TRANSMEMBRANE SIGNALING

M. Bitensky, R. J. Collier, D. F. Steiner, Organizers

February 26 – March 3, 1978

Plenary Sessions

February 27, 1978:

Down Modulation of Receptors 99-100

Mitogens 100-102

February 28, 1978:

Metabolic Modifiers 102-104

Toxins Affecting Protein Synthesis 105-107

March 1, 1978:

Adenylate Cyclase 107-109

Cholera Toxin Action and Transport 109-111

March 2, 1978:

Processing, Storage and Release of Hormones 111-112

Nervous System Receptors 113

March 3, 1978:

Steroid and Thyroid Hormones 114-115

Poster Sessions

February 27, 1978:

Technology for the Identification of Cell Surface Receptors 116-121

Receptor Mutants: Cell Lines Altered in Ligand Interactions 122-124

Sialogangliosides as Cell Surface Receptors 124-126

February 28, 1978:

Receptors in Developing Systems 126-129

Cell Surface Receptor Display 130-136

March 1, 1978:

Transmembrane Protein Transport 136-139

Cyclic Nucleotide Directed Protein Phosphorylation 140-142

Ion Transport and Protein Phosphorylation 142

Calcium as A Cellular Effector 143

Inhibitors of Adenylate Cyclase 143-149

Down Modulation of Receptors

246 REGULATION OF ADENYLATE CYCLASE COUPLED BETA-ADRENERGIC RECEPTORS BY BETA-ADRENERGIC CATECHOLAMINES. Robert J. Lefkowitz, Lewis T. Williams and Lee E. Limbird. Departments of Medicine and Biochemistry, Duke University, Durham, N.C. 27710
Direct radioligand binding studies have been used to probe the molecular mechanisms whereby agonist catecholamines regulate the function of beta-adrenergic receptors in a model system, the frog erythrocyte. The unique characteristics of agonist as opposed to antagonist action are 1) the ability to stimulate the adenylate cyclase through the receptor and 2) the ability to desensitize the system by alterations induced in the beta-adrenergic receptors. These properties of agonists are not shared by antagonists, despite the high affinity and specificity of antagonists for the beta-adrenergic receptors. An important difference between agonist and antagonist binding is that agonist binding results in the formation of a slowly dissociable, high affinity, drug-receptor complex. This high affinity receptor state has been studied using the radiolabeled agonist [^{3}H]hydroxybenzylisoproterenol ([^{3}H]HBI), and cannot be formed by the binding of antagonist, e.g. [^{3}H]dihydroalprenolol ([^{3}H]DHA). The high affinity agonist-receptor complex can be dissociated by guanine nucleotides, and the order of potency of these nucleotides (Gpp)NHp> GTP> GDP> ATP = GMP) in promoting dissociation is identical to their order of potency in affecting adenylate cyclase. The agonist-receptor complex can also be distinugished from the antagonist-receptor complex by gel chromatography on AcA 34 Ultragel. Pretreatment of frog erythrocyte membranes with [^{3}H]HBI results in an increase in apparent beta-adrenergic receptor size. Binding of antagonist ([^{3}H]DHA or [^{125}I]iodohydroxybenzylpindolol) cannot mimic this change. Binding of [^{3}H]HBI agonist in the presence of the guanine nucleotide analog, Gpp(NH)p results in the elution of the radioligand in the region characteristic of the agonist-receptor complex as well as the region characteristic of the antagonist-receptor complex. The molecular events responsible for the increase in apparent beta-adrenergic receptor size have not yet been elucidated but it has been demonstrated that this increase in receptor size does not represent a physical coupling of the receptor with adenylate cyclase activity.

Formation of a high affinity receptor complex does not appear to be the sole mechanism by which agonists desensitize the catecholamine-sensitive adenylate cyclase system. Thus, when intact frog erythrocytes are desensitized with [^{3}H]HBI the subsequent loss in [^{3}H]DHA binding sites cannot be accounted for by residually bound [^{3}H]HBI. Also membranes from desensitized cells show a comparable loss in [^{3}H]HBI agonist binding and catecholamine-sensitive adenylate cyclase activity (∿70% decrease) but a less dramatic loss in [^{3}H]DHA antagonist binding sites (∿35-40% decrease). This suggests that in addition to alterations in the ligand binding site of the receptor during desensitization alterations in the effector or coupling portion of the receptor may occur as well.

247 MITOGEN RECEPTOR DEGRADATION AND MITOGENESIS: THE INTERNALIZATION HYPOTHESIS, Manjusri Das and C. Fred Fox, Department of Bacteriology, The Molecular Biology Institute, University of California, Los Angeles, California 90024

A maximal biological response to hormones including mitogens is often achieved at a concentration which is far below the optimal concentration required for binding to cell surface receptors. Epidermal growth factor (EGF), an extremely potent polypeptide mitogenic hormone, exerts its maximal growth stimulatory effects at a concentration which is less than one-fifth the concentration required for half maximal saturation for binding to receptor sites (1). In order to explain this deviation and to gain insight into the transduction mechanism relating hormone binding to bioactivity, we examined the physiological fate of receptor following binding of EGF.

