* a GATA factor mediating nitrogen metabolite repression – are required for the expression of the structural genes *niaD* (encoding nitrate reductase, NR), *niiA* (encoding nitrite reductase, NiR) and *crnA* (encoding a nitrate permease). The linked *niiA* and *niaD* genes are transcribed divergently form an overlapping promoter region termed "Intergenic Region" (IGR).

We mapped the specific binding sites for NirA and AreA in the IGR *in vitro* and the contribution of the 4 NirA binding sites and the 10 AreA binding sites to nitrate responsive expression of NR and NiR *in vivo* was analysed by deletion studies. We showed that two central NirA sites act bi-directionally on both *niiA* and *niaD* transcription, and that these central NirA sites are located in the proximity of a cluster of 4 AreA sites (5 to 8), which are responsible for about 80% of the transcriptional activity in a $nirA^+$ background. By *in vivo* footprinting analysis of the central NirA and AreA sites we showed that binding of the specific transcriptional activator depends on the presence of nitrate and functional AreA protein. Moreover, using defined *nirA* and *areA* mutants, we showed, for the first time *in vivo,* that a GATA factor is directly involved in remodelling of chromatin. GATA sites 5 to 8 are suited in a pre-set nucleosome free region and AreA dependent remodelling occurs upon nitrate induction. Surprisingly, the repositioning of four nucleosomes out of five monitored in this region is independent from the NirA specific transcription factor.

Plant Amino Acids

Fifty years of amino acid research in retrospect

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Half a century ago, warfare seriously interrupted fundamental research, yet two techniques were developed that greatly facilitated future studies of plant amino acids. These were paper chromatography and C^{14} labelling of molecules; together with subsequent advances in other forms of chromatography, in enzymological procedures, and in m.s. and n.m.r. methodologies, they generated an explosion of knowledge concerning the number and types of amino acids found in plants, and enabled the pathways of amino acid biosynthesis to be probed in ever-increasing detail. Thus a group of "non-protein" amino acids was recognized, whilst the twenty protein amino acids could be grouped into a few distinct biogenetic families.

The long-held concept that the initial step for the assimilation of NH_3-N involved its entry into the α-NH_2 group of glutamic acid (via glutamic dehydrogenase) was toppled in the early 1970s with the discovery and acceptance of the glutamine synthetase (GS)-GOGAT pathway, which required the initial entry of inorganic-N into the amino-N of glutamine. Subsequently, the GS-GOGAT reactions were shown to be especially important for the reassimilation of NH_3 released during photorespiration.

Recent years have witnessed the widespread use of natural and induced mutants and of molecular biological methods to further elucidate amino acid metabolism. The genes encoding many enzymes have been isolated; in some situations gene isolation has revealed previously undescribed enzymes. Genetic engineering has been employed to enhance the concentration of certain amino acids, e. g. lysine, in plant tissues. Key enzymes of biogenetic pathways have been identified as critical target sites for commercially-important herbicides, and this knowledge has been crucial in engineering herbicide-resistant cultivars of major crops.

The role of certain amino acids (e. g. γ-aminobutyric acid, glycine betaine, proline and glutathione) in mitigating the effects of abiotic stresses on plants continues to attract interest.

Molecular organization of the shikimate pathway in higher plants: How many genes, how many proteins, how many sites?

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The shikimate pathway produces the three proteinogenic aromatic amino acids, i. e. phenylalanine, tyrosine, and tryptophan, which are, in addition to several intermediates of the shikimate pathway, intermediates in the biosynthesis of numerous aromatic natural products in higher plants, such as flavonoids, coumarins, lignins, alkaloids, various quinones, etc.

While the chain of reactions leading to the aromatic amino acids is identical in microorganisms and plants, considerable differences exist in the organization and regulation of the shikimate pathway in these organisms. Molecular cloning of cDNAs and genes coding for the first seven enzymes of the shikimate pathway (prechorismate pathway) have confirmed that plastids are the major, if not only site of the pathway in higher plants. However, chorismate mutase (CM), the enzyme catalyzing the first step specific for the synthesis of phenylalanine and tyrosine, clearly exists in both a plastidic and a cytosolic form, which are encoded by distinct genes and are subject to differential regulation by endogenous and exogenous factors. Analysis of CM promoter GUS fusions in transgenic *Arabidopsis* revealed completely different expression patterns for the two genes. Efforts are now directed towards the elucidation of the molecular organization of the final steps of the synthesis of phenylalanine and tyrosine, and first results will be presented on the molecular cloning of an arogenate dehydratase from *Arabidopsis*.

Analysis of the methionine biosynthetic pathway in potato

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Molecular modulations of endogenous routes by introduction of novel functions or repression in transgenic plants allow for the creation of metabolic situations enabling to study the regulation of metabolic partitioning. Metabolic pathways usually include several enzymes possibly regulating the flux of metabolites through the pathway; furthermore, cross-controls between different pathways have to be postulated, adapting metabolite fluxes according to developmental and environmental requirements. The use of mutants or transgenic plants altered with respect to the activity of a single enzyme allows to study the function and physiology of the target enzyme *in vivo*. Furthermore, ectopic expression of foreign enzymes enables the introduction of new or modulated pathways, allowing the manipulation of metabolic concentrations and/or end-products leading to tailor made products, eventually.

Sulfate assimilation in higher plants is thought to be one of the factors determining plant growth and crop yield. Inorganic sulfate will be converted in a cascade of enzymatic steps to the nutritionally important sulfur-containing amino acids cysteine and methionine. In detail, the metabolism of methionine biosynthesis in potato will be discussed. Cystathionine-gamma synthase condenses the aspartate derived metabolite O-phosphohomoserine, providing the carbon backbone of methionine, and cysteine to yield cystathionine which is cleaved to homocysteine through the activity of cystathionine-beta lyase and finally methylated by methionine synthase to generate methionine. The latter enzyme is localised in the cytosol, whereas the former are plastidial enzymes.

Manipulation of thiole contents in potato

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Higher plants use inorganic sulfate as the major source of sulfur for synthesizing the nutritionally important sulfur-containing amino acids cysteine and methionine and also a variety of other sulfur containing metabolites as vitamines, sulfolipids, glutathiones and phytochelatines. The primary step of sulfur assimilation following uptake is the ATP dependent activation of the relatively inert sulfate molecule by the enzyme ATP-sulfurylase to APS, which is in turn reduced to sulfide and finally incorporated by OAS-TL (O-acetyl-serine thiol lyase or cysteine synthase) into O-acetyl-serine, which in turn is synthesized by SAT (serine actyl transferase), to yield cysteine. Most sulfur containing organic molecules present in plants are synthesized directly or indirectly from cysteine, especially the essential amino acid methionine, the methylgroup donor S-adenosylmethionine (SAM) and the transport metabolites GSH and SMM. Moreover, nutritional quality is in some crops determined through the limiting content of sulfur containing amino acids, either free or protein bound.

Genetic engineering of sulfur-assimilation and/or amino acid

biosynthetic pathways will first provide plant mutants for the further molecular and biochemical elucidation of the plant pathways but will also provide possibilities to engineer metabolite concentrations and thus quality traits. We will report on manipulation of ATP-sulfurylase, serine-acetyl transferase (SAT) and O-acetyl-serine thiol lyase (OAS-TL) leading to different influences on thiol contents in transgenic potato plants.

Influence of heavy metals on contents of non-protein amino acids and polyamines in food plants

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Unfavourable environments, such as drought or soil salinity, increase the pool of free amino acids (protein-related and non-protein amino acids). Furthermore cadmium (Cd) promotes the formation of polyamines in plants.

Therefore in this contribution the influence of various heavy metals (Cd, Pb, Cu, Ni, Zn) on the formation and contents of free amino acids and polyamines were examined in vegetables and cereal plants.

The release of amino acids from roots of maize rose in a hydroponic culture, which was enriched with 2 ppm of water-soluble Cd. – Thus, the exudation of the non-protein amino acids β-alanine and γ-aminobutyric acid increased significantly (about 150%). Additionally, a threefold higher release of the stress indicator proline and a twofold higher exudation of the polyamine-precursor arginine were obtained.

In contaminated sandy soils the bioavailable Cd (1.5 ... 3 ppm) caused higher contents of trimethylglycine (glycine betaine) and trigonelline in barley (up to 50%). Soil salinity enhanced the uptake of Cd and other heavy metals (Cu, Ni, Zn, Pb) and these unfavourable soil conditions resulted in a 4- to 8-fold increase of non-protein amino acids such as trimethylglycine formed from free serine (serine → 2-aminoethanol → choline → trimethylglycine).

In experiments with spinach an enrichment of a sandy soil with Cd (1.2 ppm) caused an tenfold higher Cd content in leaves followed by an increased spermine concentration (up to 50%). And, a contamination of soils and bean plants with nickel induced the formation of putrescine (up to 50%; arginine is a precursor). The effects of soil pollution with heavy metals on the food quality are discussed.

Manipulation of the lysine and threonine content in plants

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Various strategies have been developed with the goal to improve the nutritional quality of plants with essential amino acid imbalance and in particular poor lysine content.

We are using biochemical, molecular and genetical approaches to study the structural and regulatory genes responsible for the production of the aspartate-derived amino acids lysine, threonine and methionine. Mutants overproducing free lysine and threonine have been isolated in various species and their biochemical characterization has shown a deregulation at the level of two key enzymes, dihydrodipicolinate synthase (DHDPS) in the lysine overproducers and aspartokinase (AK) in threonine overproducers. The cloning of AK and DHDPS encoding genes and their mutated version has allowed to analyse them structurally and functionally. Overproduction of lysine was observed after transferring a mutated *Arabidopsis* dhdps coding for a feedback-insensitive enzyme in *Nicotiana plumbaginifolia*. Similar experiments aim to increase lysine content in cereal grains (*Sorghum,* maize), legume seeds (pigeon pea) and tubers (potato) by expressing the dhdps mutated gene under the control of organ-specific promoters.

The use of immunohistochemistry to study amino acid metabolism in higher plants

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We have used the availability of specific antisera raised against both enzymes and individual amino acids, to study metabolism in the developing grape seed.

Phospho*enol*pyruvate carboxykinase (PEPCK) was shown to be located in tissues that are involved in the transfer of assimilates within the developing seed. These were in a region of the chalaza, immediately adjacent to the developing storage tissue and a single or double layer of palisade cells of the outer integument. Aspartate aminotransferase, alanine aminotransferase, glutamine synthetase, glutamate dehydrogenase and glutamate itself, were also enriched in the palisade cells. We suggest that the palisade cells function in the delivery of amino acids and sugars, which diffuse through them via plasmodesmata and then enter the nucellus by way of transfer cells. However during this transfer process, we also suggest that there is considerable metabolism, the pathway of which may well be regulated by the supply of individual amino acids.

The amount of PEPCK protein in the immature grape seed was increased 100 fold by preincubation in the presence of asparagine. This increase in PEPCK protein was far lower in the presence of glutamine (3 fold) or ammonia (7 fold) and there was no induction by aspartate alone. Data obtained from tracer studies utilising ^{14}C-aspartate is consistent with an anaplerotic role of PEPCK in the metabolism of grape seeds. Thus if the Krebs tricarboxylic acid cycle is involved in the interconversion of carbon skeletons of amino acids, this would require PEPCK or NAD(P) malic enzyme to generate acetyl CoA for the reaction catalysed by citrate synthase. It is also possible that a second role for PEPCK, may involve PH regulation.

We propose that PEPCK plays a key role in the co-ordination of carbon and nitrogen metabolism in higher plants.

Free amino acids present in edible seed sprouts sold for human consumption

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Some seedlings are sold on the European market as healthy food. The label only mentions some vitamin and mineral contents. We studied the free amino acids and particularly the free non-protein amino acids and trigonelline composition of *Pisum sativum* L. (garden pea) and *Lens culinaris* L. (lentil) by HPLC in seedlings from a commercial product (CP) and seedlings grown in the laboratory (LP). The growth conditions of commercial seedlings are unknown but the seedling axes were rather small (about 1 or 2 days of germination), in our laboratory the duration of germination was 4 days at 20° C in a constant humidity. The cotyledons and seedlings axes were studied separately.

In lentil seedlings in both conditions we found a high amount of trigonelline and some nonprotein amino acids such as α-aminoadipic acid, homoserine, β-isoxazolin-5-one-alanine (BIA), and γ-glutamyl BIA. A small amount of oxalylhomoserine is also found and γ-glutamyl-D-alanine is only found in LP.

In garden pea seedlings we found partly the same components as in lentil and a uracil-alanine derivative (isowilliardiine) is found in substantial amount. The total free nonprotein amino acid and trigonelline contents in lentil seedlings are 82.43 and 93.33 mg/100 g of fresh seedlings in CP and LP respectively and in garden pea seedlings 270.17 and 258.14 mg/100 g. The free nonprotein amino acids are higher in garden pea seedlings.

Some of these compounds such as BIA and α-aminoadipic acid are considered as rather toxic for human. For the other compounds the nutritional, pharmacological and toxicological properties are unknown.

γ-Aminobutyric acid accumulated in tea leaves

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γ-Aminobutyric acid (GABA), a hypotensive compound, is formed from glutamic acid by glutamate decarboxylase under anaerobic conditions in tea shoots. In the first three hours of anaerobic incubation, glutamate was remarkably decreased and GABA was considerably increased. But under continuous anaerobic incubation, those of amino acids remained almost constant. After three hours of anaerobic incubation, when tea leaves were released under aerobic condition, glutamate reproduced rapidly by its original amount. But GABA didn't decrease very much. After one hour of aerobic incubation, tea leaves were given three hours of anaerobic incubation again and then accumulated glutamate changed to GABA. As a result of this treatment, the amount of formed GABA increased over 1.5 times more than that of usual continuous anaerobic incubation. The longer aerobic incubation makes the more decrease of GABA. One hour of aerobic incubation is suitable. Repeated treatment of anaerobic and aerobic incubation is more effective. By 4 times repetition, GABA in tea leaves was formed 2.3 times as much as usual anaerobic incubation at the third crop of tea. By this new method, the level of GABA in tea leaves using the third crop reached to the same level in tea leaves of the first crop by the previous method. Further work is in progress to confirm the precursor of reproducing glutamate.

Regulation of lysine metabolism in rice developing seeds

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The biosynthesis of lysine and other aspartic acid derived amino acids have been well studied in several plant species. However, recently the degradation of lysine has been shown to be important for accumulation of this amino acid in cereal seeds. In rice, the regulation and characteristics of lysine metabolism has not been investigated till very recently. We have isolated, partially purified and characterized two enzymes involved in lysine biosynthesis, aspartate kinase (AK) and homoserine dehydrogenase (HSDH), and two enzymes involved in lysine degradation, lysine 2-oxoglutarate reductase (LOR) and saccharopine dehydrogenase (SDH). Two isoenzymes of AK were identified, one sensitive to lysine feedback inhibition (AK-Lys) and the other to threonine feedback inhibition (AK-Thr). The AK-Lys is predominant and has a molecular mass of 167 kDa, while the AK-Thr has a molecular mass of 186 kDa and is part of a bifunctional enzyme containing also activity of HSDH sensitive to threonine inhibition. LOR and SDH activities were detected only in endosperms and were also shown to be a bifunctional protein, with a molecular mass of 202 kDa, but producing multimeric forms. Product-inhibition studies for LOR suggests a mechanism similar to LOR from human placenta, but different from the maize enzyme.

(Research financed by FAPESP, Brazil.)

Lysine degradation in rice seeds is modulated by calcium and ionic strength

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The catabolism of lysine has only been given some special attention in recent years after two enzymes, lysine 2-oxoglutarate reductase (LOR) and saccharopine dehydrogenase (SDH), which regulate lysine degradation in plants, had been identified, isolated and characterized in a few numbers of plant species. LOR and SDH are one single bifunctional polypeptide in rice and maize. Recently, the LOR domain of the maize protein was shown to be modulated by calcium, whereas SDH was not. We have purified LOR-SDH from rice seeds and tested in the presence of calcium, EGTA, KCl, TRIS and SAM. We have also tested aminoethyl-cysteine (AEC), a lysine analogue, as substrate for LOR. The results suggest that LOR activity is modulated by calcium, but not SDH activity like the maize enzyme, since the addition of EGTA reduced LOR activity, which was recovered by the addition of calcium to the assay mixture. Ionic strength also modulates LOR and SDH. Both activities varied considerably by altering KCl or TRIS concentration in the buffer media. Although AEC can substitute lysine in proteins and was shown to inhibit the lysine-sensitive aspartate kinase activity of rice, it could only substitute lysine as LOR substrate at very high concentrations. SAM did not produce any alteration on LOR or SDH activities.

(Research financed by FAPESP, Brazil.)

Aspartate kinase isoenzymes of rice are not modulated by calcium or calmodulin

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Aspartate kinase (AK) is the first enzyme of the aspartic acid metabolic pathway catalysing the conversion of aspartate into β-aspartyl phosphate. In plants, AK has been identified, isolated and characterized from a wide variety of crops, but only recently in rice. Two AK isoenzymes have been identified in rice seeds, one sensitive to lysine feedback inhibition (AK-Lys) and the other to threonine (AK-Thr). Partially purified AK-Lys and AK-Thr were tested in the presence of calcium, EGTA, two calmodulin inhibitors, aminoethyl-cysteine (AEC), S-adenosylmethionine (SAM), KCl, methionine and valine. AK-Thr did not exhibit any significant alteration to these compounds, whereas AK-Lys activity was inhibited by AEC, a lysine analogue, but not to the same extent of lysine. SAM alone also inhibited slightly AK-Lys activity and synergistically with lysine. Although a very slight stimulation by calcium was observed for AK-Lys, the results obtained with calcium, EGTA and calmodulin inhibitors, can not support a regulatory role by calcium on AK from rice seeds. Methionine and valine produced slight increase of AK-Lys activity.

(Research financed by FAPESP, Brazil.)

Characterization and localization of cystathionine beta-lyase from potato (*Solanum tuberosum* L.)

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In plants and some bacteria, methionine biosynthesis results from the convergence of one branch of the aspartate pathway of amino acid biosynthesis and the sulphur reduction pathway. The second enzymatic step in this pathway, the cleavage of cystathionine into homocysteine, pyruvate and ammonia, is catal-

ysed by cystathionine β-lyase (CbL). We here report the isolation of a potato gene by functional complementation of the respective *E. coli* auxotrophic mutant. Detailed analysis suggests that in potato only one isoform exists, which is localised in plastids. Reversed genetic approaches were used to study the impact of the CbL on methionine biosynthesis. The results will be discussed with respect to regulation of the fluxes involved in methionine biosynthesis.

Analysis of cystathionine γ-synthase from *Solanum tuberosum* L.

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The first step of methionine biosynthesis in higher plants, the condensation of O-phospho-homoserine and L-cysteine forming the thioether cystathionine, is catalysed by the enzyme cystathionine γ-synthase (CgS). We have isolated a cDNA encoding CgS from a leaf λ ZAP II-library of *Solanum tuberosum* L. The nucleotide and deduced amino acid sequence showed homology to other sequences of higher plants from *Arabidopsis thaliana, Zea mays* and *Mesembryanthemum crystallinum*. The cDNA, when cloned into a bacterial expression vector, did functionally complement the *E. coli met*B1 mutant strain LE392. Enzyme activity of the potato CgS and *E. coli met*B were comparable in the auxotrophic background.

Transcript levels of CgS in different tissues of potato plants were determined by Northern blot analysis. Expression was found in all tissues with elevated levels in flowers and source leaves. The pattern of gene expression during a day/night period implied a light-dependent control of CgS-transcription typical for enzymes localised in plastids. The expression of CgS was shown to be light-inducible. Further aspects will be discussed.

Molecular biology of plastidic phosphorylated serine biosynthetic pathway in *Arabidopsis thaliana*

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Serine biosynthesis in plants proceeds by two pathways; the glycolate pathway which is associated with photorespiration and the pathway from 3-phsophoglycerate which is presumed to take place in plastids. The 3-phsophoglycerate pathway (phosphorylated pathway) involves three enzymes catalysing sequential reactions; 3-phosphoglycerate dehydrogenase (PGDH), 3-phosphoserine aminotransferase (PSA) and 3-phsophoserine phosphatase (PSP). We have isolated cDNA and genomic clones encoding these three enzymes from spinach [1] and A. thaliana [2–4] by means of heterologous probe screening, homologous EST clones and genetic complementation in the Escherichia coli mutant. The identity of isolated cDNAs was confirmed by functional complementation of serine auxotrophy in E. coli mutants and/or the detection of catalytic activity in the recombinant enzymes produced in E. coli. Northern blot analyses indicated the most preferential expression of three genes in light-grown roots. In contrast, the mRNAs of two proteins involved in the glycolate pathway (H-protein of glycine decarboxylase and serine hydroxymethyltransferase) were highly accumulated in light-grown shoots. Environmental stresses, such as high salinity, flooding and low temperature, induced changes in mRNA levels of enzymes in plastidic phosphorylated serine biosynthetic pathway but not in that of mitochondrial pathway. These results indicated that the plastidic 3-phosphoglycerate pathway plays an important role in supplying serine in non-photosynthetic tissues in plants and under environmental stresses.