An affinity labeling technique used previously for identification and specific radiolabeling of a membrane receptor for EGF (2) was exploited further to obtain murine 3T3 cells carrying an 'in situ' radiolabeled EGF receptor. Incubation of these radiolabeled mouse 3T3 cells at 37° resulted in a time dependent loss of radioactivity from the EGF-receptor covalent complex (M_r 190,000). The radioactivity lost could be quantitatively accounted for in three lower molecular weight bands (M_r 62,000, 47,000 and 37,000) which appeared during the degradative process. The crosslinked EGF-receptor complex (M_r 190,000) on intact cells was accessible to the action of trypsin at 4°. The degradation products were, however, inaccessible to trypsin and banded with the lysosomes upon subcellular fractionation. The kinetics of degradation of radiolabeled receptor were the same as those describing the rate of 'down regulation', i.e., the reduction of binding activity induced by EGF. An endocytic pathway for receptor removal implies that there will be a shift in the equilibrium of the binding reaction towards formation of additional hormone-receptor complex, so that even at a very low steady-state receptor occupancy, there will ultimately be a large reduction in the level of receptor on the cell surface. A study of the relationship between EGF induced 'down regulation' (or receptor degradation) and stimulation of DNA synthetic activity showed that both of these processes were half-maximally stimulated at the same low concentration of EGF (about 0.1 nM) which was insufficient to occupy even 10% of the total receptor sites. These data indicate that at concentrations of EGF, suboptimal for binding, but optimal for biological activity, there is a slow and continuous process of receptor internalization and degradation, and that this process may be responsible for the intracellular accumulation of an active substance essential for the proliferative response.

1) Carpenter, G. and Cohen, S. (1976) J. Cell. Biol. 71, 159.
2) Das. M., Miyakawa, T., Fox, C.F. Pruss, R.M., Aharonov, A. and Herschman, H.R. (1977) Proc. Natl. Acad. Sci. USA 74, 2790.

248 MODULATION OF LDL RECEPTOR ACTIVITY, Michael S. Brown, and Joseph L. Goldstein, Department of Molecular Genetics, University of Texas Health Science Center at Dallas, Dallas, Texas 75235.

When cultured mammalian cells are in need of cholesterol to support plasma membrane synthesis, they synthesize a cell surface receptor that binds low density lipoprotein (LDL) the major cholesterol transport protein in plasma. Binding of LDL to this receptor is the initial event in a process by which the cell internalizes the lipoprotein through adsorptive endocytosis, the lipoprotein is hydrolyzed within cellular lysosomes, and the cholesterol of the lipoprotein is made available for membrane synthesis within the cell. The LDL receptors are localized in discrete regions of the plasma membrane called "coated pits." Each coated pit internalizes to form an endocytic vesicle every 7 to 10 minutes. The number of LDL receptors is tightly regulated. Thus, when cells are in need of cholesterol as a result of prior growth in cholesterol - depleted medium, the cells synthesize large numbers of receptors (about 15,000 per cell). In contrast, when the cholesterol requirements of the cell are satisfied, the synthesis of the LDL receptor is suppressed. Under these conditions, the cells express fewer than 1500 receptors per cell. Although each receptor on the cell surface is internalized every 10 minutes, the half-time for decay of receptor activity in the presence of cycloheximide is on the order of 15 hours. This observation suggests that LDL receptors are reutilized. Recent studies on the mechanism of regulation of the synthesis of the LDL receptor will be discussed.

Mitogens

249 EFFECTOR-RECEPTOR RELATIONSHIPS IN MITOGENESIS. Harvey R. Herschman, Rebecca M. Pruss, Dennis S. Passovoy, and Aharon Aharonov. Laboratory of Nuclear Medicine, and Dept. of Biological Chemistry, Univ. of California, Los Angeles, Los Angeles, Calif. 90024

Mitogenic activity for individual murine embryo cell lines has been reported for cortisol, insulin, epidermal and fibroblast factors, prostaglandin $F_{2\alpha}$ and phorbol myristate acetate. In order to determine whether (i) all cell lines respond similarly to a phalanx of potential mitogens, and (ii) any of these agents are "universal mitogens", we have characterized the response of Swiss 3T3 and the two C3H derived cell lines, M2 and 10T1/2, to this group of mitogens. Each cell line demonstrated a unique response spectrum. EGF and FGF were active, albeit in varying degrees, on all four cell lines.

EGF-stimulated division of confluent Swiss 3T3 cells appears to be the most promising system for the molecular analysis of mitogen-induced proliferation of resting cells. The mitogen is easily purified in large quantities, and has been well characterized, including sequence determination. A sensitive radioreceptor assay has been developed in several laboratories, including our own, for the measurement and characterization of EGF receptors.

We have taken a genetic approach to the questions of growth factor induced mitogenesis, and developed a procedure for selecting mitogen specific non-proliferative variants of 3T3 cells [Pruss and Herschman, Proc. Nat. Acad. Sci. US 74 3918 (1977)]. Variants which do not respond to EGF but still retain a proliferative response to other mitogens have been isolated. All variants isolated are missing the EGF receptor. Alternative models for mitogen induced proliferation will be presented and discussed in the light of these data. The use of these variants in a variety of studies, including receptor specificity, surface alterations in mitogenesis and viral transformation will be described.

EGF administration to quiescent 3T3 cells leads to a rapid reduction of 60-80% of the cell surface EGF receptors. The time course and concentration dependence of this "down regulation" response will be discussed, along with an evaluation of the nature of the remaining receptors. What is the role of this rapid loss of EGF receptors when naive cells are exposed to EGF? Several experimental approaches to this problem will be discussed, suggesting that massive receptor loss is neither necessary nor sufficient for EGF induced mitogenesis.

Supported by Grant CA-15276 (NCI) and Contract EY-76-03-0012 from the Energy Research and Development Administration. A.A. is a Chaim Weizmann Fellow; R.M.P. was supported by training grants GM-0034 and T32-CA-09056.

250 REGULATION OF THE MAMMALIAN CELL CYCLE BY A PLATELET DERIVED GROWTH FACTOR, C.D. Scher, C.D. Stiles, H.N. Antoniades, and W.J. Pledger, Sidney Farber Cancer Institute, Harvard Medical School, and Center for Blood Research, Harvard School of Public Health, Boston, MA 02115

Serum can be fractionated into 2 sets of components: 1) a heat (100°) stable platelet derived growth factor (PDGF); and 2) platelet-poor plasma. We have used these components to stimulate the replication of quiescent Balb/c-3T3 cells for the purpose of analyzing the events of the cell cycle. PDGF is a polypeptide with a molecular weight of 13,000 and an isoelectric point of 9.7 (1). A radioimmunoassay demonstrates that serum contains 50 ng/ml of PDGF, while platelet-poor plasma has 10 ng/ml or less (2). Plasma does not stimulate the growth of Balb/c-3T3 cells, but does support the growth of cells transformed by either RNA or DNA tumor viruses. Although highly purified preparations of PDGF alone stimulate Balb/c-3T3 cells to synthesize DNA, plasma is required for optimal stimulation of DNA synthesis. Extracts of heat-treated human platelets function synergistically with plasma to induce DNA synthesis in quiescent cells. Cells exposed to platelet extracts become competent to enter the cell cycle, but the rate of entry into the S phase is a function of the concentration of plasma. The time required to induce competence is a function of the platelet extract concentration. The entire population becomes competent to enter the S phase after a 2 hr exposure to the platelet extract at 37°; whereas, at 4° or 25°, the cells do not become competent to synthesize DNA. The platelet extract induced competent state is stable for at least 13 hrs after removal of the platelet extract; however, in the absence of plasma, these competent cells do not progress through the cell cycle. The addition of an optimal concentration of plasma (5%) to competent cells initiates cell cycle traverse with the rapid, first-order entry of cells into the S phase, beginning 12 hr after the addition of plasma. Addition of a suboptimal concentration of plasma (0.25%) does not increase the rate of entry into the S phase. Treatment of cells with plasma (5%), before the addition of the platelet extract, does not shorten the latent period for entry into the S phase (3). Induction of DNA synthesis in quiescent Balb/c-3T3 cells can be resolved into at least 2 phases, controlled by different serum components: 1) competence, induced by PDGF; and 2) progression of competent cells into the cell cycle, mediated by factors in plasma (3). Viral-transformed cells have the ability to grow in plasma lacking PDGF.

1. Antoniades, H.N., Stathakos, D., and Scher, C.D. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2635-2639.
2. Antoniades, H.N., and Scher, C.D. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 1973-1977.
3. Pledger, W.J., Stiles, D.C., Antoniades, and Scher, C.D. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4481-4485.

251 NERVE GROWTH FACTOR RECEPTORS AND NEURONAL DIFFERENTIATION. Eric M. Shooter, Arne Sutter, R.J. Riopelle, and Ronald M. Harris-Warwick. Dept. Neurobiol. Stanford Univ. Sch. Med., Stanford, CA 94305.

Steady state and kinetic binding studies of ^{125}I-NGF to cell dissociates from sensory ganglia of 8-day-old chick embryo show two distinct, saturable binding sites with dissociation constants $K_d(I)=2.3\times10^{-11}$M and $K_d(II)=1.7\times10^{-9}$M. Dissociation of ^{125}I-NGF from the cells, even in the presence of saturating amounts of cold NGF, is biphasic. The finding that the ratio of fast to slow dissociating components varies with the relative occupancy of the two sites as predicted from the equilibrium data supports the concept of independent-receptor populations. Several lines of evidence suggest that binding of NGF to site I receptors is required for neuronal differentiation. Half maximal stimulation of neurite outgrowth from single cells by NGF occurs at 10% occupancy of site I and only 0.1% occupancy of site II. Bis desarginine NGF shows unaltered affinity for site I but a 10-fold reduced affinity for site II yet retains full biological activity. Developmentally site I appears only at the embryonic age where the sensory neurons become susceptible to the action of NGF. In addition site I does not occur on non-neuronal cells from sensory ganglia but is enriched proportionately on neurons when the latter are separated from non-neuronal cells by means of their decreased ability to absorb to plastic culture dishes. Little is known about the physiological function of the lower affinity binding sites II. They are present on non-neuronal cells as well as neurons in sensory ganglia and are found at earlier developmental stages than site I. It is possible that site II is a receptor for another, unidentified, avian hormone, with which NGF cross-reacts or alternatively it may be involved in NGF uptake.

252 REGULATION OF HEMATOPOIETIC CELL DIFFERENTIATION AND PROLIFERATION BY COLONY STIMULATING FACTORS, Antony W. Burgess, Donald Metcalf and Suzanne M. Watt, Cancer Research Unit, Walter and Eliza Hall Institute, Parkville, Australia, 3050.
Cloning techniques in semisolid medium have been developed in the last ten years that permit the clonal growth of all major hemopoietic cell types. Individual precursor cells, when specifically stimulated, can generate colonies of up to 10^4 differentiating progeny(1). Proliferation and differentiation of each blood cell type is controlled by a specific glycoprotein and recently two of these have been purified: Granulocyte-Macrophage colony stimulating factor (GM-CSF) and erythropoietin. GM-CSF stimulates the proliferation of the precursor cells for granulocytes and macrophages, but does not stimulate other blood cell types (e.g. lymphocytes) or cells from other tissues. GM-CSF initiates and determines the rate of proliferation of the precursor cells and the pattern of differentiation depends on the concentration of the hormone. At low concentrations (10^{-11}M), fewer precursor cells are stimulated, their rate of division is slower and macrophages develop instead of granulocytes(2).

Analysis of the rate of RNA synthesis by bone marrow cells has shown that GM-CSF affects the mature, non-dividing end cells (i.e. polymorphs) as well as the precursor cells. RNA synthesis was stimulated within ten minutes of adding GM-CSF to suspension cultures of mouse bone marrow cells. Under identical conditions protein synthesis in bone marrow cells was also stimulated after six hours but DNA synthesis was only stimulated after 16 hours. As yet there is no evidence to indicate the mechanism of action of GM-CSF.

Small amounts of biologically-active, radio-iodinated, purified GM-CSF (M.W. 23,000) have been prepared and studies to measure its binding to its target cells have been initiated. However, quantitation has been hampered by the heterogeneity of the target cell populations in the marrow. Highly purified populations of polymorphs (98%) have been prepared recently by light-scattering activated cell sorting of mouse peritoneal exudate cells. Iodination of the external proteins on the granulocyte membrane indicates that there are fewer than ten major proteins. The effect of GM-CSF on the accessibility and spatial distribution of these proteins will be discussed.