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Immuno Modulatory Effects of Amino Acids

Influence of glutamine on apoptosis and heat shock protein expression

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The amino acid L-glutamine (GLN) is attracting widespread attention because of its relevance to numerous metabolic processes and its potential role in the treatment and prevention of critically illness. It is a primary fuel for rapidly dividing cells, provides intermediates for biosynthesis of nucleotides and influences leukocyte phenotype and function. We show that GLN has also anti-apoptotic properties. Human myelocytic U937 cells cultured in absence of GLN exhibit an increased susceptibility to heat mediated apoptosis than cells cultured in presence of 2 mM GLN. Supplementation of GLN to GLN deprived cells at the onset of heat shock reduces apoptosis to values measured in control cells, cultured in presence of 2 mM GLN. This cytoprotective effect of GLN is accompanied by an enhanced expression of the 70 kD heat shock protein (HSP70). HSP70 is a molecular chaperon which is known to prevent apoptosis. Further experiments showed that without GLN no HSP70 is expressed after heat shock. However, the amount of HSP70 increases with the concentration of GLN in the medium. Of all other amino acids only two, namely L-alanine and L-serine, showed a similar but much weaker effect on HSP70 expression. Metabolic labeling experiments revealed that GLN specifically enhances the expression of HSPs, whereas the expression of other proteins remains unaffected. Our data show that GLN boosts the heat-mediated induction of HSP70 expression preventing thereby cell death by apoptosis.

Glutamine and cytokine production

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Glutamine has long been known to be a preferred fuel of cells of the immune system and to promote in vitro lymphocyte proliferation. More recently there has been some interest in the role of glutamine in modulating cytokine production. Maximal interleukin-1 (IL-1) production by cultured murine macrophages

and the production of IL-2 by cultured mouse lymphocytes is dependent upon the provision of glutamine [Wallace and Keast (1992) Metabolism 41: 1016–1020; Yaqoob and Calder (1997) Nutrition 13: 646–651]. Recent studies with cultured human blood mononuclear cells have found that the maximal production of IL-2, IL-10 and interferon-γ (IFN-γ) requires glutamine [Yaqoob and Calder (1998) Cytokine 10: 790–794]. There is however, very little data on the effect of dietary glutamine on cytokine production. We have recently conducted two studies to investigate the effect of increasing the dietary supply of glutamine upon the ex vivo production of cytokines by macrophages and lymphocytes, respectively. Mice were fed for two weeks on a diet which included 200 g casein/kg providing 19.6 g glutamine/kg, or a glutamine-enriched diet which provided 54.8 g glutamine/kg partly at the expense of casein. The production of all three cytokines investigated (TNF-α, IL-1β and IL-6) was greater for lipopolysaccharide-stimulated macrophages from mice fed the glutamine-enriched diet [Wells, Kew, Yaqoob, Wallace and Calder (1999) Nutrition, in press]. IL-2 production was significantly greater for concanavalin A-stimulated spleen lymphocytes from mice fed the glutamine-enriched diet [Kew, Wells, Yaqoob, Wallace, Miles and Calder (1999) J Nutr, in press]. These two studies suggest that increasing the amount of glutamine in the murine diet enhances the ability of both macrophages and T lymphocytes to respond to stimulation, at least in terms of cytokine production. These observations suggest that increasing the oral availability of glutamine could promote immune responses involving macrophage- or T cell-derived cytokines.

Glutamine and the acute phase response: The role of cell swelling

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During inflammatory processes such as infection, stimulated monocytes and macrophages release inflammatory mediators such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor α. This is associated with an increase in the consumption of glutamine, which is the main substrate of the cells. In the liver, the inflammatory mediators induce in turn the modulation of the synthesis of so-called "acute phase proteins" (APP): for example, the α2-macroglobulin (α2M) gene, encoding the major positive APP in adult rat, is expressed during inflammatory processes. Moreover, endotoxin treatment increased ten-fold the hepatic glutamine uptake. We therefore tested the possibility that glutamine *per se* may play a key role in both cell types (i. e. macrophages and hepatocytes) during the acute phase response. In lipopolysaccharide-stimulated rat peritoneal macrophages, glutamine at first accelerated the synthesis and therefore the secretion of IL-6, and then induced an increase in IL-1β production some hours later. In rat hepatocytes, the inducing effect of IL-6 on the α2M gene was about two-fold higher in the presence of glutamine than in its absence. Taken together, these results demonstrated that glutamine plays a role in the acute phase response. We therefore started experiments to specify the role of cell swelling in the effect of glutamine. Indeed, a cell swelling is induced by the sodium-dependent transport of the amino acid in hepatocytes, and cell swelling is now regarded as a novel regulatory mechanism. Moreover, we recently observed that glutamine transport into macrophages also induced cell swelling. As reported for glutamine, cell swelling *per se* reinforced the inducing effect of IL-6 on the expression of the α2M gene in hepatocytes. By contrast, cell swelling *per se* inhibited the secretion of IL-6 by LPS-stimulated macrophages. Thus, the effect of glutamine may be or not mediated by the glutamine-induced cell swelling depending on the cell type involved in the acute phase response.

Glutamine as the key amino acid in promoting cell-mediated immunity: 20 years of clinical experience

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Since the pioneering clinical study in depressed children (Cocchi, 1981) [1] normal and Down children with easiness to upper respiratory tract infections were successfully treated by Gabaergic drugs (Cocchi, 1998) [2].

Research showed that stress undermines host resistance to infections through neurendocrine mediated changes in immune competence (Boyce et al., 1995). It is the same for every kind of stress of external or internal origin or both. The adrenergic blockade improves also cellular immune responses otherwise depressed by the so called mental stress in humans as one type of internal stresses (Bachen et al., 1995).

Because the immune-suppressive action of stress via the GABA impairment (Horger and Roth, 1995) and subsequent cortisol hyper-incretion or hyper-activity (Dhabhar et al., 1996; Haessig et al., 1996; Dantzer, 1997; Friedman and Irwin, 1997), the rationale of the treatment to counteract this easiness by drugs can be explained as it follows.

The basic treatment uses Gabaergic drugs like I-glutamine as the precursor of GABA via I-glutamic acid (Laake et al., 1995; Shupliakov et al., 1997); pyridoxine as the cofactor of all decarboxylases, GAD inclusive (Baxter, 1976) and a benzodiazepine as the sensitizer of type A Gabaergic receptors (Bruni et al., 1980; Viukari, 1983; Schoch et al., 1985).

Daily doses in 1–14 years children can be so prescribed:

glutamine: 125–500 mg daily (not later than 2 pm); pyridoxine 75–150 mg daily; diazepam 1–3 mg daily in the evening (in small children better: oxazepam 5–8 mg daily, always in the evening).

This 3-drugs' prescription works in a synergistical way and can restore the glutamic-GABA pathway impaired by the stress itself. The use of a benzodiazepine aims to re-sensitize type A Gabaergic receptors, the first metabolic point where stress applies itself. Without doing it we could induce only the increasing of glutamate with hazardous effects. But this evening use of a benzodiazepine is the best way to restore sleep (Viukari, 1983) often impaired by excess adrenergic stimulation and to avoid side-effects like daily drowsiness and muscle relaxation.

On the other hand I-glutamine is directly involved in the nucleogenesis of rapid proliferating cells (Gismondo et al., 1998), being the donor of the atoms 3 and 9 of the purinic ring (Stryer, 1988). By this way it contributes to a better production of leukocytes (Heberer et al., 1996; Newsholm and Calder 1997; Yoo S. S., et al., 1997).

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Dietary supplementation with L-arginine and canola oil improves outcome following renal transplantation

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Studies in experimental animals have shown that dietary arginine and canola oil supplements can significantly increase allograft survival in rats treated with standard immunosuppres-

	Rejection episodes			Drug toxicity		BP (Mean)		S Cr (Mean)	
	Total	Pts	R'cd	OKT3	Episodes Pts	6 mos	12 mos	6 mos	12 mos
S	5	3	2	3	2	127/81	127/74	1.5	1.4
C	13	11	3	7	7	144/84	136/83	1.6	1.6

Overall rejection episodes and systolic blood pressure differences are significant.

sive drugs. To test this treatment strategy in humans, a prospective randomized clinical trial was initiated in renal transplant patients receiving standard cyclosporine A, mycophenalic acid and steroids. Study patients (S, n = 37) received 9 g arginine and 30 g canola oil daily as a supplement to a low fat diet. Control patients (C, n = 43) received a low fat diet and no supplement. Entry of patients started in September 1997, and 47 patients have been followed for at least 6 months with 19 patients followed for more than 12 months. Three S patients did not receive supplement for more than three days and were excluded. Five patients stopped taking supplement for various reasons at days 113, 122, 151, 155 and 180 and were censored at that time. Of the 24 patients on continuous supplement at 6 months, none died or lost a kidney from rejection compared to one kidney loss in the control group (n = 23). Results are given above.

We conclude that dietary supplementation with arginine and canola oil is safe, well tolerated and associated with reduced morbidity following renal transplantation.

Exercises

Amino acid measurement in atheletes

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Physical exercise strongly influences amino acid metabolism. In particular BCAA, alanine and glutamine show the most changes not only in plasma but also in muscle amino acid pools [1]. Other parameters like keton bodies, acetyl-carnitine and ammonia are useful indexes to evaluate amino acid metabolism [2, 3]. It is necessary to consider the type of exercise when measuring amino acid during physical activity, given that it deeply influences type and amount of amino acid involved [4].

Kidney has a central role in amino acid metabolism both in resting condition and during physical exercise. In fact in the resting condition the kidney guarantees an almost complete reabsorption of the amino acids filtered and it participates actively in amino acid metabolism, for example by putting serine and cysteine into the circulation, and extracting proline and glutamine [5]. In addition Claris-Appiani et al. [6] found that some amino acids like BCAA can modify renal haemodynamic such as GRF or RPF.

On the contrary during exercise a deep modification of membrane permeability occurs. In particular increase of membrane permeability and saturation of the tubular reabsorption process of filtered protein produced an intense post-exercise proteinuria [7]. The amount of proteinuria (measured as total protein, albumin, α_1-microglobulin, β_2-microglobulin, Retinol Binding Protein, creatinine) is strictly related to intensity of exercise [8]. According to the fact that during competitions urine sample collection is easier than blood collection the measurements of amino acids and proteins in urine before and after exercise can be useful to understand better the levels and types of this proteinuria.

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Influence of exercise on the serotonergic system

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The central implication of the serotonergic system on behavior, mood, fatigue as well as on mental and physical efficiency during exercise in humans has been postulated by several authors. However, the definite proof of these assumptions is difficult to provide. Conclusions based on animal trials are limited due to inevitable anthropomorphic considerations. Therefore, we examined in sedentary students the effect of a 3-week TRP administration (1.5 g/day PO) without exercise and the impact of moderate endurance training regiments for 3 weeks as well as in well-trained endurance athletes the effect of excessive increase in training volume for 4 weeks.

Methods: In these collectives, plasma concentrations of total and free tryptophan (TRP), branched-chain amino acids (BCAA) and the ratio of free TRP/BCAA were determined. Furthermore, the number of platelet serotonin transporter (5-HTT) [^{3}H]paroxe-

tine binding sites (B_{max}) and 5-HT_{2A} receptor (5-HT_{2A}R) [^{3}H]-ketanserin binding sites as well as the plasma prolactin (PRL) concentration at rest and after application of the 5-HT_{1A} agonist buspirone were measured. Behavioral changes were determined using test batteries by Nitsch (1976) as well as Abele and Brehm (1986).

Results: Basal total TRP, free TRP and the ratio of free TRP/BCAA increased significantly after oral TRP administration while no changes were found in both training groups. B_{max} of [^{3}H]paroxetine binding on 5-HTT increased after moderate training but did not change after excessive training. B_{max} of [^{3}H]ketanserin binding on 5-HT_{2A}R increased after moderate endurance training and decreased after excessive regiments. The K_d of 5-HTT and 5-HT_{2A}R binding were not changed significantly in any group. Oral application of TRP did not influence 5-HTT and 5-HT_{2A}R B_{max} or K_d. Basal PRL concentration was decreased after moderate and increased after excessive training while PRL secretion after buspirone was not significantly ($p < 0.067$) affected by training regimes. Physical efficiency was increased and mood improved by moderate training. Fatigue was increased and recovery ability was diminished by excessive training.

Conclusion: Even though oral TRP application increased plasma ratio of free TRP/BCAA, basal density values of 5-HTT or 5HT_{2A}R were not altered without exercise. Moderate training regiments in sedentary subjects increased basal values of B_{max} of 5-HTT and 5-HT_{2A}R and reduced plasma PRL concentration, coming along with improved physical efficiency and mood. On the contrary, reduced 5-HT_{2A} density and increased PRL concentration after excessive training regiments may point towards overstrain in well-trained endurance athletes.

Plasma taurine after different endurance events

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Taurine, 2-aminoethane sulfonic acid, a non-essential amino acid, is present in the muscle at relatively high concentrations, where it plays an important role in muscle function, probably being involved in calcium homeostasis.

After the completion of the Rotterdam Marathon on a 100 km run, the plasma levels of taurine were compared with the pre-race value as well as with its concentration 24 hours after the completion of the endurance event. The mean plasma level of taurine increased significantly on the completion of the Marathon and 100 km endurance event, 95 ± 23 v 52 ± 5 µmol/l and 72 ± 12 v 52 ± 8 µmol/l respectively, the changes in concentration being related to the speed or intensity of the exercise (an increase of 77% after the Marathon and by 36% after the 100 km run), rather than the duration of the exercise, which was more closely mirrored by the changes in the amino acid pool. The peak in taurine concentration occurred at the cessation of the exercise which did not parallel the increase in creatine kinase, a muscle marker of membrane disruption, which occurs approximately 24–48 hours later.

Although it is apparent that plasma volume and urinary clearance may contribute to the increase in the plasma taurine concentration, its release from the muscle could be a major factor for its elevation in the blood.

The role of glutamine for immune cells: Implications for the whole body in some conditions

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Several different types of cell, for example, lymphocytes and macrophages, are involved in defending the body against invasion by a foreign organism. The total number of lymphocytes in the body may be as high as 10^{12}, with a mass of about 300 g. Even in a quiescent state, they are metabolically active, and require large amounts of the amino acid glutamine. Not all this glutamine is fully oxidized. Most of it is converted into glutamate, aspartate, alanine and lactate. This raises questions (1) what is the significance of the high rate of utilization?; (2) what are the consequences for the whole organism in some conditions?

(1) High rates of glutamine utilization may be necessary to provide for a high energy demand; but surprisingly glutamine is not completely oxidized. Glutamine provides nitrogen for the synthesis of several important compounds (*e. g.* purine and pyrimidine nucleotides) needed for the synthesis of DNA and RNA during lymphocyte proliferation and macrophage activation. However, the rate of glutamine utilization is markedly in excess of the rates of synthesis of these compounds. A high rate of glutamine utilization may provide precision in the mechanisms that regulate changes in the rate of purine and pyrimidine nucleotide synthesis. This concept, termed "branched-point sensitivity", has a powerful mathematical basis and important consequences. Failure to make adequate provision could lead to disturbed concentrations of nucleotides to support sufficient and correct template structures of DNA and RNA.

(2) From the rate of glutamine utilization by human lymphocytes *in vitro,* it is estimated that, for all the lymphocytes in the body, about 50 g of glutamine would be used each day – even in the quiescent state. The total number of macrophages in the body and epithelial cells of the intestine might use a similar amount of glutamine. The minimal estimate is therefore 150 g/day which, because of activation and recruitment of cells, might be much higher in exercise and trauma. If these calculations are valid, they suggest that provision of amino acids that can be converted to glutamine, or glutamine itself, might be important in exercise and trauma. Muscle protein breakdown in these conditions is primarily for the provision of precursors for the synthesis of glutamine. This idea has some interesting consequences. Although glutamine utilization by these cells is large, the amount of nitrogen incorporated into DNA and RNA from glutamine is very small: a consequence of branched-point sensitivity. To provide for precision in nucleotide synthesis for DNA and RNA synthesis, the nitrogen and carbon in the glutamine end up mainly as glutamate, aspartate and alanine, which, *via* gluconeogenesis and ureagenesis, produce urea.

This theory suggests that the provision of glutamine to subjects after trauma or exercise should improve recovery and reduce infections – this has been shown to be the case.

Do changes in amino acids play a role in fatigue?

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There is an increasing interest in the mechanisms behind central fatigue, particularly in relation to changes in brain monoamine metabolism and the influence of specific amino acids on fatigue. Several studies in experimental animals have reported that physical exercise increases the synthesis and metabolism of brain 5-hydroxytryptamine (5-HT). Support for the involvement of 5-HT in fatigue can be found in studies where the brain concentration of 5-HT has been altered by means of pharmacological agents. When the 5-HT level was elevated in this way the performance was impaired in both rats and human subjects, and in accordance with this a decrease in the 5-HT level caused an improvement in running performance in rats. The precursor of 5-HT is the amino acid tryptophan and the synthesis of 5-HT in the brain is thought to be regulated by the blood supply of free tryptophan in relation to other large neutral amino acids (including the branched-chain amino acids, BCAA) since these compete with tryptophan for transport into the brain. Studies in human subjects have shown that the plasma ratio of free tryptophan/BCAA increases during and, particularly, after sustained

exercise. This would favour the transport of tryptophan into the brain and also the synthesis and release of 5-HT which may lead to fatigue. On the other hand, ingestion of BCAA was shown to balance the increase in free tryptophan level and the plasma ratio of free tryptophan/BCAA remained approximately constant or was even decreased during exercise. According to the theory, this may reduce the synthesis and release of 5-HT and therefore delay fatigue. When BCAA were supplied to subjects during standardised cycle ergometer exercise their ratings of perceived exertion and mental fatigue were lower as compared to when they were supplied a placebo. Furthermore, when subjects were supplied a drink containing BCAA during a competitive 30 km cross-country run their mental agility was improved or maintained, in terms of performance in different cognitive tests carried out after the race. The physical performance was improved during exercise in which the central component was assumed to be more pronounced, i. e. during exercise in the heat or in a competitive race. Thus, more and more evidence is presented to support the theory that changes in the plasma concentration of specific amino acids may contribute to fatigue during sustained physical exercise.

Plasma amino acids profile and catecholamines at rest and after testing swimming performance during an annual season in competitive swimmers

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Introduction: Some investigators have suggested, that amino acids (AA) metabolism is involved in athlete's fatigue and overstrain. Recently we demonstrated that availability of particular AA may be reduced by extensive running exercise and changes in some plasma AA concentrations during a treadmill step test may be predictive for later bad tolerance of increasing training load. Now we wanted to see if a swimming step test (Pansold Test) induces changes in AA profile too and if circa-annual variations in training loads affect these reactions and the catecholamine (Cat) secretion.

Subjects and methods: The Pansold swim test (100 m freestyle in intervals with increasing up to max velocity) was carried out 6 times during one competitive season in 4 female and 2 male swimmers aged 16–18 years. Test conditions were standardised. Venous blood was collected after 30 min rest in supine position, 2.5 hrs after a small lunch and within 1 min after the last (max) step of the test. Determination of Cat was performed with HPLC-A and of AA with HPLC-F after derivatization with OPA. Test phases were: 1. endurance training and team competition, 2. high load (training camp), 3. tapering, 4. after regeneration, 5. new building up endurance and athletics, and 6. like 1. (end of endurance training).

Results: Except aspartate all AA levels showed ups and downs during the season. Ala was the only one showing reproducible sign. changes during the P. test (increase), Ser, Thre, Hxl mostly tendencies to decrease. In a most remarkable manner levels of BCAAs and dopamine at rest reflected training load (intensity and/or volume) being highest in the phases 1 and 2 and decreasing during tapering and regeneration. After phases 4 and 5 resting levels of Thre, Ser and Hxl were the highest at rest and decreased during the test.

Discussion: Since metabolically connected AAs and AAs being involved in creatine synthesis and in the Meth-Homocystein cycle showed correlations, plasma levels might reflect with some restriction metabolic pathways. It seems that with increasing training load unknown mechanisms increase the availability of some AA (i. e. BCAA). Low levels in the regeneration phase of Thre, Ser, and Hxl might be due to increased uptake and turnover. Perhaps dopamine is involved in AA metabolism adaptation to increasing training load.

Examination of the relationship between central control of tryptophan and running activity

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An alteration in the uptake rate of tryptophan into the brain across the blood-brain barrier affects the serotoninergic system. 5-hydroxytryptamine is known to be involved in fatigue. In this study, the mechanisms underlying the relationship between central modulation of tryptophan and running activity were examined.

To influence the tryptophan uptake on the L-system transporter, the following were administered to Nagase Analbuminemic Rats (NAR) before the running on a treadmill: branched-chain amino acids (BCAA), 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (a specific inhibitor for the L-system transporter, BCH) and saline. A guide cannula was implanted into the left striatum; five days later, a microdialysis probe (co-ordinates: A 0.2, L 3.0, V -6.0 mm) was inserted through the cannula of the Sprague-Dawley rats (SDR) (n = 5) and NAR (n = 5), to investigate the relationship between changes in fatigue and extracellular tryptophan levels in both groups. Samples (1 ml/min) were collected every 30 min, during running to fatigue and recovery period (2 hrs).

The animals who received BCAA or BCH treatment ran for longer before becoming fatigued, relative to the saline groups: this was accompanied by a decrease in concentration of synaptosomal tryptophan and 5-hydroxytryptophan. In vivo microdialysis experiments demonstrated that there were rapid increases in extracellular tryptophan and 5-hydroxyindoleacetic acid from the basal level after running to fatigue, which returned to baseline or below during the recovery period. Thus, the serotoninergic system was activated by prolonged exhaustive running and controlled by inhibition of tryptophan uptake. The extracellular concentration of tryptophan was below 1 μM, even during the running period. This suggests that the plasma membrane tryptophan carrier in the nerve terminal was not at saturation.

We conclude that the difference between the running activity after BCAA treatment, which diminished the central action of tryptophan and running activity after saline treatment is due to fatigue in the central nervous system induced by enhanced tryptophan uptake.

Muscle efflux of glutamine in oral carnitine oversupplied sportsmen can mimic stress conditions with increased risks of athletic traumas

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Oral supply of high doses of creatine in sportsmen (up to 20 g daily) works for an increased synthesis of ATP that allows a more prolonged muscular strain. But more ATP shifts also into an increased muscular synthesis of l-glutamine that easily crosses the blood-brain barrier. In this way it can lead to an excess production of brain glutamate, the direct precursor of GABA. In absence of stress this fact could be not neurochemically relevant but, if so, the oversupply of creatine in athletes should have a poor rationale. When stress arises, even by fatigue till to overtraining stress, or by the sum of different stresses, there is a reduced GABA utilisation by type A receptor. The increased GABA availability into the synaptic cleft turns out on a larger type B receptor stimulation that inhibits also brain ACh turnover. Brain choline decreases by reduced uptake throughout the blood-brain barrier and by increased efflux from the brain. By feedback GAD reduces its work and the brain glutamate goes up because a lesser transformation into

GABA. The glutamate increasing produces several stress symptoms, among which the vagal overstimulation by hypothalamic stimulation of Vagus Nucleus Dorsalis and Tracti Solitarii Nucleus. The larger availability of choline out of the CNS makes an increased synthesis of peripheral ACh possible with a magnification of all vagal-parasympathetic brain originated answers. On heart it can lead to vagal syncope or a sudden irreversible arrest, a rare event often falsely attributed to a heart infarction. This ACh increasing seems not limited only to the vagal district, where it found experimental evidence. Our clinical results draw to an ACh end-plate increasing too, which drives to a nervous impulse magnification from end-plates to muscular fibres. This results in a magnified but wrong setting of postures, when they usually set according to their brain kinetic memory mirroring normal conditions. So postures can play at their excursion's limits, with reduced margins for recovery. In that state a provoked athletic trauma could be heavier, because the postures do not easily allow the balance to reset, being at work with poor compensating margins. In the same time the voluntary athletic gesture becomes magnified because of muscular fibres increased stimulation due to a larger amount of ACh into the end-plate. The loose of synchronism between postures working at their limits and magnified athletic gesture can end to a spontaneous athletic trauma. In such a stress condition an up synthesis of brain glutamate from a larger supply of its precursor l-glutamine does really increase the risks of similar events. An excess creatine diet can rightly do so.

Effect of aspartate and asparagine supplementation on plasma lactate concentration of rats exercised above the anaerobic threshold

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Aspartate and asparagine are amino acids metabolized at the skeletal muscle which biochemical pathways involves the synthesis of oxaloacetate and the malate-aspartate shuttle. The aim of this paper was to evaluate the possible effect of aspartate and asparagine supplementation on plasma lactate concentration of rats swimming above the anaerobic threshold (AT) until exhaustion. Sixteen male Wistar rats weighing around 215 g were tested to determine the AT and then submitted to an acute swimming exercise (above the AT) until exhaustion. The test protocol consisted of a progressive overload attached to the rat's chest which increased relatively with the rat's body weight (starting at 4%). The supplementation started at the week after the test and took place for seven days. The supplemented group (S) ingested 150 mg/kg of body weight per day of each amino acid (by gavage) for seven days. The control group (C) received water (by gavage) throughout the same period. Our results show that aspartate and asparagine supplementation decreases the total lactate concentration (mmol/l) in the plasma from 11.29 ± 2.61 (C) group to 8.58 ± 1.92 (S) group ($p < 0.03$) and the time to exhaustion (minutes) 68.4 ± 25.4 (S) group and 41.1 ± 13.8 (C) group ($p < 0.02$), close to 60% difference.

Augmentation of branched-chain amino acid (BCAA) catabolism by exercise in rats: Glycogen-sparing effect of the dietary BCAA during exercise

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Effects of a diet supplemented with free BCAA (4.8 or 6.2%) on BCAA catabolism and glycogen metabolism were examined in rats. Rats were fed the BCAA diet or control diet for 4 weeks and a part of rats were subjected to exercise training using a treadmill during the experimental period. Feeding the BCAA diet increased serum BCAA concentrations and the activity of hepatic branched-chain α-keto acid dehydrogenase complex, the rate limiting enzyme in the catabolism of BCAA, suggesting that dietary BCAA promotes the BCAA catabolism. Although serum glucose concentration and the glycogen contents in liver and gastrocnemius muscle of rested rats were not significantly affected by feeding of the BCAA diet, those in rats moderately exercised for 30 min were 3–4-fold higher in rats fed the BCAA diet than in rats fed the control diet. The activities of pyruvate dehydrogenase complex in liver and gastrocnemius muscle after exercise showed reverse trends; the complex activities (especially in liver) tended to be less in the former than in the latter. The total amount of muscle glucose transporter IV measured by Western blotting was not altered by the BCAA diet. These results suggest that dietary BCAA spare a glycogen store in liver and skeletal muscle during exercise and that the decrease in pyruvate dehydrogenase complex activities in these tissues by dietary BCAA are involved in the mechanisms.

Synthesis of Amino Acids

Synthesis of optically active fluorinated analogues of glutamic acid and glutamine with potential biological activity

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In living cells, glutamine represents one of the main storage forms of nitrogen and is a major physiological source of ammonia for the biosynthesis of amino acids, amino sugars, purine and pyrimidine nucleotides and coenzymes.

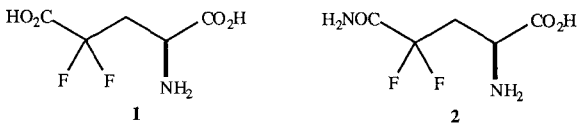
Glutamine-dependent amidotransferases perform nitrogen transfer from the amide group to various electrophiles. This migration results from the cleavage of the amide bond by an active site cysteine residue to give a covalent γ-glutamyl thioester and ammonia. Ammonia is then transferred to the electrophile and hydrolysis of the thioester gives glutamate and regenerates the catalytically competent cysteine.

Fluorinated analogues of glutamic acid and glutamine are expected to interfere with such biological processes due to the strong electron withdrawing effect of fluorine atom (without significant steric consequence), inducing modulation of binding and/or electronic properties. These compounds might therefore behave as reversible or irreversible active site-directed enzyme inhibitors.

Particularly, 4,4-difluoroglutamic acid **1** and 4,4-difluoroglutamine **2** could lead to anticancer agents resulting from the in-

hibition of purine and pyrimidine biosynthesis or to compounds active against pathogenic fungi by inhibition of cell wall edification.

First results in the synthesis of optically active derivatives of **1** and **2** from serine will be described.



1 **2**

Asymmetric synthesis of aminophosphonic acids

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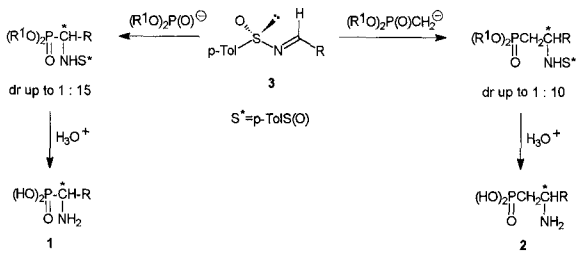
Aminophosphonic acids – phosphonic analogues of naturally occuring amino acids – are important class of compounds that exhibit a wide spectrum of biological activity. As the bioactivity of aminophosphonic acids strongly depends on the chirality at the stereogenic carbon atom, there is a continuous interest in the synthesis of optically active compounds. Herein, we disclose a new, highly efficient asymmetric synthesis of α- **1** and β-aminophosphonic acids **2** which is based on the stereoselective addition of dialkyl phosphite anions and dialkyl phosphonate carbanions to enantiopure sulfinimines **3** as chiral auxiliaries [1, 2].



1 **2**

Isolation of major diastereomers formed and deprotection of the amino function and phosphonate ester moiety afford enantiopure aminophosphonic acids **1** and **2** in high yields. The steric course of the asymmetric addition shown above is proposed.

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Synthesis and transformations of cyclic dehydroamino acid derivatives

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Dehydroamino acids are constituents of various important natural products that display an array of interesting biological activities: some of them are antibiotics or antibacterials (novobiocin, vancomycin, lantibiotics, etc.), antifungals, cytotoxins, calcium antagonists, etc. They are also synthetically challenging targets and intermediates for the preparation of optically pure amino acids. For these reasons their isolation from natural sources as well as their synthesis have attracted much attention [1, 2].

We have developed new methodologies for the preparation of a wider series of heterocyclic nonproteinogenic dehydro-α-amino acid derivatives containing a dehydroamino acid moiety designed in the cyclic form in various ways. In some cases, either amino or carboxy group is a part of the heterocyclic ring, or both the amino and the hydroxy groups are a part of the ring system. In this manner, 2*H*–pyran-2-ones and fused pyran-2-ones, fused pyridin-2(1*H*)-ones, oxazol-5(4*H*)-ones, 3-hydroxypyrazoles, fused pyrimidines and some other ring systems were prepared. We also investigated solution structure of some products. α,β-didehydroamino acid derivatives in the defined pure isomeric form can be prepared by various methods, though some of them result in *E*/*Z* mixtures of isomers. The synthesis of pure (*Z*)-isomers can be performed easier and with higher yields than the synthesis of their (*E*)-analogs, most probably due to the higher thermodynamic stability of the former. We introduced a new cheap and convenient method for the preparation of (*E*)-α,β-didehydroamino acid derivatives containing a pyrazolyl residue that might serve as substrates for the synthesis of new enantiomerically pure amino acids [3–8].

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Synthesis of various N-quinonyl amino acids and their transformation to six membered azlactones

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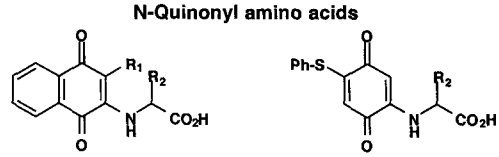
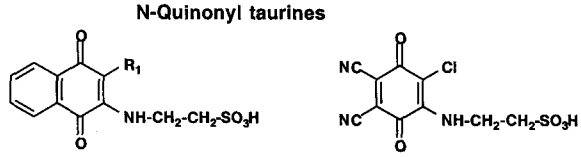
The chemistry and properties of N-quinonyl amino acids gained importance as being building blocks in the synthesis of biologically active cytotoxic peptides. A flora of N-quinonyl amino acids was prepared by the direct Michael-like reductive addition to the appropriate quinones or by substitution of a good leaving group. Various benzo- and naphthoquinones with different reduction potential were used. Natural α-amino acids including taurine (sulfonyl amino acid) were attached to the quinones.

N-Quinonyl amino acids

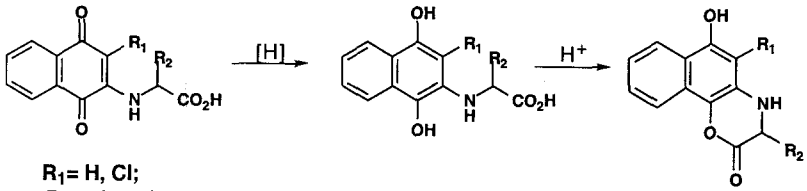


N-Quinonyl taurines



R_1= H, Cl;
R_2= side chains of natural amino acids

N-quinonyl amino acids were reduced to the corresponding hydroquinones which were easily cyclised in acidic conditions to give the six membered azlactones:



Preparative methods, as well as physical and spectral data (NMR, IR and ESR) of the new compounds, will be presented.

Stereoelectronic properties of α,β-dehydroamino acids incorporated in a peptide chain

M. A. Broda and **B. Rzeszotarska**

Department of Organic Chemistry, University of Opole, Poland

α,β-dehydroamino acid residues have been found to occur naturally, e. g. in a class of antibiotics known as lantibiotics and in other peptides of microbial and fungal origin. Their properties turn out to be special. Viz. the double bond in the α,β position provides conformational constraint to the peptide backbone and to the side-chain orientation at the same time. Therefore α,β-dehydroamino acids proved useful peptide modifiers. However, the stereoelectronic properties of α,β-dehydroamino acids still remain insufficiently recognized. Based on FTIR spectra in the region of ν_s(N-H), AI, AII and $\nu_s(C^{\alpha}=C^{\beta})$ modes and on *ab initio* B3LYP/6-31G* calculations, we studied the solution conformational preferences and the amides' electron density perturbation of the series of Ac-ΔAA-NHMe, where ΔAA=ΔAla, *(E)*-ΔAbu, *(Z)*-ΔAbu, ΔVal in relation to the parallel series of saturated analogs.

Each of studied dehydroamides adopts C_5 structure, which in Ac-ΔAla-NHMe is fully extended, and accompanied by the strong C_5 hydrogen bond. Ac-*(E)*-ΔAbu-NHMe has in dichloromethane two conformers in equilibrium. One of them is almost fully extended and like Ac-ΔAla-NHMe is C_5 hydrogen-bonded. The other adopts a warped C_5 structure similar to that assumed by *(Z)*-dehydroamides. Ac-*(E)*-ΔAbu-NHMe shares then conformational features with both Ac-ΔAla-NHMe and *(Z)*-dehydroamides.

The experimental as well as theoretical evidence shows electron interaction between the double bond $C^{\alpha}=C^{\beta}$ and both flanking amide groups. This interaction for *N*-terminal amide group differs largely from that for *C*-terminal one. The *N*-terminal $C^1=O^1$ bond is noticeably shorter and the amide bond $C^1(O^1)$–N^1 is longer and the $\pi^*(C^1=O^1)$ antibonding orbital decreased in occupancy as compared with the corresponding bonds and occupancy in the saturated entities. This proves a lesser amidic resonance at the *N*-terminal amide group than that in the common amides. At the same time the delocalization of electrons from the N^1 lonepair into the $\pi^*(C^{\alpha}=C^{\beta})$ antibonding orbital occurs. The *C*-terminal $C^2=O^2$ bond is longer and the $\pi^*(C^2=O^2)$ antibonding orbital increased in occupancy in all unsaturated amides as compared with the saturated analogs. Moreover, the sum of the occupancies of $\pi^*(C^2=O^2)$ and N^2 lone pair is larger than that in saturated counterparts. This indicates the donation of π electron density by the double bond $C^{\alpha}=C^{\beta}$ and testifies the existence of cross-conjugation between this bond and both amide systems.

Quinone-amino acid conjugates bridged via an ureido or carbamato spacers

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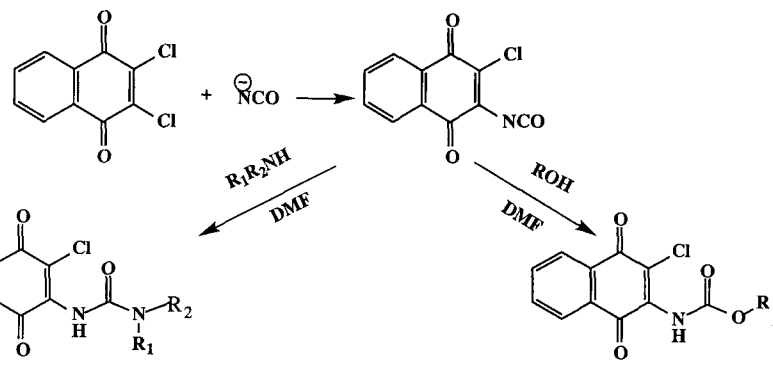
Quinones are widely distributed in nature and are produced by the chemical industry. The principle aspects of the application of quinones is their utilization as organic dyes, luminophors in colour photography, lasers, photochromic materials and scintillators. Quinones have very important biological roles and many find use in medicine. More and more types of quinones are needed for research and for applications, and we contributed the synthesis of ureideo- and carbamatoquinones.

We found that sodium isocyanate can react with 2,3-dichloro-1,4-naphthoquinone in DMF or DMSO to yield the quinonyl isocyanate. This isocyanate can now react either with alcohols and form carbamates or with amines to yield ureidoquinonyl derivatives. The quinone isocyanate can also react with amino acids and their esters or even peptides, thus yielding quinonyl amino acid conjugates bridged via an ureido or carbamato moieties. Such compounds may be important for further research in the synthesis of biologically active cytotoxic peptides.



R_1	C_2H_5	C_2H_5			
R_2	C_2H_5	C_6H_5			COOH
R_1	$(CH_2)_2SOOH$		4-EtCOOC$_4H_6$	CH_2COOEt	
R_2	H		H	H	

R	CH_3	C_2H_5	$CH(CH_3)_2$
R	C_4H_9	$CH_2C_6H_5$	cyclohexyl
R	$CH_2CH(NHt\text{-}BOC)COOH$		
R	$CH(CH_3)CH(NHt\text{-}BOC)COOH$		

Preparative methods, as well as physical and spectral data of the new modified quinones will be presented.

Enzymatic resolution of Fmoc- and Boc-protected amino acid racemates

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[1]Industrial Chemistry Research Institute, Warsaw, Poland

[2]Research Institute for Food Science, Kyoto University, Kyoto, Japan

Biological studies on synthetic peptides containing non-protein amino acid residues is the most explored fields in biology and medical sciences. The availability of non-protein amino acids often is the critical point in such approach. The stereoselective synthesis and resolution (enzymatic or chemical) of racemic amino acids are two methods, most commonly used for preparation of free amino acids. Nevertheless, N-protected amino acids are used in peptide synthesis. In order to obtain the N-protected amino acids used directly in peptide synthesis, we have developed a method of resolution of N-protected racemic amino acids by selective enzymatic hydrolysis of their esters. In our experiments Boc- and Fmoc-protecting groups were used as the mostly used in peptide synthesis.

Boc- and Fmoc-amino acids and their alkyl esters are very lipophylic. Therefore enzymatic reactions have to be carried out in water containing high concentration of organic solvents. We have optimized reaction conditions for hydrolysis of Fmoc- or Boc-amino acid methyl esters catalyzed with a-chymotripsin or trypsin in water/acetonitrile or water/DMF solutions. Enzymes can be successfully absorbed on solid support (silica gel) what create possibility to carry out this reaction as a continuous process.

[This work was supported by Industrial Chemistry Research Institute and the Polish Scientific Research Committee (Grant 3 T09B 04711).]