1. Metcalf, D., "Hemopoietic Colonies", Recent results in cancer research, 61, Springer-Verlag, Berlin, 1977
2. Burgess, A.W. and Metcalf, D., chapter 15, in "Experimental Hematology Today", edited by S.J. Baum and G.D. Ledney, Springer-Verlag, New York, 1977

Metabolic Modifiers

253 BINDING AND INTRINSIC DEGRADATION OF INSULIN BY HEPATOCYTES. D. F. Steiner, C. Hofmann and S. Terris, Department of Biochemistry, University of Chicago, Chicago, IL 60637
Earlier studies from this laboratory have shown that the degradation of insulin in isolated hepatocytes and perfused livers is dependent upon prior association of the hormone with cell surface recognition sites which are indistinguishable from the receptors believed to mediate biological activity [JBC, 250, 8389 (1975), JCI, 57, 885 (1976)]. Degradation proceeds after a brief lag of approximately 10 minutes which may represent the time required for transmembrane penetration of the hormone to site(s) of degradation. Degradation, but not binding, is markedly suppressed at temperatures below 20° and by several lectins. The latter findings suggest that lateral movement of recognition sites within the plane of the plasma membrane may be necessary either for internalization or degradation of the hormone. Incubation of isolated hepatocytes with monofluorescein insulin (B-1 substituted) leads to diffuse surface labeling of viable hepatocytes, but prolonged incubation at 37° leads only to some clustering of the fluorescent material at the cell surface without definite capping or lysosomal uptake. (Non-viable cells rapidly accumulate the derivative in the nucleus and to a lesser extent, cytosol.) These results are consistent with the failure of cytochalasin B, an inhibitor of pinocytosis, to influence insulin binding or degradation. On the other hand, colchicine as well as colcemid and vinblastine significantly inhibited insulin degradation, suggesting the possible participation of microtubules. The most reasonable model for degradation to account for these observations is one in which the hormone penetrates the cell membrane by a nonendocytotic mechanism to enter the cytosol where degradative activities are localized, rather than undergoing uptake into pinocytotic vesicles which later associate with lysosomes as has been proposed in the case of several other proteins or polypeptide hormones that interact with cell surface receptors. In this respect, the behavior of insulin more closely resembles that of several toxic proteins, such as diphtheria toxin, which are able to penetrate into cells in an active form. Uptake and degradation of insulin may in turn lead to the generation of active fragments of the hormone. Almost no definitive information is available on the cleavage products of insulin that are generated by its association with various tissues; the B chain especially merits consideration as a possible locus of biologically active fragments, i.e., sequences that may modify the activity of protein kinase and phosphatases within the cell, thus regulating the phosphorylation-dephosphorylation state of membrane components and enzymes. These mechanisms will be discussed in further detail [Diabetes, 26, 322 (1977)]. The recognition of the role of receptor binding in the degradation of insulin explains many previous observations that had suggested that derivatives of the hormone with lower biological activity have prolonged metabolic half-lives. Recent studies in several laboratories have shown that the participation of the liver in degradation is the important determinant of biological half-life of insulin and various analogs such as proinsulin. Thus intrinsic degradation may explain the existence of the so-called "spare" receptors. (Supported by NIH grants AM 13914 and AM 17046.)

254 CHARACTERIZATION OF INSULIN RECEPTORS IN ADIPOCYTES, Jørgen Gliemann and Ole Sonne, Inst.Med.Physiol.C, Univ. of Copenhagen, Blegdamsvej 3C, DK 2200 Copenhagen N.

Binding of ^{125}I-labelled insulin was measured in rat adipocytes at 37°, pH 7.4 using the following technique: 0.5 ml cell suspension (Krebs-HEPES buffer, albumin 50 mg/ml, 50-70 µl packed cells/ml) was incubated in 14 ml roundbottom tubes. The incubation was stopped by the addition of 12 ml 8° NaCl (0.15 mol/l) and 1.2 ml silicone oil (D 0.99) followed by centrifugation. The cells concentrated above the oil and were recovered on adsorbent material. The high concentration of albumin did not change insulin binding, whereas that part of insulin degradation, which is unrelated to insulin binding, was markedly reduced. The wash removed most of the "non-specific" binding resulting in a ratio "specific"(trace)/"non-specific" binding of about 15. The amount of trapped medium was negligible.

The binding of tracer [^{125}I]monoiodoinsulin (labelled in the A-chain) in the presence of unlabelled insulin 1 µmol/l ("non-specific" binding) was constant from time zero to 180 min. A constant level of binding of 100 pmol/l [^{125}I]iodoinsulin was obtained from 40 min to 180 min, and the ratio cell-bound/free radioactivity was about 0.01. Addition of unlabelled insulin 1 µmol/l at any time caused a dissociation of ^{125}I-activity to the level of "non-specific" binding. Cell-associated ^{125}I-activity was extracted completely by acetic acid 3 mol/l, urea 6 mol/l and about 80% of the activity coeluted with tracer [^{125}I]iodoinsulin on Sephadex G50F. This fraction titrated with anti-insulin as the tracer. A small fraction eluted in the void volume and a small fraction was retarded on the column.

The fate of cell-associated [^{125}I]iodoinsulin (100 pmol/l in the incubation step) was studied by resuspension in fresh buffer (37°, pH 7.4) of cells recovered from the oil phase. Essentially all activity was eluted from the cells after 60 min. 40-50% was insulin as judged by gelfiltration and titration with anti-insulin. About 50% was soluble in 12% trichloroacetic acid, coeluted with iodotyrosine and migrated as iodotyrosine on paper chromatography in two solvent system. A small fraction appeared in the void volume.