Synthesis of RGD analogues incorporating the salicyl-residue and their effect on human platelet aggregation *in vitro*

V. Magafa[1], **M. Liakopoulou-Kyriakides**[2], **Y. Sarigiannis**[3], **G. Stavropoulos**[3], and **Z. Sinakos**[4]

[1]Department of Pharmacy, [3]Department of Chemistry, University of Patras, [2]Department of Chemical Engineering and [4]Department of Medicine, University of Thessaloniki, Greece

Platelet aggregation is generally mediated by fibrinogen, an extracellular matrix protein, which is specifically bound to the platelet receptor glycoprotein IIb/IIIa. The RGD (Arg-Gly-Asp) sequence is an important component in the recognition of fibrinogen by the platelet receptor. Extensive research studies have revealed the property of RGD-containing peptides to inhibit platelet aggregation and thrombus formation by interfering with fibrinogen-GPIIb/IIIa [1, 2]. On the other hand, aspirin treated platelets failed to aggregate in response to arahidonic acid, indicating inhibition of platelet cyclooxygenase [3]. We have already reported that combination in the same molecule of dipeptide amides containing amino acid(s) of RGD sequence with salicylic-residue {o-RO-C_6H_5–CO~, where R=H or CH_3CO} at their N-terminal amino group have shown inhibitory activity on human platelet aggregation [4, 5]. With the present report we carry further this investigation by synthesizing a series of RGD analogues incorporating the salicyl-residue {o-RO-C_6H_5-CO-Arg-X-Asp(OR_1)-NH_2, where X=Gly or Leu and R_1=H, Me or Bzl}. The synthesis was carried out by conventional solution techniques and/or by solid phase. The synthesized salicyl-peptides were tested for inhibitory activity on human platelet aggregation *in vitro,* by adding the aggregation reagent (collagen or ADP) to citrated platelet rich plasma (PRP). Platelets were obtained from venous blood of healthy volunteers and the PRP was isolated by centrifugation at 200 g for 5 min at 37° C. The aggregation was determined using a dual channel electronic aggregometer by recording the increase of light transmission. Strong inhibition of platelet aggregation initiated with collagen presented the compounds with lack in the acetyl group from their salicyl-moiety and they proved more potent inhibitors than the corresponding compounds carrying this group. Comparing the side ester group of aspartyl-residue it was found that the methyl ester analogues are more active than those having the benzyl ester on their side chain. The IC_{50} values of the synthesized and tested compounds, as well as their MS, IR and NMR spectra will be discussed.

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Transformation of aromatic amino acids by reduction and ozonolysis. A novel approach to the synthesis of unnatural α-amino acids

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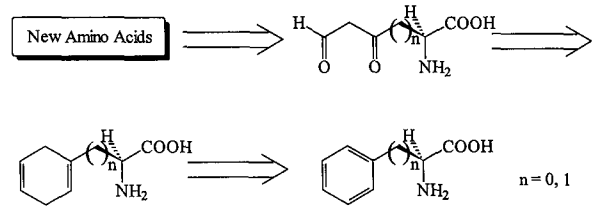
Many non-proteinogenic α-amino acids have been found to have important biological and pharmaceutical activities. A number of naturally occurring and synthetic non-proteinogenic heterocyclic amino acids were found to excite neurons in the mammalian central nervous system. Several other unnatural heterocyclic amino acids possess antibiotic activity or they are incorporated in the side chains of penicillins, cephalosporins and biologically active peptides. Pyrimidoblamic acid is an important constituent of *Bleomycins* which are used in cancer therapy. In pyrimidoblamic acid the amino acid amide is linked through an amino group to a substituted pyrimidine ring.

In this presentation a new methodology, by which heterocyclic α-amino acids can be synthesized, is described. It consists of using readily available aromatic amino acids by modifying their aromatic ring into an open chain polyfunctional intermediate. The basis for this manipulation is the Birch reduction of the aromatic rings, followed by ozonolysis of the cyclohexadiene system which is obtained. The scheme below shows the retrosynthetic perspective for obtaining new amino acids, from phenylalanine and phenylglycine, by this methodology. The preparation and ozonolysis of 1,4-cyclohexadienyl amino acids will be discussed. Approaches to the synthesis of pyrazolylalanine derivatives, isoxazolylalanine derivatives, pyrimidylalanine derivatives, and synthesis of heterocyclic derivatives of glycine will be presented.



Derivatives of glutamic acid as new surfactants

L. Rodehüser, H. Chaumette, A. Meyers, E. Rogalska, Ch. Gérardin, and **C. Selve**

Laboratoire de Chimie Physique Organique et Colloïdale, Université Henri Poincaré – Nancy I, Fac des Sciences, INCM, UMR 7565 CNRS, Vandoeuvre-lès-Nancy, France

Starting from glutamic acid, different types of surfactants have been synthesized by using original modular strategies. Monosubstituted zwitterionic amides of glutamic acid obtained with excellent yields show good surface activity. The grafting of a second hydrophobic side-chain leads to bicatenar cationic surfactants or to disubstituted nonionic cyclic compounds. In order to reduce the hydrophobic character of the bicatenar surfactants, a second synthetic method has been developed, allowing the introduction of a polar sugar group into these molecules. The surfactant properties of several of the products have been determined by physico-chemical methods such as surface tension measurements and compression isotherm studies by means of a Langmuir balance.

Simple and efficient synthesis of silylated monomers on the basis of amino acids for the preparation of grafted silica

F. Hamdoune, C. Gérardin-Charbonnier, L. Rodehüser, and **C. Selve**

Laboratoire de Chimie-Physique Organique et Colloïdale, UMR n° 7565, Faculté des Sciences-Université Henri Poincaré – Nancy I, Vandoeuvre-les-Nancy, France

The production of grafted silica has largely expanded during the last fifteen years due to the multitude of potential applications for this kind of new materials: chemistry, biology, nonlinear optics, environment, ... Our objective is not only to prepare but also to characterize modified silica, which can selectively complex bivalent or trivalent metallic ions such as Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+} or Cr^{3+}. For this purpose, we propose grafted silica of the structure ***I***. Reaction of aminopropyltriethoxysilane with amino acid or peptide like carnosine (β-alanine-histidine) used as chelating moiety allows to obtain silylated monomers which are copolymerised with tetraethoxysilane (TEOS) using a method previously described by Stöber et al.

This simple method is applicable to different structures of amino acids and peptides. Several preparations of modified silica have been realised and their characterization (RMN ^{13}C, ^{29}Si, size determination) is under study.

Optimization of the synthesis of cross-linked amino acids and a ^{13}C-NMR method for their diastereomeric characterization

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Dipartimento di Scienze Molecolari Agroalimentari, Università di Milano, Italy

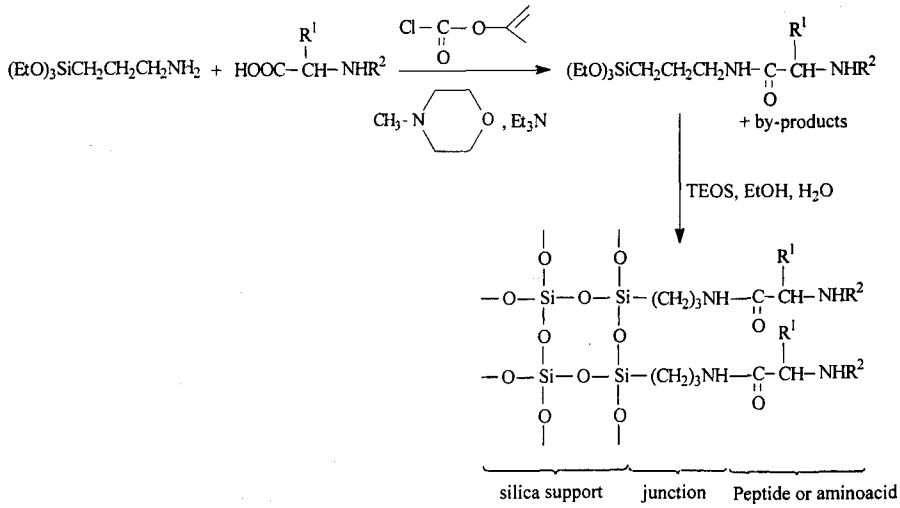
When foods are submitted to alkaline treatments and/or heating, some reactions can modify the structure of the protein chains. Besides the Maillard reaction, another important interaction is the formation of dehydroalanine, through elimination of good leaving groups from *O*-phosphoryl serine, *O*-glycosyl serine or cystine residues. Dehydroalanine can undergo a Michael addition by a nucleophilic group, such as ε-NH_2 of lysine, δ-NH_2 of ornithine or an imidazolic N of histidine. In the first case the product is lysinoalanine (LAL), the trivial name for N^{ε}-(*R,S*-2-amino-2-carboxyethyl)-*S*-lysine [1]. The most important property of these products is their stability in acidic conditions, that allows to recover them after protein hydrolysis.

Many papers have been published about the nutritional and toxicological consequences of the formation of LAL in foods. LAL provokes lesions in rat kidney cells (nephrocytomegaly), a discovery that is particularly important, because LAL can be detected in baby formulas and other baby foods or in enteral solutions.

LAL is a mixture of two diastereoisomers: *(S,S)*-LAL and *(S,R)*-LAL, because the configuration of lysine is preserved during the reaction, while the Michael addition is not stereospecific. We have, therefore, developed an analytical method based on ^{13}C-NMR to determine the stereoisomeric ratio of LAL without any derivatization [2].

Another possible cross-linked amino acid is ornithinoalanine (OAL, N^{δ}-(*R,S*-2-amino-carboxyethyl)-*S*-ornithine), that has been investigated much less, because it is not commercially available. We have developed a synthetic procedure for its production that, to our knowledge, is the best ever reported. The production of a large quantity of OAL will allow a systematic study of its toxicity, and the availability of a very pure standard will permit the determination of OAL in foods.

In order to improve our knowledge about other cross-linked amino acids, we have also synthesized histidinoalanine, usually



Structure I. Synthesis of sylilated monomer and structure of the modified silicia

called HAL. In this case, due to the presence of two nucleophilic N on imidazole ring of histidine, the Michael addition gives two isomers. High field ^{13}C-NMR is a good tool for the determination of the diastereoisomeric ratio for these compounds.

Finally, we have determined the amount of LAL in various kinds of foods, in particular baby formulas and solutions for enteral nutrition.

References

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[Study carried out with financial support from the Commission of the European Communities, Agriculture and Fisheries (FAIR) specific RTD program, contract CT96–1080. "Optimisation of the Maillard reaction in Foods. A way to improve food safety and quality."]

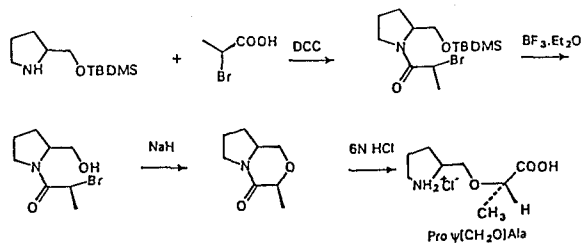
Synthesis and application of methylene-oxy and methylene-thio isosters in chemistry of amino acids and peptides

J. Hlaváček[1], **J. Mařík**[1], **B. Bennettová**[2], and **R. Tykva**[2]

[1]Institute of Organic Chemistry and Biochemistry, Academy of Sciences, Praha, and [2]Institute of Entomology, Academy of Sciences, České Budějovice, Czech Republic

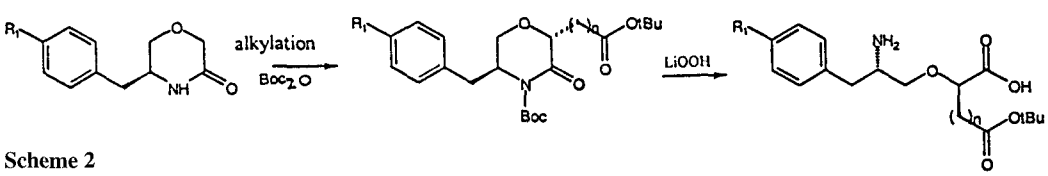
Isosteric replacement of peptide bond can lead to metabolically stable peptidomimetics. In our study on utilization of non coded amino acids in peptide chemistry the interest was focused to methylene-oxy (ψ[CH_2O]) and methylene-thio (ψ[CH_2S]) isosters of peptide bond which exhibit enhanced lipophility, flexibility and resistance to proteolytic enzymes; the [CH_2O] surrogate is moreover similar in its geometry to extended space structure of peptide bond. The changed spectrum of biological activities could be therefore expected in corresponding peptides containing such mimetic structures. We have used two alternative synthetic methods for the stereoselective preparation of the [CH_2O] containing pseudodipeptides:

1. The Proψ[CH_2O]Ala was synthesized by the modified Williamson etherification (Scheme 1). No racemization at C-2 was observed during the formation of the morpholinone ring. Acidic hydrolysis of the lactam followed by Boc protection afforded Boc-Proψ[CH_2O]Ala-OH.



Scheme 1

2. The Tyr(Phe)ψ[CH_2O]Asp(Glu) were prepared by synthetic route utilizing the 5-substituted morpholin-3-one ring which is formed by coupling of amino alcohols with 2-chloropropionic acid ethyl ester (Scheme 2). The key step of the synthesis is the alkylation of the (5S)-N-protected-5-substituted morpholin-3-one; this reaction provides only one diastereomer (2R,5S)-2,5-substituted morpholin-3-one. Protected 2,5-substituted morpholin-3-one is hydrolyzed using LiOOH in the last step to yield desired side-chain protected pseudodipeptides.



Scheme 2

The sulfur pseudodipeptide Boc-Proψ[CH_2S]Ala-OH was prepared by the alkylation of thiolactic acid disodium salt using O-mesyl derivative of Boc-prolinol in DMSO. The pseudodipeptide units were inserted into molecules of tetra- and pentapeptide analogs of the insect oostatic hormone H-Tyr-Asp-Proψ[CH_2O]Ala-OH, H-Tyr-Asp-Proψ[CH_2O]Ala-Pro-OH and H-Tyr(Phe)ψ[CH_2O]Asp(Glu)-Pro-Ala-OH which, compared to the corresponding tetra- and pentapeptides, exhibited an accelerated and enhanced oostatic effect in reproduction of the flesh fly *Sarcophaga bullata*. The sulfur containing pseudopeptide analogs H-Tyr-Asp-Proψ[CH_2S]Ala-OH and H-Tyr-Asp-Proψ[CH_2S]Ala-Pro-Oh were less active.

(This work was supported by grants of the Grant Agency of the Czech Republic, No. 203/98/1330 and No. 203/98/0754.)

Synthesis and biological evaluation of *p*-boronophenylalanine derivatives

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p-Boronophenylalanine (BPA) has been used in clinical level as a boron carrier for boron neutron capture therapy (BNCT) of human melanoma cancer, since BPA is a kind of phenylalanine and tyrosine analogue that are precursors of melanine biosynthesis. However, there is a particular need to develop new BPA analogues having the high water-solubility and selectivity of boron uptake by cancer cells. Therefore, two kinds of new amino acids, *p*-borono-β-hydroxytyrosine (BPS) and syn-*O*-(*o*-carboran-1-yl)-methyl-3-hydroxytyrosine (CMHT), were designed and synthesized by an aldol-type condensation of isocyanoacetate with boron-containing aldehyde. Furthermore, three amino alcohols, BPA-ol, BPS-ol and CMHT-ol, were prepared from the corresponding amino acid. We wish to report the synthesis of these boron compounds and a relationship between the molecular structures and their biological properties (water solubility, cytotoxity, and cellular uptake) toward human glioma and melanoma cells.

Azaamino acids and azapeptides; synthesis and application

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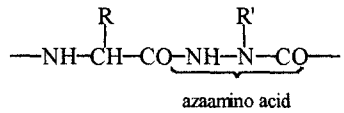
Azapeptides are peptide analogs in which the α-CH group of one or more amino acid residues in the peptide chain is replaced by a nitrogen atom.



The α-CH/N exchange leads to changes in local conformation of the modified peptide and also to the increased -NH- acidity. Those effects doubtlessly affect the biological action of the azaanalogs of the natural substances in the organism. That is the reason of the fact that azapeptides could be used as the inhibitors of the enzymes (e. g. the ACE and renin inhibitors) and active peptide analogs (azaanalogs of the oxytocin, LHRH) [Gante J (1989) Synthesis 405].

In last years in our laboratory we were interested in the synthesis of the new, active inhibitors of cysteine proteases. Azapeptides fulfil all requirements for inhibition of proteases, and therefore we have synthesized some potential inhibitors of cysteine proteases that contain the azaglycine, azaphenylalanine and azatyrosine residues (Agly, Aphe, Atyr, respectively) instead of the appropriate amino acid residues. The sequence of some of the azapeptides is based on the structure of the N-terminal segment of cystatin C – natural inhibitor of cysteine proteases. Those substances, having the azaglycine residue in a place of the expected hydrolysis, were incorporated into the peptide by means of the carbonyldiimidazole. The results of the initial assays of inhibition show the potentials of "azainhibitors". In our communication we will discuss some synthetic aspects of azaamino acids and azapeptides and their application in the peptide chemistry.

[Supported by Polish Scientific Research Committee (KBN).]

Protease-catalyzed kinetically controlled peptide synthesis using carbamoylmethyl esters

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Enzymatic peptide synthesis based on proteases has been recognized as an alternative or complement to chemical synthesis. The advantages of enzymatic methodologies are, among others, freedom from racemization, high regio- and stereoselectivity, and minimal side-chain protection. On the other hand, a narrow substrate specificity and the secondary hydrolysis of a growing peptide are counted as major disadvantages. In the α-chymotrypsin-catalyzed kinetically controlled approach in organic media with low water content, we found recently that the coupling efficiency was greatly improved by the use of activated esters such as the 2,2,2-trifluoroethyl ester instead of the conventional methyl ester [1]. Furthermore, the extremely low efficiency during the coupling of an inherently poor amino acid substrate, e. g., Ala which lacks a large hydrophobic side chain, using the methyl ester as acyl donor proved to be significantly improved using the carbamoylmethyl ester [2]. This was attributed to the ameliorating effects of this ester both on the binding of the substrate ester onto the enzyme and on the acyl-enzyme formation. The superiority of this ester over other activated esters was also demonstrated in the couplings of sterically demanding non-protein amino acids and also in a number of segment condensations: extremely high yields without racemization and the secondary hydrolysis. Moreover, this approach was successfully applied to the construction of the Leu-enkephalin sequence. These results mean that the above-mentioned disadvantages of protease-catalyzed peptide synthesis can be overcome by the use of this particular ester without impairing its advantages. The profound effect of the carbamoylmethyl ester was observed also in the couplings mediated by another serine protease subtilisin and the cysteine protease papain.

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A new protecting group for serine and threonine in peptide synthesis

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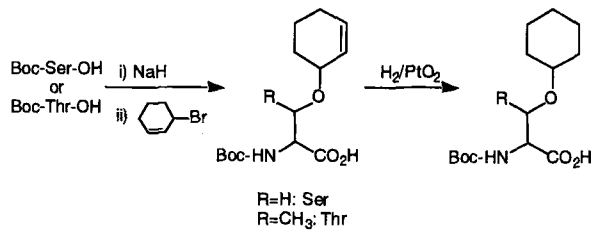
The cyclohexyl group has been developed as a new hydroxy-protecting group for Ser and Thr in peptide synthesis. The Chx group was introduced to the hydroxy functions of Ser and Thr in two steps from N^{α}-Boc-derivatives, as shown in the following scheme. The Chx ether has proved to be much more stable to TFA than the benzyl (Bzl) ether. The Chx group could be removed readily with 1 M TFMSA-thioanisole/TFA. Unlike Bzl, Chx was not removed by catalytic hydrogenation, and this enabled independent removal of the Bzl group in the presence of Chx and Boc groups. Applications of the Chx protection to solid-phase synthesis of an antimetastatic peptide, to solution-phase synthesis of some analogs from an anti-HIV-1 peptide, and to solid-phase synthesis of protected peptide segments for use in convergent solid-phase peptide synthesis were demonstrated. An improved synthesis of Boc-Tyr(Chx)-OH will be also reported.



Design of peptidomimetic inhibitors of the osteoclast-specific cysteine protease, cathepsin K

D. S. Yamashita, R. L. DesJarlais, H.-J. Oh, X. Dong, W. W. Smith, B. Zhao, C. A. Janson, T. A. Tomaszek, M. J. Bossard, D. G. Tew, T. J. Carr, S. K. Thompson, K. F. Erhard, R. C. Haltiwanger, M. S. McQueney, K. J. D'Alessio, B. R. Smith, K. L. Salyers, K. W. Ward, M. A. Levy, D. Zembryki, L. V. Rykaczewski, I. James, B. Lechowska, J. A. Vasko-Moser, G. B. Stroup, M. W. Lark, S. Abdel-Meguid, and **D. F. Veber**

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Reversible, selective, and potent inhibitors of the papain-related, osteoclast-specific cysteine protease, cathepsin K, were rationally designed by overlaying leupeptin (Ac-Leu-Leu-Arg-H) and Cbz-Leu-Leu-Leu-H as observed in inhibitor/papein co-crystal structures. The symmetrical 1,3-bis-(acylamino)-2-propanone, Cbz-Leu-$NHCH_2COCH_2NH$-(Cbz-Leu), had a $K_{i,app}$ of 22 nM against cathepsin K. It was highly selective with respect to the other members of the papain family ($K_{i,app}$papain, >10 μM, cathepsin L, 0.34 μM; cathepsin B,1.3 μM; cathepsin S, 0.89 μM) and was also selective relative to serine proteases ($K_{i,app}$ >50 μM, trypsin and chymotrypsin). Its bound conformation to cathepsin K was determined by x-ray crystallography. A solid-phase synthesis of unsymmetrical 1,3-bis-(acylamino)-2-propanones was also developed. Peptidomimetics of Cbz-leucinyl were incorporated into potent inhibitors and x-ray co-crystal structures of these inhibitors bound to cathepsin K were solved. The *in vitro* and *in vivo* antiresorptive activities of cathepsin K inhibitors were characterized.