The half-time of elution of insulin and non-insulin from the cells was nearly the same at 37° (about 8 min). At insulin concentrations higher than 0.5 µmol/l the eluted ^{125}I-activity, was precipitable in trichloroacetic acid and presumeably represents loosely adsorbed insulin. The receptor-bound insulin (total minus "non-specific") was eluted with the same ratio: insulin/non-insulin over a wide range of occupancies.

The following is concluded: A steady-state of insulin binding to adipocytes can be maintained for several hours at 37°. Receptor bound insulin is the substrate for insulin degradation as shown in hepatocytes by Terris and Steiner (1). A hypothetical initial degradation product cannot be detected in the cells. The final labelled degradation product is presumeably iodotyrosine; this is released into the medium and is only to a very small extent accumulated in the cells.

255 CHARACTERIZATION OF THE PARTIALLY PURIFIED, RECONSTITUTED INSULIN-SENSITIVE D-GLUCOSE TRANSPORT SYSTEM OF THE RAT ADIPOCYTE PLASMA MEMBRANE. Michael P. Czech, Michael F. Shanahan and Dennis J. Pillion, Div. of Biology and Medicine, Brown University Providence, Rhode Island 02912.

One approach to the investigation of insulin action on membrane transport involves the direct chemical identification of the covalent or conformational modification of the complex. This approach entails the purification and isolation of the membrane component(s) directly involved in membrane transport and its modulation by insulin. We have initiated such studies using our recently developed methodology which allows reconstitution of D-glucose transport activity from a cholate-solubilized plasma membrane fraction highly enriched in a 94,000 glycoprotein species (1). The extrinsic plasma membrane proteins were first released from adipocyte plasma membranes using dimethylmaleic anhydride (DMMA) (2). The particulate fraction was extracted with 2% sodium cholate at 4°C, and the soluble fraction combined with cholate-dispersed phospholipids and chromatographed on a column of Sephadex G-50. In the present studies, the reconstituted liposomes collected in the void volume were subjected to electron microscopy and found to consist of vesicles (0.02 microns dia.) bounded by a single lipid bilayer structure. The vesicles were too small to be assayed for transport activity on Millipore filters. A rapid freeze-thaw cycle resulted in extensive fusion of the vesicles into very large bilayer structures which did not take up D-glucose by a cytochalasin B-sensitive process. Sonication of these large structures for 10 or 20 sec resulted in spherical vesicles (about 0.2 microns dia.) which exhibited stereospecific, cytochalasin B-sensitive, D-glucose transport activity. Omission of $MgCl_2$ in the buffer used to elute the Sephadex column and in the incubation buffer during the transport assay resulted in the abolition of hexose transport activity. Addition of excess EDTA to reconstituted vesicles prepared and incubated in the presence of $MgCl_2$ partially inhibited D-glucose uptake. Reconstituted vesicles exhibited increased specific (^{3}H)cytochalasin B binding capacity when compared to protein-free phospholipid vesicles. We also observed that insulin-activated hexose transport activity is retained in plasma membranes stripped of extrinsic proteins by DMMA. Attempts to reconstitute this insulin-activated transport system activity following solubilization and purification are currently in progress.

1) Shanahan,M.F.,and Czech,M.P. J.Biol.Chem.252: 8341-8343, 1977.
2) Shanahan,M.F.,and Czech,M.P. J.Biol.Chem.252: 6554-6561, 1977.

256 ROLE OF PROTEIN PHOSPHORYLATION IN INSULIN ACTION, Joseph Avruch, Lee A. Witters, Maria Alexander, Michael A. Bush and Lawrence M. Crapo, Diabetes Unit, Massachusetts General Hospital, Boston, MA 02114.

Insulin, like glucagon and beta adrenergic catecholamines, rapidly alters target cell function by effecting a series of coordinate and relatively stable alterations in the activity of key rate limiting enzymes and transport systems. While certain actions of insulin, such as inhibition of epinephrine stimulated lipolysis, may be explained by insulin's ability to inhibit epinephrine stimulated cAMP accumulation, many other actions of insulin appear to be unrelated to insulin induced alterations in cyclic nucleotide accumulation or action. Several of the enzymes whose activity is altered by insulin are known to be regulated by phosphorylation and dephosphorylation. In order to evaluate the role of these interconversions in the expression of insulin action we examined the effects of insulin on the phosphorylation of cellular proteins in adipose tissue and liver, as compared to the effects of glucagon and beta adrenergic catecholamines.

In adipocytes, incubated with extracellular $^{32}P_i$ to steady state, epinephrine stimulates the incorporation of ^{32}P into a large number of cellular peptides, through a mechanism that appears to involve cAMP and the cAMP dependent protein kinase. Insulin has two types of effects on protein phosphorylation in these cells. First, insulin antagonizes certain epinephrine stimulated protein phosphorylations, consistent with insulin's known effects on cAMP metabolism. Second, insulin stimulates the phosphorylation of a major adipocyte peptide (m.w. 123,000, located in the endoplasmic reticulum and cytoplasm) through a pathway that appears not to involve cAMP (1-3). Studies in adipocyte cytoplasmic extracts suggest the presence of a cAMP independent protein kinase specific for this peptide. However, attempts to isolate the 123,000 dalton peptide have been thwarted by the scant amounts present in adipose tissue.

Consequently, we have examined the hormonal control of cytoplasmic protein phosphorylation in isolated hepatocytes. In these cells, glucagon stimulates ^{32}P incorporation into four major cytoplasmic peptides, in association with an increase in cAMP and an activation of the cAMP dependent protein kinase. Insulin modifies protein phosphorylation in a manner entirely analogous to that observed in adipose tissue. In addition to inhibiting certain glucagon stimulated phosphorylations, insulin selectively stimulates the phosphorylation of a 46,000 dalton peptide through a cAMP independent mechanism.

Insulin stimulated protein phosphorylation may reflect an intermediate step in certain actions of insulin perhaps mediated by an unidentified second messenger, other than cAMP.

1. Benjamin, W.B. and Singer, I. (1975) Biochemistry 14:3301-3309.
2. Avruch, J., Leone, G.R. and Martin, D.B. (1976) J. Biol. Chem. 251:1511-1515.
3. Forn, J. and Greengard, P. (1976) Arch. Biochem. Biophys. 176:721-733.

257 REGULATION OF PYRUVATE DEHYDROGENASE BY INSULIN
P. J. Randle, N. J. Hutson, A. L. Kerbey, P. H. Sugden, Oxford University, U.K.

Flux through pyruvate dehydrogenase is apparently the major determinant of rates of pyruvate oxidation in animals. In adipose tissue, insulin increases and lipolytic hormones decrease the rate of oxidation of pyruvate and the synthesis of fatty acids from glucose. In other tissues, *in vitro* effects of insulin on flux through pyruvate dehydrogenase have not been shown. *In vivo*, insulin deficiency (in diabetes or starvation) reduces the rate of flux through pyruvate dehydrogenase in many tissues. This change may be related to enhanced oxidation of lipid fuels which has been shown to inhibit oxidation of pyruvate in heart, some skeletal muscles, liver, kidney and brain. The pyruvate dehydrogenase reaction is inhibited by its end products, acetyl CoA (competitive CoA) and NADH (competitive NAD^+). The pyruvate dehydrogenase complex is also regulated by a phosphorylation-dephosphorylation cycle. An intrinsic kinase catalyses phosphorylation and inactivation with $Mg\text{-}ATP^{2-}$, an associated phosphatase catalyses dephosphorylation and reactivation. The kinase incorporates phosphate into α chains of the decarboxylase component of pyruvate dehydrogenase which is a tetramer of subunit composition $\alpha_2\beta_2$. Pig heart complex is inactivated by incorporation of one phosphate per $\alpha_2\beta_2$; thereafter two more phosphates may be incorporated without further change in activity. These additional phosphorylations inhibit reactivation of the complex by the phosphatase reaction. Thus the extent of the kinase reactions can regulate reactivation of the complex by the phosphatase reaction. Current evidence indicates that the kinase and phosphatase reactions operate simultaneously *in vivo* and that the relative activities of kinase and phosphatase determine the proportion of active complex. The kinase reaction is enhanced by high concentration ratios of acetyl CoA/CoA, $NADH/NAD^+$ and ATP/ADP, and inhibited by pyruvate. The phosphatase reaction is activated by Mg^{2+} and Ca^{2+}. *In vivo*, the proportion of active complex is reduced in tissues by insulin deficiency (starvation, diabetes). In perfused heart, oxidation of lipid fuels decreases the proportion of active complex and there is evidence that effects of starvation and diabetes are based on enhanced oxidation of lipid fuels. In adipose tissue insulin increases and lipolytic hormones decrease the proportion of active complex. Current evidence suggests that insulin deficiency and oxidation of lipid fuels may activate the kinase through altered metabolite concentrations (e.g. ratio of acetyl CoA/CoA). This may lead to multi-site phosphorylations in the complex and inhibit reactivation by the phosphatase. The same mechanism could be involved in effects of lipolytic hormones and of insulin in adipose tissue.

Toxins Affecting Protein Synthesis

258 RELAXIN AS AN INSULIN-RELATED GROWTH FACTOR, Hugh D. Niall and Ralph A. Bradshaw,
Howard Florey Institute, Melbourne, Australia, 3052 and Dept. Biol. Chem. Washington Univ. Sch. of Med., St. Louis, Mo. 63110.
It is now appreciated that there exists a family of peptide growth factors regulating cell growth and activity. The distinguishing feature of this group is their ability to exert long, as well as short, term effects on their target tissues. Hormones of this class include insulin, insulin-like growth factor (IGF), the somatomedins, nerve growth factor (NGF), epidermal growth factor, multiplication stimulating activity and relaxin. While the physiological role of many of these is currently uncertain, structural studies have defined a sub-set related to insulin. Relaxin, an ovarian peptide hormone, is particularly closely related to insulin with regard to sequence identities and disulfide bond disposition, two chain structure and, most probably, mode of biosynthesis and three-dimensional structure. The sequences of IGF and NGF also reveal a homologous relationship to insulin. Thus insulin, IGF, NGF and relaxin have evolved from a common ancestral gene. In addition, it is suggested that this common origin may also reflect similarities in mode of action. Studies on NGF have shown that binding to cell surface receptors of responsive neurons leads to intracellular up-take and intra-axonal transport of the peptide to the cell body. Nuclear binding sites provide a locus for the transported hormone to act. The presence of similar receptors for insulin suggests a more general application of internalization as a major feature of the mechanism of action of this sub-set of hormones, including IGF and relaxin.
(Supported in part by USPHS research grant NS10229).

259 TRANSPORT OF DIPHTHERIA TOXIN A-FRAGMENT ACROSS THE PLASMA MEMBRANE, A. M.
Pappenheimer Jr., Biological Labs., Harvard University, Cambridge, MA 02138
The 60,000 dalton diphtheria toxin molecule is synthesized and released from the bacteria as a single polypeptide chain which may be subdivided into three functional regions of approximately equal length. There is an enzymically active 21,150 dalton A fragment extending from the N-terminal glycine residue to the first of the two disulfide bridges. This hydrophilic, negatively charged polypeptide must cross the plasma membrane of the target cell and reach the cytoplasm in order to inactivate EF2 by ADP-ribosylation and thereby block protein synthesis. There is a C-terminal positively charged polypeptide sequence of 10-20,000 daltons which interacts with specific receptors present on the membranes of sensitive cells and which includes the second cystine disulfide. Between these two hydrophilic regions there is an hydrophobic zone which, when "unmasked" is capable of binding ca 44 molecules of the non-ionic detergent Triton X100 and readily becomes inserted into membrane vesicles. It is suggested that the entry process involves an initial reversible interaction with membrane receptors, followed by an irreversible process in which the C-terminal region is released by a proteolytic cleavage, thus permitting the hydrophobic portion of the molecule to enter the lipid bilayer and form a channel through which the A fragment is drawn in an extended form to reach the cytoplasm.