The efficient conversion of α-amino acids to chiral cyclic amines and β-lactam

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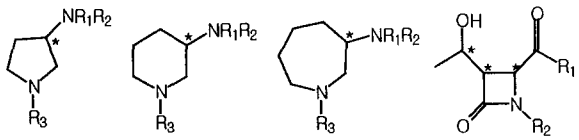
Cyclic amines such as 3-amino pyrrolidine, 3-amino piperidine and 2,3,4,5,6,7-hexahydro-1H-azepine and β-lactam such as (1-hydroxyethyl)-4-acyl-1-p-methoxyphenyl-2-azetidinone are pharmacologically active and constitute essential parts of molecular structures of some important drugs, e. g. antibacterials, antitumor agents and antiulcer agents. Therefore, it is of interest to develop methods by which enantiomerically pure cyclic amines and β-lactam could be readily formed with the desired absolute configuration.



1 2 3 4

In our research, it was envisioned that chiral cyclic amine derivatives might be obtained from amino acids. Enantiomerically pure cyclic amines such as 3-amino pyrrolidine **1,** 3-amino piperidine **2** and 2,3,4,5,6,7-hexahydro-1*H*-azepine **3** and β-lactam such as (1-hydroxyethyl)-4-acyl-1-p-methoxyphenyl-2-azetidinone **4** have been synthesized in high yields from the optically active natural α-amino acids such as L-aspartic acid, L-glutamic acid, L-2-aminoadipic acid, L-threonine and their enantiomers. This talk will discuss the synthesis of these chiral cyclic amine derivatives using by α-amino acids.

Study of the importance of [Pro^{B28}] on the self association of insulin semisynthesis of des[Pro^{B28}] insulin

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A new class of human insulin analogues has been developed and produced by biosynthetic and semisynthetic methods. These analogues are characterized by the replacements or deletion of a single amino acid residue close to the C-terminal of the B-chain. The analogues have been evaluated by measuring the in vitro biological potency in mouse fat cells, by osmometric determination of the association state in solution at neutral pH and the blood glucose lowering effect found after subcutaneous injection in pigs. The in vitro biological potencies relative to human insulin were found to be 218% for des[Phe^{B25}]-human insulin, 157% for des[Tyr^{B26}]-human insulin 168% for des[Thr^{B27}]-human insulin. In conclusion, human insulin analogues with an amino acid residue deleted close to the C-terminal of B-chain are new candidates for improved fast acting insulin injection preparation.

To study the importance of [Pro^{28}] on the self association of insulin, insulin des[Pro^{28}] was semisynthesized. To get free insulin, Zn-insulin was passing through gel column of G25/10% AcOH, which was splitted off by Trypsin to give des $B_{23\text{-}30}$-human insulin (DOI) and octapeptide $B_{23\text{-}30}$ insulin fragment. DOI was protected at the free amino groups by MSC-group.

Hepta peptide des Pro^{28}[$B_{23\text{-}30}$] Boc-Gly-Phe-Phe-Tyr(OBt)-Thr(OBt)-Lys(Tfa)-Thr(OBt) was synthesized by solid phase method, then enzymatically coupled to DOI-MSC to form insulin-des[Pro^{28}]. All processes of purification were tested and evaluated by HPLC.

Self association of the end product (insulin des[Pro^{28}]) is under investigation regarding fast acting, and biological potency.

Effect of amino acids on the biosynthesis and activity of some enzymes

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β-glucosidase which catalyses the hydrolysis of cellobiose to glucose was produced by the yeast *Kluyveromyces lactis.* When the nitrogen content of the growth medium (i. e. yeast extract) was replaced by each of 20 of different amino acids, only four amino acids namely arginine, lysine, phenylalanine and valine were correlated with moderate growth and higher enzyme formation, while a lower growth with an inhibition effect for the enzyme biosynthesis was noticed in the presence of other amino acids. When all or some of these four amino acids were mixed and added with different concentrations to the growth medium, β-glucosidase formation was quantitatively much higher reaching its maximum level in the presence of a mixture containing both arginine and lysine. On the other hand, *Streptomyces venezuelae* β-galactosidase which catalyses the hydrolysis of lactose to glucose and galactose was highly affected and correlated with sever inhibition when the nitrogen content of the growth medium (i. e. $NaNo_3$) was replaced by each of 20 amino acids. On the contrary, when each of these amino acids was added to the growth medium containing $NaNo_3$, the growth was generally decreased while β-galactosidase biosynthesis was highly increased in the presence of each of arginine, proline and phenylalanine.

Cold-adapted trypsin catalyzed peptide synthesis using inverse substrate

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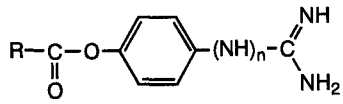
Protease-catalyzed peptide coupling reaction has some advantages compared with chemical synthesis. The reaction is stereoselective, racemization-free, and requires minimal side-chain protection. The serious defect of the enzymatic peptide synthesis, however, are the loss of the product due to hydrolysis by the protease, and the restricted substrate specificity.



1: n=0
2: n=1

Previously we reported that "inverse substrate", *p*-amidino-**1** and *p*-guanidinophenyl ester **2**, behave as specific substrates for trypsin. It is supposed that the acyl moieties are transferred to the enzyme's catalytic residue in the form of acyl trypsin intermediates during the course of the hydrolysis, and these intermediates are expected to be useful for trypsin-catalyzed peptide synthesis. It was found, in fact, that bovine and *Streptmyces griceus* (SG) trypsin catalyzed peptide condensation reaction by using inverse substrate was successful. We can expect that the trypsin from cold-adapted species is advantageous catalyst of the peptide synthesis, since the reaction can be performed at low temperature.

Chum salmon trypsin was purified from pyloric ceaca of chum salmon *(Oncorhynchus keta).* Atlantic cod trypsin was purchased from Sigma Chemical Co. Peptide coupling reaction was performed in analytical scale as follows. The reaction mixture of acyl donor (inverse substrates **1** or **2**, having Boc-amino acid as acyl moiety; 1 mM) and acyl acceptor (amino acid amide or *p*-nitroanilide; 20 mM) was incubated with trypsin (10 μM) in aqueous DMSO. The progress of the coupling reaction was monitored by HPLC. Both trypsins were effective for the condensation reaction, even at 0° C. The effects of acyl donor and acyl acceptor on the coupling reaction will be discussed.

N-6-Phenylacetyl-L-ornithine, its synthesis and utilization in the construction of a peptide chain

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The substitution of L-lysine by L-ornithine in a peptide chain leads to increased resistance of peptide to the hydrolytic action of

trypsin [1]. The substitution of ε amino group of lysine by a phenylacetyl group (PAC) made it possible to synthesize cyclic peptides, from which the PAC group could then be removed by penicillin amidohydrolase cleavage under mild conditions [2]. Using a similar approach, we designed the B^{23}-B^{30} sequence of human insulin, in which L-ornithine occupied position B^{29} instead of L-lysine. N-δ-PAC-L-ornithine was synthesized using a procedure that has been used earlier for the synthesis of N-δ-PAC-lysine [2], consisting in the formation of a cupric complex of L-ornithine (involving its α-amino group) followed by the substitution of the δ-amino group by phenylacetyl chloride. The cupric complex was decomposed by boiling it shortly in a solution of EDTA and the δ-amino group of ornithine was then substituted by a Boc group. The protected form of L-ornithine was characterized by MS and amino acid analysis. Protected ornithine was then used in the construction of the B^{23}-B^{30} sequence of insulin (for the substitution of lysine). The resulted octapeptide was used in the semisynthetic preparation of B^{29}-Orn human insulin. During the various phases of the procedure, we checked whether the PAC group could be removed from ornithine by means of penicillin amidohydrolase. Human insulin and B^{29}–Orn (PAC) human insulin were characterized by HPLC, MS and biological assays.

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(Supported in part by grants GA AV CR 75 543, GA CR 303/93/1012; 505/93/2140; 303/95/1247 and MPO CR PZ-Z2/32.)

Amino acids in cluster rhenium compounds as ligands: Synthesis, structure, some chemical and biological properties

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Biological activity of amino acids together with low toxicity and essential lability of rhenium substances arose an interest to study the process of complexation of binuclear clusters Re_2^{6+} with amino acids. We have obtained three types of clusters as result of such investigation. First were octachlorodirhenates where amino acids existed as intraspheric cations $(AH)_m[Re_2Cl_8]nH_2O$ (I) A=Gly, DL-Ala, L-Val, α-aminobutyric acid, GABA, L-Pro, DL-Lys; m = 1-2; n = 0-1. They were synthesized according to an original method with great yields from 75–95%. Due to high lability of chlorine ions during dissolving I in nitrometane solutions led to formation of compounds of another type cis-$[Re_2A_2Cl_4]Cl_2$ (II). In these clusters amino acids were coordinated to fragment Re_2^{6+} with carboxylic bridges in cis-positions according to Re-Re bond while amino groups left protonized and didn't take part in coordination. Products of thermal treatment (195–225° C) of I in inert atmosphere were compounds with structure trans-$[Re_2A_2Cl_4]Cl_2$ (III) which differed from II by steric position of amino acids ligands. All types of the complexes had different velocity of hydrolysis and cis-trans isomerization; some of them caused the morphological changes of red blood cells. Mechanisms of cell surface interactions with the complexes are speculated.

Arginine

Regulation of NO production and NO-mediated apoptosis by arginase isoforms

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Nitric oxide is synthesized from arginine by nitric oxide synthase (NOS), and citrulline which is generated can be recycled to arginine by argininosuccinate synthetase and lyase, forming a cycle termed "citrulline-NO cycle". We showed *in vivo* that this cycle is important in NO synthesis in activated macrophages. On the other hand, arginase may down-regulate NO synthesis by decreasing availability of arginine for the NOS reaction. We found that iNOS and liver-type arginase (arginase I) are coinduced by LPS in rat tissues and peritoneal macrophages. In addition to arginase I, a non-hepatic isoform (arginase II) is present. In contrast to rat macrophages, both arginases I and II were induced in activated mouse macrophages. When mice were injected intraperitoneally with LPS, iNOS and arginase II mRNAs were induced rapidly in the lung, whereas arginase I was induced more slowly. When mouse macrophage-like RAW 264.7 cells were treated with LPS and interferon-γ, iNOS was induced, NO production was increased, and apoptosis followed. On the other hand, when dexamethasone and dibutyryl cAMP were further added, arginase II as well as iNOS was induced, NO production was decreased, and apoptosis was prevented. Furthermore, the cells transfected with an arginase I and II expression plasmid were rescued from apoptosis. These results indicate that induced arginase competes with iNOS for arginine, suppresses NO production, and prevents NO-mediated apoptosis.

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The role of arginase in heat shock protein induction and nitric oxide regulation

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Arginase and nitric oxide (NO) synthase use L-arginine as their substrate to synthesize polyamines and NO, respectively. Polyamines are required for vascular smooth muscle (VSM) growth; however, NO is known to inhibit VSM growth. Although the upregulation of arginase during heat stress, wound healing and angiogenesis has been reported, the signaling mechanism for arginase regulation remains to be elucidated. Whether this upregulated arginase can modulate NO produc-

tion, via substrate competition, is also unclear. To address these issues, rat arterial smooth muscle cells in culture were subjected to heat (43° C) or chemical (sodium arsenite) stress for arginase induction. These interventions produced an increase in both arginase protein expression and activity by 2 folds. The increase in arginase activity was associated with an increase in heat-shock protein 70 (hsp70) expression. Forskolin and 8-Br-cAMP mimic the effect produced by heat and chemical stress. PKA inhibitors KT5720 and Rp-8-Br-cAMPS abolished the increases in arginase and hsp70, while serine-theroine and tyrosine kinase inhibitors had no effect on arginase and hsp70 induction. Furthermore, the increased arginase activity was not altered by PKC activators or inhibitors. It appears that overexpression of arginase and hsp70 occurred at protein translation level since cycloheximide, but not actinomycine D, abolished this process. Endotoxic stress increased NO production from VSM in an L-arginine dependent manner. Inhibition of arginase by L-norvaline lead to a further increase in NO production. In the presence of endotoxin, NO production from VSM treated with heat or chemical stress was significantly reduced; however, this reduction was reversed by arginase inhibitor L-norvaline. L-norvaline also abolished the hsp70 expression, indicating that hsp70 is the downstream metabolic product of arginase. These results suggested that arginase activation in VSM is mediated by the cAMP/PKA pathway. This activation can reduce NO production and increase hsp70 expression. Thus, arginase may play an important role not only in cell growth but also in the protection of cell from injury elicited by environmental stress.

Effect of oral administration of arginine on the short-term storage of memory in the elderly

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Introduction. Many reports suggest that nitrogen monoxide (NO) plays an important role in learning and storage. It is also reported that the concentrations of tissue NO and arginine decrease with aging. In the present study, we investigated the effect of oral administration of L-arginine on the short-term storage of memory (STM) in the patients with senile dementia.

Methods. The patients were 65–88 years old (mean: 83.3). Each of them suffered from some internal diseases and was hospitalized. We gave L-arginine with daily doses of 1.6 g for 3 months orally. Before and after the treatment, we evaluated the level of dementia with a method of HDS-R scale, and also measured the levels of superoxide dismutase (SOD), lipid peroxides and plasma amino acids.

Results. In all cases the scaling scores of dementia were improved, especially it was notable that the STM and the ability of calculation were improved. Moreover, all the patients showed very expressive faces and quick responses. Plasma levels of lipid peroxides decreased significantly after treatment. No remarkable side effects of arginine administration were observed.

Discussion. The possible mechanisms how STM was improved are as follows:

1. increase in concentrations of nitrogen monoxides as neurotransmitters
2. increase of brain blood flow by the increased nitrogen monoxides
3. reduction of oxidative stresses by antioxidative effect of arginine.

Conclusion. Arginine was effective for improvement of short-term storage (STM) in the elderly with dementia.

The effects of arginine administration on the amino acid levels and the acute cellular inflammatory response. Experimental study after the implantation of prosthetic material in the rat abdominal wall

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Aim. To investigate the fate of administered arginine (ARG) and the histological response up to 48 h after the implantation of polypropylene meshs in the rat.

Methods. Polypropylene material (Marlex^r) was inserted at the abdominal wall of 4 m rats. Rats (n = 48) were divided into Control (C) and Study (S) group. The S group received a continuous perfusion of Arg 1.8 g · Kg^{-1} · d^{-1}. The C group was perfused with an equal volume of 0.9% NaCl (19.201 · Kg^{-1} · d^{-1}). Rats were killed 6 h, 12 h, 24 h, or 48 h after surgery. Amino acids were quantified by HPLC (nmol/L; nmol/G). Nitrate/nitrite: ELISA (uM). Light microscopy and immunohistochemistry: Collagens (I/III) and lymphocytes (CD4/CD8). Kruskall-Wallis test.

Results. Arg levels increased in most of the tissues studied. Peak concentrations were reached at 6–12 h and were maintained until 48 h: Plasma (153 ± 6 vs 358 ± 20), tissue-prothesis interfaces (3.03 ± 0.44 vs 7.27 ± 1.60), liver (0 vs 0.456 ± 0.02), psoas (2.88 ± 0.74 vs 18.42 ± 3.21), $P < 0.05$. No increase was detected in the kidney. Ornithine (Orn) levels increased in all tissues studied (peak concentrations at 12–24 h, decreasing at 48 h), $P < 0.05$. Changes in citrulline levels were not significant. Arg perfusion also affected a number of amino acids in the plasma and tissues. No differences were detected in plasma nitrate/nitrite levels between the S and C group (e. g.: 48 h: S: 9.43 ± 1.8; C: 8.9 ± 2.0). 6 h after surgery polymorphonuclear leukocytes appeared around the mesh; at 24–48 h: macrophages, histocytes and lymphocytes could be quantified, but there were no differences between the C and the S group. 48 h after surgery, Type III collagen fibers were higher in the S group ($P < 0.05$).

Conclusions. 1. Higher values of Arg and Orn could be detected in the organs studied; 2. Arg metabolism through the arginase pathway seemed to predominate; 3. The prosthetic material elicited an acute-phase inflammatory cellular response (peak: 12–24 h); 4. Arg supplementation seemed to improve collagen synthesis in the wound space; 5. Also, longer periods of study and tensiometric measurements results will additionally be presented.

(FIS 97/1054. J. M. Ferrando is recipient of a grant from Bard®, Spain.)

Differential regulation of arginase genes in macrophage cells

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Depending upon cellular context, arginase can play a role in regulating substrate supply for the synthesis of many key metabolites, including urea, nitric oxide, polyamines, proline, glutamate, agmatine, and creatine. The precise roles of arginase will depend upon its patterns of tissue-specific expression, inducibility, and distribution of activity between the arginase isozymes. There are two arginase isozymes: type I (cytosolic) and type II (mitochondrial). As part of our approach to elucidate the metabolic roles of the arginases, we are investigating the regulation of arginase gene expression in the RAW 264.7 macrophage cell line. This line is capable of expressing both arginases and also the inducible isoform of nitric oxide synthase (iNOS). We have shown that type I arginase, type II arginase, and iNOS can be regulated indepen-

dently by a wide variety of inflammatory cytokines and other stimuli. Conditions have been identified in which either or both arginases are induced. In fact, the arginases are induced more promiscuously in this cell line than is iNOS. Interestingly, a key inducer of both arginases in the RAW cells is cAMP, either alone or in combination with other stimuli such as bacterial lipopolysaccharide. The noncoordinate regulation of the arginases indicates differential metabolic roles for the two isozymes. In order to more precisely elucidate these roles *in vivo,* a genetic "knockout" of type II arginase expression in mice has been created. Initial results obtained with the genetic "knockout" mice also will be presented.

Supplemental arginine on immune function in streptozotocin-induced diabetic rats

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Infection is a common problem in diabetes mellitus. The aim of the present study is to investigate the effect of supplemental L-arginine on diabetic rats for improving immune function. Streptozotocin (STZ) was used to induce diabetes in Wistar rats (100 mg/kg body wt., i.v.). The diabetic rats received either no supplement or supplementation of drinking water with 2%(w/v) L-arginine, or 2%(w/v) glycine. The control rats (injected with the vehicle) received no supplement. At eight weeks after the injection, rats were sacrificed and cervical lymph nodes were collected.

The injection of STZ resulted in 20% decreased body wt. ($p < 0.01$), 73% increased food intake ($p < 0.01$), 162% increased water intake ($p < 0.01$) and 70% increased urine secretion ($p < 0.01$). Blood glucose level also increased by about 90% in diabetes ($p < 0.01$). STZ-injection also resulted in significantly decreased ratio of lymph nodes wt. to body wt. (L/B ratio; by 45%, $p < 0.01$), decreased lymph nodes cellularity (by 44%, $p < 0.01$), and decreased total lymph node monocytes (TLM) number (by 76%, $p < 0.01$).

The supplementation of L-arginine to the diabetic rats increased the L/B ratio by 46% ($p < 0.01$), the cellularity by 45% ($p < 0.01$) and the TLM number by 113% ($p < 0.01$). The supplementation of glycine to the diabetic rats also increased the TLM number, but did not affect the cellularity. Furthermore, lymphocytes prepared from diabetic rats showed significantly lowered proliferative response to concanavalin A compared to cells from the controls, while arginine, but not glycine, significantly increased this response in diabetes to a level higher than that of the controls. Thus, the results of the present study suggest a beneficial effect of supplemental arginine to the immune function in diabetes.