260 SURFACE CHANGES IN A *RICINUS COMMUNIS* TOXIN-RESISTANT MURINE LYMPHOMA CELL LINE.
Garth L. Nicolson, Department of Developmental and Cell Biology, University of California, Irvine, California 92717

A variant of the murine lymphoma cell line BW5147 that was 250 times more resistant than the parent to *Ricinus communis II* agglutinin (RCA$_{II}$, ricin) toxicity was selected by repeated exposure to increasing toxin concentrations. Quantitative binding, however, was decreased by only 30-40% in the variant. Most surface glycoproteins on the parental and variant cell surfaces were similar, as judged by SDS-PAGE after lactoperoxidase-catalyzed iodination and RCA$_{I}$-affinity chromatography. Studies showed that and RCA$_{I}$- and RCA$_{II}$-binding protein of ~80,000 MW on the surfaces of parental cells is altered on the variant cells to a form with apparent lower MW. This protein is probably important for RCA$_{II}$ entry in parental cells, but its altered form on the variant cells no longer mediates efficient uptake imparting toxin resistance. In addition, a protein of ~35,000 MW, which does not bind toxin, is weakly iodinated on parental but not variant cells. Low concentrations of RCA$_{II}$ inhibit protein synthesis in cell-free extracts but not in intact cells of the RCA$_{II}$-resistant line. The concentration dependence of inhibition by RCA$_{II}$ is the same in cell-free extracts of both resistant variant and parental lines, while intact parental cells are 250X more sensitive to RCA$_{II}$ toxicity. The onset of RCA$_{II}$ inhibition of cell-free protein synthesis was extremely rapid in both cases, being complete in a few minutes. RCA$_{II}$ inhibits protein synthesis in intact toxin-sensitive parental cells, but the maximum inhibition requires several hours. These results support EM observations using ferritin-^{125}I-RCA$_{II}$. Fer-^{125}I-RCA$_{II}$ is indistinguishable from native RCA$_{II}$ in quantitative binding and cytotoxicity experiments. When RCA$_{II}$-sensitive and resistant cells were labeled with Fer-^{125}I-RCA$_{II}$ at various toxin concentrations (1-10 μg/ml), no differences in quantitative toxin binding were observed. At low Fer-^{125}I-RCA$_{II}$ concentrations (1-3 μg/ml RCA$_{II}$), where only the parental cells are sensitive to RCA$_{II}$, toxin receptors on the sensitive cells were endocytosed. In parallel experiments Fer-^{125}I-RCA$_{II}$ concentrations (>7 μg/ml RCA$_{II}$), where both parental and resistant cells are sensitive to the cytotoxic effects of RCA$_{II}$, ferritin-toxin was bound, and subsequent endocytosis occurred to a similar degree in both cell types. Endocytosis of Fer-Con a was indistinguishable on parental and resistant variant cells at all concentrations tested. The results suggest that a specific defect on the selected cells prevents RCA$_{II}$ entry at low toxin concentrations rendering them more resistant.
Supported by NCI contract CB-33879 and ACS grant BC-211A.

261 ENTRY AND ACTION OF ABRIN, RICIN AND MODECCIN, Sjur Olsnes, Norsk Hydro's Institute for Cancer Research, Montebello, Oslo, Norway

Abrin, ricin and modeccin are plant lectins which are highly toxic to animal cells due to their ability to inactivate ribosomes and thus inhibit protein synthesis (1,2). The toxins have molecular weights between 62,000 and 65,000 and consist of two dissimilar peptide chains. A prerequisite for the toxic action is binding to cell surface receptors containing terminal galactose residues. Abrin and ricin appear to bind to the same receptor, whereas the receptor for modeccin is different. On a HeLa cell there are about 3 x 10^7 receptors for abrin and ricin and about 10^5 receptors for modeccin. Neuraminidase treatment of the cells increased the number of receptors for abrin and ricin about 3 fold and the number of modeccin receptors 10 fold. The sensitivity of the cells to the toxins increased correspondingly (3).

At least in the case of abrin and ricin the binding properties are confined to the one polypeptide chain, the B-chain, whereas the ability to inactivate ribosomes is confined to the A-chain. Since abrin and ricin A-chain inactivate ribosomes enzymatically and since no cofactor is involved, it is clear that the A-chain must somehow penetrate through the cell membrane to gain excess to the ribosomes (1). Studies with toxin resistant mutants have indicated that if endocytosis is involved in the uptake mechanism it cannot be the rate limiting step (2).

The target for abrin and ricin A-chain is the 60S ribosomal subunit which is somehow modified, apparently at a site close to the binding site for the elongation factor EF 2. The ribosomes appear to be sensitive only in a particular phase of protein synthesis. All three toxins inhibit peptide chain elongation as well as initiation of new chains.

Different cell lines show different sensitivity to the toxins. The anti-cancer properties of the toxins will be discussed (4).

1. S. Olsnes, A. Pihl (1976) Receptors and Recognition Series B: "The specificity and action of animal, bacterial and plant toxins". (Ed. P. Cuatrecasas) Chapman and Hall, London, pp. 130-173.

2. K. Refsnes, T. Haylett, K. Sandvig, S. Olsnes. Biochem.Biophys.Res.Commun. In press.

3. K. Sandvig, S. Olsnes, A. Pihl (1978) Eur.J.Biochem. In press.

4. Ø. Fodstad, S. Olsnes, A. Pihl (1977) Cancer Res. In press.