Thermostability, structure-function relationships and patterns of DNA interactions of the arginine regulatory protein in thermophilic bacteria

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Hyperthermophilic procaryotes occupy the deepest branches on the phylogenetic tree and represent an important source in studying evolution of metabolic pathways and regulatory networks in microorganisms. Analysis of available genomic sequences from hyperthermophilic archaea and bacteria has not yet revealed a putative gene coding for a regulatory ArgR protein. However, in this study the *argR* gene from hyperthermophilic bacteria of the genus *Thermotoga* (*T. neapolitana* and *T. maritima* with a growth temperature 90° C) was cloned and found to govern its own expression (autoregulation) and perhaps a putative biosynthetic *argHCJBD* operon expression. The *T. neapolitana* ArgR exhibited an extreme intrinsic thermostability (>103° C) and tolerance against urea. Moreover, ArgR binding increased the target DNA melting temperature by approx. 15° C. Our *in vitro* data showed that at high temperatures DNA denaturation, rather than protein denaturation is atcrucial factor limiting the ArgR DNA-binding ability. Arginine decreased the affinity of the *T. neapolitana* ArgR protein toward its own *argRo* operator *in vitro,* whereas it acts as a co-repressor in other bacterial systems. Even if Arg boxes of the *T. neapolitana argRo* operator did not share a strong similarity with those of other bacteria, the *T. neapolitana* ArgR protein was able to bind the operator DNA of the moderate thermophile *Bacillus stearothermophilus*. Further comparison of structural and functional properties of thermophilic ArgRs with regard to different target DNAs showed that substitutions of conserved amino acids in α helices of the winged helix-turn-helix DNA-binding motif diminished their repression effect; some distinct leucine substitutions by other hydrophobic amino acids in the oligomerization domain caused either derepression or strong superrepression. Physiological behaviour of thermostable ArgRs were strengthened by circular dichroism and structure modelling analysis.

Our data underlined that thermophilic ArgR, in contrast to its mesophilic analogue, exhibits higher adaptibility towards homologous and heterologous target DNAs. We postulate its involvement in a global regulatory network in native thermophilic hosts.

Ornithine acetyltransferases involved in the acetyl cycle of arginine biosynthesis undergo an internal processing of the single precursor in thermophiles

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Arginine biosynthesis proceeds via acetylated intermediates from L-glutamate to L-ornithine. In many microorganisms the acetyl group is recycled by N^2-acetyl-L-ornithine: L-glutamate *N*-acetyltransferase (E.C. 2.3.1.35, ArgJ). The corresponding enzyme was comparatively studied in thermophilic procaryotes, in the archaeon *Methanocossus jannaschii* and in the eubacteria *Thermotoga neapolitana* and *Bacillus stearothermophilus*. The *argJ* genes were cloned in a system allowing a stable expression of the deleterious products in *Escherichia coli* hosts cells. The ArgJ proteins were proved to be composed of two non-identical subunits, issued from a single precussor gene product. The native molecular masses of the enzymes were determined close to 110 KDa. *N*-acetyltransferases were found processed at the same position between Ala and Thr as it was also found in *Saccharomyces cerevisiae* (Liu et al., 1995). The purified *B. stearothermophilus* and *T. neapolitana* ArgJ enzymes acetylate L-glutamate using as the acetyl-donor AcetylCoA and *N*-acetyl-L-ornithine and, therefore, are considered as bifunctional enzymes catalyzing both the first and the fifth steps in the acetyl cycle of arginine biosynthesis. In contrast, *M. jannaschii* ArgJ only catalyzed acetyl transfer from *N*-acetyl-L-ornithine to L-glutamate and is considered as a monofunctional enzyme. *N*-acetyltransferase activities towards *N*-acetyltornithine and L-glutamate were retained within pH 6–10 with the highest activity at pH 8.0 and at 75° C, 90° C or over 95° C respectively for *B. stearothermophilus, T. neapolitana* and *M. jannaschii* enzymes. Kinetics studies of the recombinant enzymes strongly suggested a ping-pong BiBi mechanism for the *N*-acetyltransferases studied.

Hydroxy-L-arginine inhibits recovery of rat lungs from hyperoxic injury

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Arginase expression is increased in rat lung during hyperoxic injury and recovery. Because arginase is co-induced with or-

nithine decarboxylase, an enzyme involved in tissue repair, we hypothesized that arginase induction during hyperoxic injury would contribute to repair of injured lung cells. Rats were exposed to 100% O_2 or room air (RA) for 60 hours followed by recovery in RA for 48 hours. Animals received 2 doses of either hydroxy-L-arginine (HOA, 1 mg per dose), a potent arginase inhibitor, or saline by intratracheal instillation at 0 and 24 hours of recovery. At 48 hours of recovery, lungs were harvested for wet-to-dry weight ratio (W/D) and inflation fixed for proliferating cell nuclear antigen (PCNA) staining. HOA inhibited lung arginase activity by 40%. In saline-treated lungs, 60 hours of hyperoxia increased W/D to 5.9 ± 0.1 which normalized after 48 hours of RA recovery (4.8 ± 0.1). HOA-treated lungs, however, still had increased W/D after 48 hours of recovery (5.1 ± 0.1, p = 0.02 vs. saline-treated). HOA-treated lungs also showed persistent inflammation and hemorrhage, and increased staining of PCNA in bronchial epithelium, alveolar macrophages and endothelium. HOA itself had no effects in RA rats. These results indicate that enhanced expression of arginase during hyperoxic lung injury may contribute to cessation of cell proliferation during recovery in RA.

Activity of arginase isoforms and NO production in 293NOS cells

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The mitochondrial isoform of arginase (AII), like the cytosolic AI, can regulate NO synthesis presumably by limiting L-arginine available to nitric oxide synthase (NOS). It is unclear, however, if AII exerts its effect exclusively in the mitochondria via its mature form. In this study, 293 cells expressing rat neuronal NOS gene (293NOS cells) were transfected with control expression vectors (pEGFP-C3), vectors expressing rat AI (pEGFP-AI) and AII (pEGFP-AII) genes or incubated with dibutyric cAMP (DBcAMP) for 24 hours. Nitrite in cell media was measured and cells harvested for immunoblotting, arginase activity and urea measurements. pEGFP-AI- and pEGFP-AII-transfected cells increased GFP-AI and GFP-AII expression by 9 and 6 fold and arginase activity by 10 and 3 fold, respectively. No increase in GFP-mitochondrial transporter fragment was noted on GFP-AII immunoblotting, indicating the AII remained as immature form. Nitrite production (nN/min) is shown below (*: P < 0.01 vs pEGFP-C3):

Expression vectors	L-arginine (mM) in cell media		
	0.0	0.1	1.0
pEGFP-C3	2.4 ± 0.3	2.8 ± 0.3	2.9 ± 0.6
pEGFP-AI	0.5 ± 0.2*	1.1 ± 0.2*	2.6 ± 0.5
pEGFP-AII	1.9 ± 0.5	2.9 ± 0.6	3.1 ± 0.6

Urea production increased in pEGFP-AI-transfected cells (0.2 ± 0.03 μmol/mg protein/hr), but not in pEGFP-C3- or pEGFP-AII-transfected cells. DBcAMP induced mature and immature AII, but dose-dependently inhibited nitrite production. These results indicate that the immature AII is inefficient in regulating NO synthesis and the AII effects may occur primarily in the mitochondria.

Genetics

Can quantum information be used to study life science such as gene, brain and consciousness?

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In theory of complexity, there are mainly two approaches, one of which is due to chaotic aspects of natural or nonnatural phenomena and the other is to ask the steps needed to work out a certain job like computation. The former is strongly related to a work finding a quantity measuring chaos of dynamical systems. The latter is recently focused on the research of computational complexity with computer.

There exist several mathematical tools to describe chaos such as (1) entropy and dynamical entropy, (2) Chaitin's complexity, (3) Lyapunov exponent, (4) fractal dimensions, (5) bifurcation, (6) ergodicity.

The author proposed Information Dynamics (ID for short) to synthesize the dynamics of state change and the complexity of a classical or quantum system in 1991, and it is applied to define new chaos degree, which have been used to study several different fields such as quantum physics, fractal theory, quantum information and genome sequences.

Here we review our former works on life sciences, in particular, the information genetics, and apply ID to study the structure and function of brain and consciousness.

Reconstruction of large-scale phylogenetic trees: Problems and solutions

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Thanks to the rapid progress of technology for the determination of DNA sequences and genome sequencing projects for various organisms, researchers can get a large amount of sequence data. The reconstrucution of phylogenetic trees from sequence data became a fundamental method for the study of molecular evolution, the classification of genes and the identification of organisms, etc. Some institutes start collecting the sequence data of such ubiquitous genes among organisms as rRNA and gyrase in order to find the origin of existing organisms or to study the diversity of life. To survey relationships of genes or organisms, researchers need to draw large-scale phylogenetic trees as possible as they can be based on a reliable method. The maximum likelihood method is a candidate because it is the most robust algorithms. However, it requires so huge computational power that it is impossible to be applied to the reconstruction of the large-scale phylogenetic tree even if with super computers. The shortage of the computational power is also a serious problem in other methods than the maximum likelihood. It is because the number of CPUs and size of memory are not infinite. What all we need is to design a new procedure which reduces data size in a step of computation;

1. To draw a small scale phylogenetic tree by a core set of population and expand the tree to the whole set;
2. To only use a partial region of sequences to reconstruct trees effectively after alignment.

In this paper, we introduce an efficient algorithm based on information theory to realize above two ideas. We also show the robustness and the quality of our method by using the computer simulation.

Analysis of the disease course for HIV by chaos degree

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There exist several different quantities to measure chaotic aspects of dynamical systems. A new measure has been introduced in [1], which is called the entropic chaos degree and it was successfully applied to some dynamics in physics [2]. Here we use this entropic chaos degree to analyze the variation of human immunodeficiency virus as we did in [3, 4]. That is, we calculate the entropic chaos degree of the dynamics reduced from the variation of V3 regions of HIV which are obtained from patients infected with HIV-1 at various times after seroconversion or infection. If the variations of genome have chaotic aspects, it can be considered that the state of the disease progression is characterized by the entropic chaos degree. As a result, the chaos degree for the dynamics changing V3 region shows the specific variation patterns throughout from primary infection to death after having AIDS. The variation patterns indicate that the entropic chaos degree is useful to infer patient's condition of disease progression.

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Oxidative stress and DNA-repair-deficiency, potential causes for neurodegeneration in Down syndrome?

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Oxidative stress in combination with a DNA-repair-deficiency results in severe DNA-damage. This failure has been proposed to be a trigger for the cell loss and for neurodegenerative changes like regional cortical atrophy in brain of patients with Down syndrome (DS, Trisomy 21) and Alzheimer's disease (AD). SOD, catalase, gluthatione- and thioredoxin-peroxidase are known to be important for cell defence against reactive oxygen species. Data about possible failures in the activity of these antioxidant systems in blood and brain of DS patients are controversial including the "gene dosage effect" by the SOD-activity. Some controversial data have been also obtained by measuring the products of lipid-peroxidation in the brain of these patients. DNA has been found to be damaged via fragmentation. The products of DNA oxidation, however, have not been found in the brain of DS patients and AD [1].

Thus, the molecular mechanisms of cell loss and neurodegenerative changes in the brain of DS and AD patients are still unclear. Using the "gene hunting" method we were looking for the deviations in the gene regulation in DS-fetal brain and consequently for the metabolic pathways involved in this pathology. We have found upregulated DNase I which fragments DNA, but X-ray-repair-cross-complementing gene (XRCC1) generally important for DNA repair has been found to be highly upregulated as well. Moreover, very important antiapoptotic factors (NFκB, ADF, NAIP, hsp70) have been found to be crucially downregulated in comparison to the control fetal brain. These results explain the monitored DNA-damage (fragmentation) in the brain of DS patients through the highly upregulated DNase I. DNA repair is highly activated in response to the DNA damage. Decreased activities of the important antiapoptotic factors could be a cause for increased apoptosis of neurons with subsequent neurodegeneration.

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A stochastic model for the cooperative behaviour of biological systems

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The cooperativity in biological systems involves the concerted formation or rupture of many similar weak chemical bonds. A stochastic model for these transitions is proposed. This model involves only two parameters, the mean probability p and the coupling capacity Δp, but it offers a surprising wealth of different qualitative behaviours when the two parameters are varied. This model, originally devised to describe protein folding, can be applied to more general biological systems which undergo transitions following a sigmoidal curve and it seems to improve the qualitative description of these systems with respect to the more familiar deterministic models. The advantages of this stochastic model are the following: a) its simplicity, b) the fact that it depends only on two parameters, c) the relatively short simulation time required.

The cooperative systems are characterized by a sharp transition, indicating that a large variation of the *output variable* y takes place in a very small interval of the independent *state-variable* x. Significant examples of these systems are: i) the binding of four oxygen molecules to one hemoglobin molecule; ii) the thermal transition from *gel* to *sol* phase of artificial and natural membranes; iii) the unfolding of macromolecules like DNA, proteins.

All these phenomena are empirically described by a *sigmoidal curve*:

$$y = \frac{x^{\alpha}}{1 + x^{\alpha}} \tag{1.1}$$

where α is related to the *cooperativity* of the change of y. When $\alpha = 1$, the transition is non-cooperative, while for α *large* the transition tend to become *all or none* and the total change in y takes place in a very narrow interval of x.

The mean probability p, is related to $exp(-\Delta G/RT)$, (ΔG represents the mean activation free energy for the formation of a chemical bond, T is the absolute temperature and R is the Rydberg constant) so it is controlled by macroscopic variables such as temperature, *pH*, etc.

The parameter Δp, which is related with the cooperativity of real biological transitions, measures the probability of forming a new weak bond and depends on the number of similar bonds already formed: the higher the number of bonds already formed, the greater the probability that additional bonds can be formed, and vice versa. Δp can be calculated using the fact that it is relat-

ed to the steepness α, i. e. the slope of the tangent line to the sigmoidal curve at the middle point of the transition. Experimental data concerning sigmoidal curves relative to cooperative transitions in these systems have been compared with our stochastic model showing that it provides a good approximation for them. This allowed to estimate the cooperativity parameter Δp.

A method of sequential analysis by 2D-pattern formation with coloration

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The work is motivated by the demand for efficient analysis of a very long sequential data such as a DNA sequence consisting of 4 nucleotides and a protein sequence consisting of 20 amino acids. Characteristics to be searched in such a sequential data:

1. (local periodicity) Periodicity of letters observed in a relatively short segment in the long sequential data.
2. (tandem repeat) Periodic appearance of a small block of letters (both direct repeat $\rightarrow \rightarrow \rightarrow \rightarrow$ and inverted repeat $\rightarrow \leftarrow \rightarrow \leftarrow$).
3. (long period) Local periodicity with a rather long period (more than 100).
4. (distribution of particular letters) Relative frequency of observed letters and its uneven distribution in the sequential data.
5. (randomness) Randomness of the appearance of observed letters.
6. (global structure) Beyond numeric characteristics though not yet well formulated.

These are clues for (i) distinguishing particular regions in the sequence; (ii) searching duplicated regions; (iii) searching regions which are expected to play a particular role; (iv) similarity or dissimilarity among sequential data.

In this talk we shall propose a new method (patent pending in Japan 1997; in USA and Europe 1998) for searching the above mentioned characteristics. Our method consists of two steps: rearranging the sequential data into a 2-dimensional table and assigning a particular colour to each letter. Then some characteristic properties can be visualized with certain patterns to be seen by eyes, for example, the difference of coding and non-coding regions of DNA sequences, and tandem repeat sequences with different repeat length in DNA sequences. The density of colour spectrum gives a distribution of particular letters and some hierarchy of randomness is observed.

Our method possesses flexibility from some technical aspects. In particular, the way of assignment of colours is important in order to visualize a particular pattern and should be improved. On the other hand, as a future direction the mathematical theory of "global structure" will be interesting to discuss and might give a new aspect to the analysis of sequential data appearing in various research fields.

On multiple alignment of genome sequences by quantum algorithm

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Alignment is the most important operation in comparing the amino acid sequences of biological species. The computational complexity for the alignment when dynamic programming is used is $O(L^N)$, when L is the length of the sequence and N is the number of sequences; it is tremendous when N is increased. In [1, 2] we apply the simulated annealing to the multiple alignment. Here we report a new algorithm for the multiple alignment by means of quantum algorithm [3].

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A hypothetic physical mechanism for the folding of protein structural classes

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Although the number of protein sequences is extremely large, the number of their folding patterns is quite limited. Actually, owing to the very high degenerate nature of the sequence-structure relationship, proteins are generally folded into one of only a few structural classes that are closely correlated with the amino acid composition. This suggests that the interaction among the components of amino acid composition might play a considerable role in determining the structural class of a protein. To quantitatively test such a hypothesis, three potential functions were formulated that respectively represent the 0^{th}-order, 1^{st}-order, and 2^{nd}-order approximations for the interaction among the components of the amino acid composition in a protein. It was observed that the correct rates in recognizing protein structural classes by the 2^{nd}-order potential are significantly higher than those by the 0^{th}-order and the 1^{st}-order potentials, indicating that an algorithm that can more completely incorporate the interaction contributions will yield better recognition quality, further demonstrating that the interaction among the components of amino acid composition is an important driving force in determining the structural class of a protein during the sequence folding process.

Gene structure of *S*-adenosylmethionine decarboxylase and its localization on mouse chromosome

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Gene structure of mouse *S*-adenosylmethionine decarboxylase (AdoMetDC) has been determined. The mouse genome have three AdoMetDC genes (AMD1, AMD2 and AMD3). The AMD1 gene consisted of 8 exons and 7 introns, similar to the gene of rat AdoMetDC, and it was mapped to chromosome 10. The AMD2 gene was intronless gene that was previously reported and the AMD3 gene was pseudogene. AdoMetDC encoded by the intronless AMD2 gene had two amino acid replacements (Met to Ile at codon 70 and Ala to Val at codon 139), compared with the protein encoded by the AMD1 gene, and exhibited decreased cytalytic activity and decreased processing activity when expressed in AdoMetDC-deficient *E. coli*. When Ile-70 of the protein encoded by the AMD2 was converted into Met-70, both the catalytic and processing activities recovered markedly. The strength of the promoters of AMD1 and AMD2 gene was measured using the reporter assay in various cell lines. In all cell lines, the AMD1 promoter was much stronger than the AMD2

promoter. The region of the AMD1 promoter for the maximal transcriptional activity was located in 400 nucleotides upstream the transcriptional initiation point. In this region, TATA box and two GC box were included. The upstream open reading frame in the 5'-untranslated region of AdoMetDC mRNA functioned as a negative regulatory element as reported previously, and the accumulated polyamines strengthened the negative effect.

Exogenous nucleotides vs. *de novo* synthesized nucleotides: Incorporation in RNA from Caco-2 cells

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Background: Dietary nucleotides have been suggested to have beneficial effects on the development and repair of the gastrointestinal tract. Tissues with a rapid turnover, such as the gut and the immune system cells, may utilise preformed nucleotides (coming from the diet), in situations in which there is a high demand of nucleotides for nucleic acid synthesis. Therefore, nucleotides could be considered as conditionally essential nutrients.

Aim of the work: (1) To establish the fractional synthesis rate of RNA purine nucleotides in Caco-2 cells, grown in culture medium containing different concentrations of glutamine, in the presence or absence of added nucleotides and (2) to determine if the presence of nucleotides could partly replace the glutamine dependence of Caco-2 cells proliferation.

Results: Fractional synthesis rate (%/d) of RNA-bound purine nucleosides (guanosine and adenosine) in Caco-2 cells, grown in cultures containing different concentrations of glutamine (0, 0.5, 1 or 2 mmol/L) with nucleotides (NT) added or not (10 mg/L each: AMP, CMP, GMP and UMP). Cells were stimulated or not by the addition of interleukin-1β to the culture medium.

	Guanosine		
Group	IL 1β–	IL 1β+	t test (p)
Gln 0 mM, NT–	21.9 ± 1.5	21.2 ± 0.2	0.41
Gln 0 mM, NT+	11.6 ± 3.0	17.6 ± 0.3	0.11
Gln 0.5 mM, NT–	33.5 ± 3.5	34.9 ± 4.2	0.41
Gln 0.5 mM, NT+	26.9 ± 2.5	32.2 ± 1.8	0.11
Gln 1 mM, NT–	38.4 ± 4.0	36.2 ± 0.6	0.36
Gln 1 mM, NT+	28.9 ± 3.8	25.4 ± 1.2	0.28
Gln 2 mM, NT–	40.0 ± 3.6	40.1 ± 5.4	0.49
Gln 2 mM, NT+	27.6 ± 4.0	36.8 ± 4.5	0.10

	Adenosine		
Group	IL 1β–	IL 1β+	t test (p)
Gln 0 mM, NT–	24.9 ± 2.3	30.0 ± 0.1	0.14
Gln 0 mM, NT+	13.1 ± 2.9	26.6 ± 2.0	0.01
Gln 0.5 mM, NT–	34.5 ± 2.9	39.7 ± 2.0	0.15
Gln 0.5 mM, NT+	28.7 ± 1.7	37.1 ± 2.6	0.01
Gln 1 mM, NT–	40.3 ± 3.8	45.9 ± 3.7	0.19
Gln 1 mM, NT+	29.7 ± 4.2	35.7 ± 2.7	0.19
Gln 2 mM, NT–	45.6 ± 3.3	47.5 ± 4.8	0.38
Gln 2 mM, NT+	27.3 ± 3.8	38.9 ± 2.1	0.04

Conclusions: (1) The presence of nucleotides cannot replace the glutamine dependence of Caco-2-cell proliferation. The incorporation of exogenous purine nucleotides into RNA of Caco-2 cells is rather limited, and it becomes important when cells are stressed by glutamine deprivation. (2) Stress by addition of interleukin-1β resulted in the maintenance or the increase in the *de novo* synthesized RNA-purine nucleotides, even in the presence of nucleotides.

Functional expression of heterologous genes enconding key enzymes in phenylalanine biosynthetic pathway by *Brevibacterium flavum*

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Three heterologous genes, *aroG pheA* and *tyrB,* were obtained by PCR from desensitized mutants of *Escherichia coli* K12. In the phenylalanine biosynthetic pathway, *aroG* gene encodes 3-deoxy-D-arabino-heptulonate-7-phosphate (DAHP) synthase (DS) which catalyzes the important reaction from erythrose-4-phosphate (E-4-P) and phosphoenolpyruviate (PEP) to form DAHP, *pheA* gene encodes a bifunctional enzyme: chorismate mutase (CM)/prephenate dehydratase (PD) which catalyzes two key reactions from chorismate to phenylpyruvate, and *tyrB* gene encodes aromatic transaminase (TA) which catalyzes the last step reaction of phenylalanine synthesis. The heterologous genes were inserted into a shuttle plasmid pJL42, and introduced into the phenylalanine producers of *Brevibacterium flavum* 3721 by conjugation. The specific activities of relative enzymes DS, CM/PD and TA increased by 12.3, 2.3/5.6 and 1.5 folds in the engineering strains compared with that of the host strain, respectively. The functional expression of heterologous genes in the phenylalanine-producing strains brought about further improvement of the phenylalanine production. The yields of phenylalanine increased in varying degrees, when the strains were cultured in the medium containing the glucose and molasses in shaking flask broth at 30° C for 3 days. Six types of recombinant strain were constructed. The results are shown in Table 1.

Table 1

Recombinant strains	Heterologous genes	Yields of phenylalanine compared with that of the host strain
B. flavum 3721/pJLA	*phe*A	2.97
B. flavum 3721/pJLG	*aro*G	2.36
B. flavum 3721/pJLGA	*aro*G-*phe*A	3.93
B. flavum 3721/pJLGB	*aro*G-*tyr*B	1.19
B. flavum 3721/pJLAB	*phe*A-*tyr*B	1.23
B. flavum 3721/pJLGAB	*aro*G-*phe*A-*tyr*B	2.92
B. flavum 3721/pJL42 (Control)	–	1.00

DNA dumping in patients with low serum cobalamin

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Nuclear appendages consisting of blebs in the nuclear membrane which may either break loose on one end to form a stalk or be extruded into the cytoplasm as rings have been described in patients with low serum cobalamin (vitamin B12 deficiency), but the function of these appendages is unknown.

Eleven patients (ages – 58–88 years, mean – 77.4 years; 8 males and 3 females) with normal serum folate and low serum cobalamin levels (range – 13–202 pg/ml, mean – 138.6 pg/ml) were studied; the mean homocysteine was 44.8 micromoles/l with a range from 8.8 to 197 micromoles/l in nine patients tested; six had elevated levels and three had normal levels. Granulocytes from these patients as well as those from normal controls were isolated and prepared for routine electron microscopic study. Grids were then labeled using electron microscopic in situ DNA end-labeling techniques (ISEL) prior to examination; .8 nanometer gold particles were silver enhanced to 5–15 nanometers. Nuclear appendages were found in all patients ranging from 2.0% to 21.2% of all granulocytes examined. Labeled DNA (range – 2–30 granules) was seen in all of these appendages including the cytoplasmic rings.

DNA synthesis and repair has been studied extensively, but only scant attention has been given to the fate of DNA which is fragmented or beyond repair. Although it is not certain that the labeled DNA in these nuclear appendages represents DNA fragments, it has been shown that DNA fragmentation occurs in this condition when uracils are misincorporated into DNA and subsequent unrepaired excision of these uracils occurs. The production of nuclear appendages and subsequent cytoplasmic dumping may be an active mechanism for the removal of fragmented DNA from the nucleus.

Conformational studies of model peptides

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A relationship between amino acid sequence and the native structure of peptide and protein is still far from being fully understood. However, it has become possible to design and obtain some model polypeptides containing some structural elements (helixes, turns and sheets). Very recently, an enormous progress has been made in designing peptides and proteins containing even more complicated structural motifs like 2 or 4 interacting helixes [Sikorski A, Kolinski A, Skolnick J (1998) Biophys J 75/1: 92–105].

In our communication we present synthesis and conformational studies of model, helical peptides:

Dns-AAAAKAAAAKAAAAKA-D-Nal-NH_2

DNS-AKAAKAKAAKAKAAKA-D-Nal-NH_2

Tyr-GELEELLKKLKELLKGPRRGELEELLKKLKEL-LKGE-NH(9-methylantracene)

Tyr-GELEELLKKLKELLKG-NH_2

The peptides were prepared by SPPS (continuous flow, Fmoc methodology) and their homogeneities were assessed by HPLC, FABMS and amino acid analysis. To establish the conformation of the peptides we used circular dichroism (CD), infrared spectroscopy (IR) and fluorescence. We found that the helical structures of our compounds are sensitive to microenvironment used in investigations and are less sensitive to peptide concentration itself. Additionally, in our communication we will discuss parameters influencing conformations of our peptides.

(Supported by grant BW/8000-5-0275-9.)

Chimeric hepapitis B core particles as a carrier of foring peptides, problems of its self-assembley and possible ways of solving these problems

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Hepatitis B core antigen is one of the most promising protein carriers for exposure of foreign epitopes of different of human and animals pathogens. Chimeric HBcAg particles can be used as an effective artificial immunogens. We have constructed several vectors that allow to insert foreign epitopes into munodominant loop of HBcAg. The chimeric HBcAg bearing epitopes of FMDV, VEE, HBV, HCV and other viruses were obtained. Unfortunately, not all of chimeric proteins were able to be particulated. The chimeric HBcAg proteins with some insertions turned out water insoluble and lost an ability to form a VLP structure. The dependence of correct or incorrect folding of chimeric proteins on physical-chemical properties of insertions with the help of computer program <ProAnWin> was studied. The recommendations concerning the design of insertions with the purpose of obtaining a correct folding of chimeric HBcAg particles have been done. Some of these recommendations were practically realized (for example construction of mosaic particles, creation of substitution vector, using spacer and others).

Using random peptide libraries displayed on phage for searching peptides, mimicking epitopes of the protein E tick-born encephalitis virus

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Four phage peptide libraries, consisting of random amino acids inserted in major or minor coat protein (pIII or pVIII) of filamentous phage were screened with monoclonal antibodies (mAbs) E6B, 10H10 and 14D5 against major envelope protein E of the tick-borne encephalitis virus (TBEV). The epitopes of protein E, whose three-dimensional structure is known, recognized by mAbs E6B and 10H10 have been shown to be discontinuous. Positive phage clones selected to both mAbs had consensus sequence of peptides displayed on phage KCCY. To find those discontinuous epitopes we designed the computer program that is searching for similarities between selected peptides and protein E surface, whose three-dimensional structure is known. By means of this program amino acids that form discontinuous epitopes for mAbs: 6' and 10H10 were identified (T303, Y304, C307, K309, C338 and R73, C74, T76, C105, L107 accordingly). Two phage clones highly reactive with mAb 14D5 were selected from the library consisting of 9 random amino acids insertions into pVIII phage protein flanked by Cis. Their binding to the mAb could be competed for by TBEV, showing that these peptides bind to the antigen-binding site of the mAb and are antigenic mimic of natural antigen. The clones were used to immunize mice. The obtaining sera have specifically reacted with TBEV, suggesting that selected peptides are also immunogenic mimic.

Pharmacology

Interaction of pharmacologically active drugs with OCTN2, an organic cation carnitine transporter

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OCTN2 is a member of the organic cation transporter family and transports organic cations in the absence of Na^+. It also transports carnitine, a zwitterion, in the presence of Na^+. Since OCTN2 transports several xenobiotics and since the transporter is expressed ubiquitously in the body, OCTN2 may play a significant role in the pharmacodynamics of certain therapeutically active drugs. We investigated the interaction of β-lactam antibiotics with OCTN2, either by using human cell lines which express OCTN2 constitutively or by using cloned human and rat OCTN2s expressed heterologously in mammalian cells. The ability of these drugs to compete with OCTN2-mediated transport of carnitine and the organic cation tetraethylammonium (TEA) was assessed. Thirteen different β-lactam antibiotics (penicillins and cephalosporins) were tested. Three of them (cephaloridine, cefepime and cefoselis) interact with OCTN2 with appreciable affinity. A common feature in their structures is the presence of a quarternary nitrogen. Furthermore, all of them are zwitterions. These structural features are found in carnitine, the physiological substrate for OCTN2. Ceftazidime, which contains a quarternary nitrogen but is an anion, is not recognized by OCNT2. Kinetics of the interaction were investigated using cephaloridine. Cephaloridine inhibits OCTN2-mediated carnitine transport with an IC_{50} of 300 μM. The inhibition is competitive. The role of Na^+ in the interaction was studied by assessing the potency of cephaloridine to inhibit OCTN2-mediated TEA transport in the presence and absence of Na^+. The ability of cephaloridine to inhibit TEA transport is reduced significantly in the absence of Na^+, suggesting that Na^+ is needed for the interaction.

Conclusion: OCTN2 is likely to mediate Na^+-dependent active transport of certain cephalosporins and thus may play a significant role in the pharmacodynamics and disposition of these drugs in the body.

Novel composite lotion of medicinal plants extract for treatment of hair loss

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This investigation prescribes a new topical treatment for preventing hair loss and for stimulation and induction of hair growth.

A total of 68 volunteers suffering from hair loss (age 20–60 years) were included in this study. A new composite lotion was prepared by mixing the following components:

a) Polar solvent extract of medicinal plants formulation comprising: coriander, acrid lettuce, *Nigella sativa* and garden cress;

b) Polypeptide compound including keratin and keratin derivatives.

c) Methionine; in the ratio of 5 : 0.5 : 0.1 respectively.

The new lotion was continuously applied on the scalp skin every other day for a period of 4–6 months. The results are elucidated by colored photographs and video tape prior and after treatment with the new composite lotion and revealed cessation of hair loss while starting stimulation of inactive hair follicle and hair growth.

Effect of physical and pharmacological stimulation on branched-chain amino acids (BCAAs) in cardiac muscle of rats

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BCAAs (leucine, isoleucine and valine) have metabolic properties and some other not too well recognized biological features. Their metabolism occurs mainly in the sceletal muscle, rather than in the liver. They may exert specific regulatory effects on the rates of protein synthesis and degradation in the skeletal muscle. Some data exist that BCAAs influence neurotransmitters synthesis in the brain of mammalians and their behavior. The level of total FAAs and BCAAs after 4 hours swimming in the water, 4 hours hypoxia (686 hecto Pascals), IP injection of reserpine (3 mg/kg), isoproterenol (24 mg/kg) or caffeine (3 injections of 80 mg/kg, one hour apart) were essayed by gas-liquid chromatography in cytosol of rats' arterial and ventricular muscle tissue cardiomyocytes. It was shown that all above stimulators decreased of the total FFAs in the arterial and ventricular cardiomyocytes as compared to the control rats. The decrease of BCAAs after physical stimulation and caffeine expressed as a percentage value of the total FFAs was relative lower as compared to the control group (mainly in the arterial muscle). These results demonstrate that in the heart muscle BCAAs play the regulatory and metabolic roles rather than solely serving as an energy-producing substrate.

Effects of some non-proteinogenic amino acids on nociception in rats

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The number and structural types of protein and non-proteinogenic amino acids, and particularly their application in understanding fundamentally important biological questions, has increased over the past years.

This study was aimed to examine the analgesic activity of L-canavanine (Cav), L-canaline (Can), L-arginine (Arg), L-ornitine (Orn) and L-citrulline (Cit). The experiments were carried out on male Wistar rats. The changes in the mechanical nociceptive threshold of the rats were measured by the Randall-Selitto paw pressure test using an analgesimeter (Ugo Basile), tail flick and hote plate. The amino acids were administered intra cerebroventricularly (i.c.v.) at a dose of 20 μg/rat. The naloxone was administered intraperitoneally (i. p.) at a dose of 1 mg/kg, L-Cav – 30 μg/rat and D-Arg – 20 μg/rat. Experiments began 10 min after i.c.v. injection.

L-Cav, L-Cit, L-Arg, and L-Orn exerted an antinociceptive effect, whereas L-Can induced hyperalgesia in rats. According to antinociceptive activity the range order was L-Cav > L-Cit > L-Arg > L-Orn, L-Cav being the most potent.

The antinociceptive effect of L-Cav, L-Arg and L-Orn but not L-Cit is antagonized by naloxone (1 mg/kg i.p.).

The analgesic effect of the studied amino acids were reversed by L-Cav to hyperalgesia, while D-Arg applied 10 min before the testing of the amino acids slightly decreased its antinociceptive effect.

(This study was supported by Grant X-805 of the National Science Fund, Sofia, Bulgaria.)

Effects of thiazole-containing peptidomimetics on smooth-muscle contractile activity

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Amino acids, containing thiazole residues, have been synthesized and their effects on smooth muscles were studied in vitro on tissues with predominant adrenergic or cholinergic neurotransmission. Some acetaminothiazoles, structurally related to the newly-synthesized compounds, act as H_1-agonists, hence, the effects of the novel thiazole-containing analogue on smooth-muscle contractile activity were compared to those of histamine (HA). Isolated smooth-muscle preparations were used in the experiments: prostatic parts of rat vas deferens (RVD) or rabbit vas deferens (RabVD), proximal segment of guinea-pig ileum (GPI). Smooth-muscle contractions were evoked by low-frequency electrical stimulation (ES). HA exerted a biphasic effect (a slight stimulation followed by a strong inhibition) on the predominantly adrenergic contractions of RVD and RabVD in response to ES. The synthesized thiazole derivatives of proline [(Pro)Thz] and alanine [(Ala)Thz] only inhibited the ES-contractions. HA had also a biphasic effect on the ES-evoked cholinergic contractions of GPI. (Pro)Thz exerted a stimulating effect only while (Ala)Thz was without effect. The blocker of the histamine H_1-receptors diphenhydramine (DPH) inhibited the direct contractile action of (Pro)Thz on the GPI longitudinal layer. The canavanine (Cav)Thz derivative also evoked a bisphasic effect on the GPI contractions in response to ES: low concentrations slightly potentiated and high concentrations tended to inhibit the contractions. However, the new (Cav)Thz derivative only inhibited the ES-evoked neurogenic contractions, the effect being concentration-dependent. L-Leucine (Leu) and its thiazole-containing analogue (Leu)Thz had only a concentration-dependent inhibitory effect on the GPI contractions in response to ES. The results obtained reveal the modulatory action of the newly-synthesized amino acid derivatives on the smooth-muscle neurotransmission and might change the contractions in response to ES. A similarity in the effects of HA and some of the new compounds was observed but their mechanisms of interaction with smooth-muscle neurotransmission remains to be clarified by further investigations.

(This work has been supported by Grant X-805 and by Grand B-601 of the National Science Fund, Sofia, Bulgaria.)

Comparative studies in the analgesic effects between MIF-analogue and morphine in rats

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Melanocite-inhibiting factor (MIF-1) tripeptide (Pro-Leu-Gly-NH_2) was discovered in the hypothalamus and exhibits a range of behavioral and pharmacological effects after central and peripheral administration.

MIF-1 blocks the analgesic effects of the enkephalins and morphine in mouse tail-flick assay.

MIF-1 and synthesized analogue Pro-sLys-Gly-NH_2 were tested for analgesic activity in the rat using paw-pressure test.

MIF-1, Pro-sLys-Gly-NH_2, naloxone and morphine were administered intraperitoneally (i.p.) at a dose of 1 mg/kg.

The aim of the present study was to compare the analgesic effects of MIF-1, Pro-sLys-Gly-NH_2, naloxone and morphine. MIF-1 exerts weak analgesic effect, while a newly synthesized MIF-1 analogue had a significant analgesic effect in respect to MIF-1. Morphine exerts a stronger analgesic effect in respect to MIF-1 and Pro-sLys-Gly-NH_2. Naloxone antagonized the analgesic effect of morphine in rats. MIF-1 antagonized the analgesic effect of morphine too, while the newly synthesized MIF-1 analogue potentiated the analgesic effect of morphine.

The present results show that substitution of leucine in position **2** of MIF-1 molecule by non-protein amino acid sLys increased the pain-threshold in rats.

(This work has been supported by Grant X-805 of the National Science Fund, Sofia, Bulgaria.)

Mechanisms of interaction between kyotorphin and histaminergic system in nociception

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Histamine acts as neurotransmitter and neuromodulator in the CNS and according to recently obtained data affects nociceptive processes. Kyotorphin (Kyo) is an endogenous neuropeptide isolated from the bovine brain. Kyo elicits a naloxone (NAL)-reversible antinociception by enhancement of the [Met^5]-enkephalin release in the brain stem and spinal dorsal horn. Recent literature data give grounds to the idea that Kyo and HA might realize their effects on nociception by affecting a common pathway.

Therefore, our investigation was aimed at revealing whether H_3-receptors are involved in the mechanism of Kyo analgesic action. We studied in rats the nociceptive effects of Kyo, of FUB 94 (a precursor of the H_3-receptor agonist R-α-methyl histamine) and of their combination. The Randall-Selitto paw-pressure test was applied and an Ugo Basile analgesimeter was used with a cut-off value of 500 g for preventing paw damage.

In a dose of 5 mg/kg (i.p.) both Kyo and FUB 94 exerted a moderate antinociceptive activity. Administered together, Kyo + FUB 94 elicited an enhanced analgesic effect, as compared to the neuropeptide alone. NAL (1 mg/kg, i.p.) reversed the effect into hyperalgesia. Applied 60 min before Kyo + FUB 94, the inhibitor of the guanylate cyclase methylene blue (MB, 500 μg/rat, i.p.), reversed the analgesic action of the combination to a stronger hyperalgesia than that of NAL.

The present results suggest that (i) H_3receptors contribute to the modulation of the Kyo analgesic effect by the histaminergic system, and (ii) cyclic GMP might also play a role in the antinociceptive action of the neuropeptide.

(This study was supported by Grant B-601 of the National Science Fund, Sofia, Bulgaria.)

Role of the nitric oxide system in the nociceptive effect of the neuropeptide Tyr-MIF-1

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TYR-MIF-1 is a member of a family of structurally related peptides, endogenously found in humans. It is present in the bovine hypothalamus and parietal cortex and binds to specific non-opiate receptors and to the μ-receptors in the brain. This suggested that TYR-MIF-1 plays a role in nociception and it has been proved that it elicits a strong naloxone (NAL) reversible analgesia. Recent observations indicate that histamine (HA) could also modulate nociception. On the other hand, it is well known that nitric oxide (NO) is involved in various physiological and pathological processes in the CNS. Therefore, it is of particular interest to find out if NO plays a role in the HA and TYR-MIF-1 effects on nociception. The hypothesis was tested by studying the effects of methylene blue (MB), and inhibitor of the NO synthase, on the nociceptive action of TYR-MIF-1 and of

histaminergic agents: H_1-antagonist diphenhydramine (DPH), H_2-agonist dimaprit (DMP), H_2-blocker cimetidine (CIM) and FUB 94 (a precursor of the H_3-receptor agonist R-α-methyl histamine). The changes in rat nociception were examined by the Randall-Selitto paw-pressure (PP) and the tail-flick (TF) tests.

TYR-MIF-1 (1 mg/kg, i.p.) exerted a marked NAL-reversible antinociception in both tests used. Applied i.p., DPH (100 μg/kg), DMP (5 or 10 mg/kg), CIM (50 or 100 mg/kg) and FUB 94 (5 mg/kg) showed analgesic actions, varying at the different drugs. The combined injection of the histaminergic agents with TYR-MIF-1 elicited antinociceptive effects, usually stronger than those of the separate drugs alone. After NAL (1 mg/kg, i.p.) the analgesic effects of the combinations were decreased or reversed to hyperalgesia. MB (500 μg/rat, i.p.), applied 1 h before the co-administration of histaminergic drugs with the TYR-MIF-1, reversed their antinociceptive effects even stronger than NAL. The results obtained confirmed the hypothesis that the NO, being part of the cell second-messenger system, might be involved in the nociceptive action of the endogenous neuropeptide TAR-MIF-1 (and in its modulation by the histaminergic system?).

(This study was supported by Grant B-601 of the National Science Fund, Sofia, Bulgaria.)

The effect of L-canavanine on yeast *Saccharomyces cerevisiae*

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This study reviews our analysis of the effect of L-canavanine on yeast *Saccharomyces cerevisiae*. The presented results demonstrate that yeast cells are resistant to canavanine in a IPD-medium. This can be explained by the fact that the IPD-medium contains the whole set of amino acids. The L-canavanine added to it competes with the L-arginine for entering the cells and being included in proteins instead of arginine. The presence of arginine in a IPD medium reduces the lethal effect of canavanine while its absence in the SC-medium could be the most probable reason for the decrease of the endurance and the adaptive properties of the cells from the two strains. The anti-toxic effect of L-arginine against L-canavanine (Rosenthal et al., 1995) fully corresponds with our results.

As with the higher concentrations of canavanine in an SC-medium, the quantity of the total protein extracted from yeast increases, we think that this is due to coagulants of denaturated proteins whose enzyme hydrolysis rate is lower because of the poor function of the hydrolytic enzymes.

The results from the electronic microscope analysis give us reason to state that, in canavanine-treated cells, the permeability of the cell membrane changes. With the introduction of canavanine in them, canavanine-containing proteins are being synthesized. The ones incorporated higher quantities of canavanine form granules clusters of denaturated proteins which are accumulated in the cytoplasm next to the cell membrane. They are similar to the granules reported in *E. coli* cells, treated with 20 μg/ml of canavanine (Prouty et al., 1975); in *Chlamydomonas reinhardii* (McMahou et al., 1975); in cells from people suffering from the Heindley and thalassaemia (Schachtell et al., 1965).

(This study was supported by Grant X-805 of the National Science Fund, Sofia, Bulgaria.)

Synthesis and antibacterial activity *in vivo* of some new non-proteinogenic amino acids containing oxazole residue

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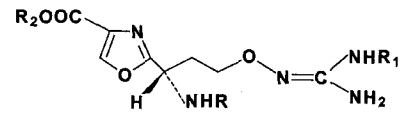
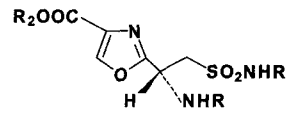
Optically-active natural and unnatural α-amino acids are important precursors for the synthesis of pharmaceuticals, agrochemical, and food ingredients. For example, oxazole-containing amino acids are a group of important modified amino acids which have been widely used in medicinal chemistry and biochemistry to change the conformation, restrict the flexibility and enhance the potency of molecules. Moreover, the synthesis of new non-protein amino acids and their incorporation into devours natural biologically active peptide might become a powerful method for the design of peptide and peptidomimetics therapeutics.

Following our current interest in synthetic application of unnatural amino acids we report now the synthesis of some new canavanines and S-cysteinesulfonamides containing oxazole residue **(1, 2).**



1 DL



2 DL

R = H, Boc, Fmoc, Z; R_1 = H, Boc, Fmoc, Tmob; R_2 = H, Et

For the synthesis of (Cav)Oxa and ($CySO_2NH_2$)Oxa suitable protected canavanine and S-cysteinesulfonamides amides were easily converted into iminoethers using Bergeron's procedure in high yields. The oxazoline derivative was obtained by the cyclization of the iminoethers and serine methyl ester followed by oxidation to yield oxazole **(1, 2)**. Resolution of the racemates was achieved by using alkaline protease from *Bacillus subtilis* DY strain.

Preliminary results showed that (Cav)Oxa and ($CySO_2NH_2$)Oxa derivatives exerted antibacterial activity *in vitro* against various gram-positive (*Staphylococcus aureas, Bacillus cereus* etc.), gram-negative microbial strains.

(This work has been supported by Grant X-619 of the National Science Fund, Sofia, Bulgaria.)

Is histamine involved in the Tyr-MIF-1 antinociceptive effect?

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Histaminergic system exerts neurotransmitter and neuromodulatory effects in the CNS, mediated via three receptor subtypes (H_1-, H_2- and H_3). It has been proved that both H_2-agonists and antagonists, applied i.c.v., exert antinociceptive effects. Recent studies have also shown that most of the H_2-antagonists (cimetidine, ranitidine, famotidine) potentiate non-opiate as well as opioid-induced analgesia while the H_2-blocker zolantadine abolishes the latter. Furthermore, it has been suggested that the opioid-receptors stimulation is followed by brain H_2-receptors activation. Hence it might be supposed that HA and neuropeptides might have a common pathway in their actions on nociception. Therefore, we investigated the effects of H_2-agonist (dimaprit – DMP) and antagonist (cimetidine – CIM) on the antinociceptive activity of Tyr-MIF-1, an endogenous peptide, isolated from bovine hypothalamus and

human parietal cortex. This neuropeptide can bind to the μ-opioid receptors as well as to specific non-opiate receptors in the brain, thus evoking a naloxone (NAL)-reversible analgesia. The experiments were carried out on male Wistar rats with the Randall-Selitto paw-pressure test, using an Ugo Basile analgesimeter and applying a cut-off value of 500 g to prevent paw damage. Tyr-MIF-1 (1 mg/kg, i. p.) exerted a strong NAL-reversible antinociception. CIM 50 mg/kg, i. p.) exerted a similar analgesic effect that was inhibited by NAL. However, an increase in the pain threshold after the combined application of CIM+Tyr-MIF-1 was not observed. DMP (5 and 10 mg/kg, i. p.) also elicited a marked dose-dependent antinociceptive effect that was increased by the combined administration of DMP+Tyr-MIF-1. This combination induced analgesia that was stronger than that of the neuropeptide alone. NAL and CIM exerted similar inhibitory effects on the DMP-induced analgesia. Our results suggest that *(i)* CIM and DMP exert analgesic effects that might be due to interactions with the opiate system (NAL abolishes their antinociceptive action); *(ii)* CIM-evoked antinociception is not related to its histaminergic H_2-activity; *(iii)* DMP analgesic effect is at least partly mediated via direct H_2-receptor activation (H_2-blockade antagonizes it) and contributes to the Tyr-MIF-1 opiate-mediated analgesia.

(This study was supported by Grant B-601 of the National Science Fund, Sofia, Bulgaria.)

Invited Lectures

Immunological evidence for hypochlorite/HOCl)-modified (lipo)proteins in human kidney disease

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An analogy between atherosclerosis and glomerulosclerosis suggested that factors that contribute to the development of atherosclerosis, i. e., oxidation of lipoproteins, may also participate in glomerular injury. Previous studies suggested that H_2O_2 can cause glomerular injury by reaction with halides in the presence of the neutrophil cationic enzyme myeloperoxidase (MPO) to form oxidants, e. g., hypochlorous acid (HOCl), which can oxidize and halogenate tissues. HOCl reacts with a wide range of biological target molecules including lipids, antioxidants, and proteins to form N-Cl-derivatives. The most sensitive amino acids prone to be modified by HOCl or the MPO-H_2O_2-Cl-system in vitro and in vivo are Cys, Met, Tyr, followed by Phe, Lys, His, and Arg. We have obtained several lines of evidence that the MPO-H_2O_2-Cl-system is functional in vivo. First, HOCl-modified proteins are recognized by specific mouse monoclonal antibodies (raised against HOCl-modified low density lipoproteins [HOCl-LDL]) that do not cross-react with native or other oxidative modifications of LDL or other proteins. Immunoreactive hypochlorite-modified epitopes were detected in renal tissues biopsies obtained from patients suffering from acute or chronic kidney disease by immunostaining and immunoblot analysis. Finally, HOCl-modified proteins are present in glomeruli of renal biopsies of patients with membranous glomerulonephritis. HOCl-modified LDL or albumin effectively competed for antibody binding to vascular and tubulointerstitial compartments of human kidney. Second, MPO, a sensitive marker for neutrophil activation was demonstrated in renal tissue biopsies by immunostaining in inflammatory cells which suggested excessive amounts of MPO present in human leukocytes. The 56 kDa MPO protein that was immunoreactive with rabbit polyclonal antibodies for MPO was present in detergent extracts of human kidney biopsies; the apparent molecular mass of the immunoreactive protein and MPO were indistinguishable by Western blotting. Third, immunohistochemical colocalization of HOCl-modified epitopes and MPO strengthen the assumption that the MPO-H_2O_2-Cl system participates in glomerular dysfunction in patients with renal disease.

Ozone-induced lung injury: role of protein oxidation

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Ozone is one of the most toxic air pollutants we are exposed to. As a "fair weather" pollutant, ozone formation requires a combination of anticyclonic weather with lots of sunlight, together with emissions of hydrocarbons and oxides of nitrogen. During spring and summer, in late morning and early afternoon, these interact to give ground-level ozone (as opposed to the ozone layer in the upper atmosphere). Background concentrations of ozone vary between 20–60 ppb depending on seasonal and geographical factors. The striking feature about ozone is that the background concentration is only a factor of 3 or 4 below that which gives rises to a range of symptoms including compromised lung function, inflammation and increased blood/gas permeability in susceptible individuals.

Despite the wealth of information regarding the impact of ozone on pulmonary function, little is known about its underlying mechanism of tissue injury. Ozone is a powerful oxidising agent and although not a free radical species itself, it is thought to mediate many of its toxic effects through free radical reactions. On inspiration, ozone first comes into contact with respiratory tract lining fluid (RTLF), which covers the cells of the respiratory tract. This layer of mucus and lining fluid forms the first line of defence against ozone. RTLF is rich in a number of small molecular weight antioxidants including glutathione, ascorbic acid, uric acid and vitamin E. Ozone preferentially reacts with these antioxidants before other targets, such as protein and lipid. In this way RTLF antioxidants help protect the underlying epithelial cells. However, protection is not always adequate and under certain circumstances (high ozone and/or low antioxidant levels) ozone does elicit reactions in the lung as described above. In these circumstances however, it is not ozone itself (because it is much too reactive to cross the compartment), but rather secondary and tertiary reaction products arising from its interactions with RTLF proteins or lipids that are responsible for the pulmonary responses.

The damage that ozone inflicts upon protein is the result of its ability to oxidise a range of functional groups, sulphydryls, amines, alcohol and aldehydes, either directly or through free-radical mediated reactions. Ozone is particularly reactive toward a limited number of amino acids including cysteine, methionine, tryptophan, phenylalanine histidine and tyrosine. Ozone reacts with each of these giving rise to a specific product such as dihydroxyphenylalanine in the case of tyrosine. Importantly how-

ever, the rate of amino acid oxidation varies widely between proteins. The actual rate of ozone-inducted oxidation appears to be dependent upon the size, amino composition and the secondary and tertiary structure of the protein. These differences, along with the fact that only certain amino residues are susceptible to ozone ensures a high degree of specificity in the reaction products generated following ozone inhalation.

The crucial role of amino acids and their polymers in our understanding of immunological phenomena, and the different roles that D-amino acids are playing

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Polymerization of N-carboxyamino acid anhydrides yields polyamino acids of a narrow molecular weight distribution. In contrast to a free amino acid which possesses the positive electrical charge of the ammonium ion and the negative charge of the carboxylate ion, polymers or proteins are composed of amino acid residues devoid of these charges, and this affects their solubilities: L- and D-alanine are water-soluble, but poly-L-alanine and poly-D-alanine are alpha-helices utterly insoluble in water. Poly-DL-alanine is not only water-soluble, but – when attached to a protein is a great solubilizer, e. g. to myosin. The realization that amino acid copolymers are good and specific synthetic antigens permitted the elucidation of the molecular basis of antigenicity, including the role of optical configuration. Poly-alpha-amino acids built exclusively of D-amino acids lead to antibody formation only at a relatively low concentration, otherwise they provoke immunological paralysis. The specificity of the immune response toward peptides containing D-amino acid residues is exquisite and often D-amino acids play a dominant role in defining the specificity. Polypeptides composed exclusively of D-amino acids are thymus-independent antigens. Nevertheless, it is possible to prepare against them highly specific T cell hybridomas. In future plans for synthetic vaccines against infectious or autoimmune diseases, the inclusion of D-amino acids may be an advantage in terms of both specificity and efficacy, the latter because of longer persistence in an undigested form as they resist enzymatic degradation.

Amino acid changes in cerebral ischemia: Their significance as markers for ischemic deterioration and recovery

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Recent results support the concept of the ischemic penumbra (defined as still viable but not functioning regions surrounding the core of an ischemic focus) as a dynamic process of impaired brain perfusion and metabolism and finally cell damage extending with time from the centre to neighbouring tissue.

Since the discovery of excessive ischemic accumulation of extracellular Glu and Asp by microdialysis, excitotoxicity has been discussed to play a major role in this process of deterioration through activation of NMDA and Non-NMDA receptor operated ion channels, in particular influx of Ca^{2+}, and possibly through induction of electrophysiological disturbances such as transient, spreading depression-like tissue depolarizations. The excitotoxic cascade may be augmented by a variety of factors, e. g. by ischemically elevated Gly and D-Ser through binding sites on the NMDA receptor. Hyperthermia has been shown to increase ischemic Glu accumulation dramatically. NO released after onset of ischemia has also recently been assumed to amplify acute excitotoxicity by facilitating Glu efflux. In experimental models of cerebral ischemia, a variety of medications that antagonize excitatory effects at post-synaptic receptors have been shown to ameliorate ischemic damage, but the time window for their effective use is limited, and they have serious side effects.

Microdialysis has not only revealed ischemic extracellular elevations of excitatory but also of inhibitory amino acids such as GABA or Tau. There is recent interest in medications that increase inhibitory responses in cerebral ischemia, particularly, because the used agents have less side-effects than Glu antagonists and seem to be effective for longer periods after onset of ischemia. GABAergic agents, similar to adenosine agonists, seem to have neuroprotective properties not only in grey, but also in white matter ischemia, and significant GABA elevations have been found in both grey and white matter compartments, the elevation in white matter being delayed and lasting longer. The ischemic increase of Tau has been discussed as a compensatory response to the release of Glu and to local edema formation. Studies on neuroprotective roles of Tau have yet to be accomplished. In the context of inhibitory mechanisms, the specific role of cerebral hypothermia for reduction of ischemic Glu release and of ischemic injury should be considered as well.

To date, however, expectations regarding the search for effective neuroprotective strategies for the treatment of acute stroke evolving from mechanistic studies on the role of excitatory or inhibitory amino acids are still limited. In parallel to this search, another perhaps more promising field emerges from the work on amino acids in cerebral ischemia: In recent studies, intracerebral microdialysis has been used to monitor metabolic disturbances in the brain of patients suffering from severe head injury or subarachnoid hemorrhage. Liberated excitatory amino acids in the extracellular fluid of the brain are most probably early indicators for ischemic and traumatic brain damage, moreover, their release was found to be related to the patients outcome. Perhaps in combination with amperometric techniques which improve time resolution, microdialytic monitoring of amino acids might therefore serve as an important tool even for intensive care patients encountered for example with malignant edema formation in the course of acute stroke and thrombolysis treatment.

Amino acids in the ventral medulla and spinal cord: Role in the control of blood pressure

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The organism defends itself against increases, or falls, in arterial blood pressure (BP) by modulating the activity of medullospinal sympathoexcitatory (SE) neurons in the ventral brainstem. The targets of SE neurons are sympathetic preganglionic neurons (SPN) controlling the heart, blood vessels and release of adrenaline and noradrenaline from the adrenal gland. SE neurons control both tonic and reflex control of blood pressure, since their inactivation results in large falls in blood pressure and loss of sympathetic baroreceptor reflexes.

The principal excitatory neurotransmitter synthesised by SE neurons is glutamate, although other compounds such as neuropeptide Y, adrenaline and serotonin are also present. The inhibitory amino acids GABA and glycine also play a critical role in BP regulation. Interneurons in the caudal medulla inhibit SE by releasing GABA that acts on GABA-A receptors causing a fall in SE activity, and a fall in blood pressure. These caudal inhibitory interneurons are activated by glutamate released from excitatory neurons in the nucleus tractus solitarius in response to increases in the activity of arterial baroreceptors that respond in turn to increased BP. SE neurons are also inhibited by glycine. A possible source of this inhibition are the inhibitory expiratory Bötzinger neurons in the ventral respiratory group. Bötzinger neurons lack the glutamate synthesising enzyme PAG but con-

tain message for the glycine transporter GLYT2. This may account for the expiratory inhibition seen in many SE neurons.

GABA also tonically inhibits SPN. Application of bicuculline (GABA-A antagonist) but not strychnine (glycine receptor antagonist), elevates arterial blood pressure. These local spinal inhibitory inputs to SPN are not important for reflex control of BP since the baroreflex is equally effective even in the presence of bicuculline. The physiological role of the spinal "non-baroreceptor" inputs remains to be determined.

Beneficial and adverse effects of methionine derivatives

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L-Methionine is an essential amino acid for humans and for animals. It needs to be present at optimal amounts in our food chain. A derivative of methionine, S-adenosylmethionine (SAM-e) was discovered in the 1950s, being formed from methionine and adenosine triphosphate (ATP) through the interaction of a specific enzyme. Since that time, there have been numerous studies in a number of fields to describe the interesting, and indeed the essential properties of SAM-e. One area is that of mood and depression in humans, that has been associated with lower prevailing blood levels of SAM-e, and where intake of supplements of SAM-e controlled the depression to some extent. It is possible to suggest that depression, in part, may originate with a progressive failure in humans to synthesize adequate levels of SAM-e. This chemical has also been found useful in lowering the risk of liver disease in chronic alcoholics. Furthermore, SAM-e is involved, through mechanisms yet to be defined in more detail, in the aging of cartilage and also, therefore, in bone health. It seems plausible that the biosynthesis of SAM-e declines with age, and may explain the problems in cartilage and bone structure, including arthritis. SAM-e, and relevant mammalian enzymes, acts as a key biochemical methylating agent, and through decarboxylation leads to functional amines and diamines, controlling cellular process. Therefore, this methionine derivative deserves much more research on its formation and mechanisms of action in a series of important diseases affecting humans.

In a distinct area, that related to etiological factors associated with stomach cancer, and perhaps head and neck cancer, it has been found that the intake of salted, pickled fish is associated with these types of cancers. We have discovered that analysis of such processed fish gave rise to powerful, direct-acting mutagens in the Ames test. We have identified one such mutagen by appropriate analytical techniques, including HPLC, MS and NMR spectrometry and found that this mutagen was 2-chloro-4-methylthiobutanoic acid (CMBA). Furthermore, we discovered that this mutagen was formed by the reaction of L-methionine with salt and nitrite at pH 3, the conditions under which fish is usually salted and pickled. This chemical, being a direct-acting mutagen may well be one of the unknown carcinogens associated with stomach cancer. In many parts of the world, where salting and pickling is no longer needed and used for food preservation purposes, with introduction of refrigerated storage for food preservation, cancer of the stomach has declined sharply. Our conclusion is that salted and pickled food should be consumed sparingly, especially since high blood pressure and stroke is also associated with this bad dietary habit.

In conclusion, one methionine derivative, SAM-e is an important promoter of good health, but the other one, CMBA, should be avoided.

Verleger: Springer-Verlag KG, Sachsenplatz 4–6, A-1201 Wien. – Herausgeber: Prof. Dr. Gert Lubec, Universitäts-Kinderklinik, Währinger Gürtel 18–20, A-1090 Wien. – Redaktion: Sachsenplatz 4–6, A-1201 Wien. – Hersteller: Satz- und Umbruch: Vogel Medien GmbH, A-2102 Bisamberg; Offsetdruck: Novographic, Ing. W. Schmid, Maurer-Lange-Gasse 64, A-1238 Wien. – Verlagsort: Wien – Herstellungsort: Wien. – Printed in Austria.

Offenlegung gemäß § 25 Abs. 1 bis 3 Mediengesetz: Unternehmensgegenstand: Verlag von wissenschaftlichen Büchern und Zeitschriften. An der Springer-Verlag KG ist beteiligt: Bertelsmann Fachinformation GmbH, Carl-Bertelsmann-Straße 270, D-33315 Gütersloh, als Kommanditist zu 74,04%. Geschäftsführer: Rudolf Siegle, Sachsenplatz 4–6, A-1201 Wien.