262 MODES OF ACTION OF DIPHTHERIA TOXIN AND EXOTOXIN A FROM PSEUDOMONAS AERUGINOSA,
Collier, R.J., Dept. Bacteriology, UCLA, Los Angeles, CA 90024
Diphtheria toxin (Mr 60,000) and exotoxin A from Pseudomonas aeruginosa (Mr 66,000) show similarities in structure and intracellular mode of action. Both toxins catalyze attachment of the ADP-ribose moiety of NAD to the same site on elongation factor 2 (EF-2), thereby inactivating EF-2 and blocking protein synthesis. Both toxins are secreted as single chain proenzymes which must undergo modifications of covalent structure prior to expression of this enzymic activity. Diphtheria toxin must be exposed to both limited proteolysis and reduction before release of the enzymically active, amino terminal fragment A (Mr 21,145). The enzymic activity of exotoxin A, in contrast, is expressed by the intact, 66,000 dalton chain after partial reduction or by a 26,000 dalton peptide, devoid of half-cystine residues, which is released upon limited proteolysis. Results of recent studies on the structures and activities of the two toxins and their modes of penetration into sensitive animal cells will be presented.

Adenylate Cyclase

263 THE REGULATORY COMPONENTS OF ADENYLATE CYCLASE SYSTEMS,
Martin Rodbell, Michael C. Lin, Constantine Londos, Werner Schlegel, Pramod M. Lad, Laboratory of Nutrition and Endocrinology, National Institutes of Health, Bethesda, MD 20014
The current perception of adenylate cyclase systems in animal cells is that these membrane-bound systems are composed of several functionally distinct "units" which control the activity of the catalytic unit. In addition to receptors for hormones, neurotransmitters, and prostaglandins, these include GTP binding units that control independently the binding and activity state of hormone receptors (1,2); metal ion binding sites (Mn, Ca, Mg in descending order of affinity) that control enzyme activity independently of the actions of GTP and hormones (3); and inhibitory and stimulatory adenosine binding units that are linked differentially to cyclase systems (4,5). The structures and physical relationship of these units in the membrane remain unknown. Also unknown is the nature of the molecular transitions responsible for conversion of the catalytic unit to its various activity states. Various methods used to explore these questions and recent promising results will be discussed.

1) Lad, P.M., Welton, A.F., and Rodbell, M., (1977) J. Biol. Chem. 252, 5942-5946.
2) Welton, A.F., Lad, P.M., Newby, A.C., Yamamura, H., Nicosia, S., and Rodbell, M., (1977) J. Biol. Chem. 252, 5947-5950.
3) Londos, C. and Preston, M.S. (1977) J. Biol. Chem. 252, 5957-5961.
4) Londos, C. and Preston, M.S. (1977) J. Biol. Chem. 252, 5951-5956.
5) Londos, C. and Wolff, J. (1977) Proc. Natl. Acad. Sci. (USA). In Press.

264 HYBRIDIZATION OF HORMONE RECEPTORS WITH ADENYLATE CYCLASE FROM DIFFERENT CELLS,
Michael Schramm, Department of Biological Chemistry, Hebrew University, Jerusalem, Israel

A hormone receptor in one type of cell can be coupled to adenylate cyclase in another type of cell by fusing the two cells with the aid of Sendai virus (1). Prior to fusion the adenylate cyclase is selectively inactivated in the cell contributing the hormone receptor. A functional hybrid system is thus formed in the fused cell membrane between the receptor and the enzyme originating from two different cells. The experimental approach could be used to analyze the functional state of the receptor and the enzyme in mutants and in malfunctioning situations. For example, hormone receptors discovered by ligand binding could be tested for function by hybridization with a response system in the cell membrane of another type of cell. This novel experimental approach could be extended to study other dissociable multicomponent systems residing in biological membranes.

(1) M. Schramm, J. Orly, S. Eimerl, and M. Kanner, Nature, 268, 310-313 1977.

265 SIMILARITIES BETWEEN HORMONE SENSITIVE ADENYLATE CYCLASE & LIGHT ACTIVATED PHOTORECEPTOR PHOSPHODIESTERASE,
M.W. Bitensky and G.L. Wheeler, Dept. of Pathology, Yale Univ. Sch. of Med., New Haven, Connecticut 06520

The finding by Rodbell of a GTP requirement for hormone activation has provided an additional dimension to the adenylate cyclase paradigm. In our studies of rod phosphodiesterase we also found that GTP was required for light activation. We have found a remarkable number of similarities between the two systems, in relation to the involvement of GTP as an activator: 1) GTP is indespensible for activation and cannot function without a system specific signal(i.e., light or a hormone); 2) In both systems a signal-specific GTPase has been found; for hormone sensitive cyclase Zehlinger has identified epinephrine activated GTPase; for light activated phosphodiesterase we have detected light activated GTPase; 3) In both instances non-hydrolyzable analogues such as GMP-PNP provide more persistent activation than GTP itself; 4) In both cases the concentrations of GMP-PNP which substitute in the activation step also function as inhibitors of the associated GTPase; 5) Both signal-specific GTPases exhibit a km for GTP in half micromolar range; 6) Inactivation of the signal-specific GTPases results in a persistant activated state with GTP; 7) In both cases the catalytic moiety appears activable by F alone. Questions still exist concerning the number of components involved in the modulation of enzyme activity and whether the GTPase is the same as, or different from, the GTP modulator locus. We anticipate that each of the systems will continue to provide information useful for analyzing the other. Purified photoreceptor components may allow reconstitution of a functional system.

266 RECONSTITUTION OF HORMONE-SENSITIVE ADENYLATE CYCLASE USING RESOLVED SUBCOMPONENTS OF THE ENZYME, Elliott M. Ross and Alfred G. Gilman, Dept. Pharmacology, Univ. Virginia, Charlottesville, VA 22903. Adenylate cyclase (AC) consists of at least two proteins needed for basal or F^-- or Gpp (NH) p-stimulated activity with Mg/ATP as substrate. A component retained in a phenotypically AC-deficient S49 lymphoma cell variant is proposed to be the catalytic moiety because of its apparent ability to catalyze Mn^{++}-dependent AC activity. One or two regulatory components which confer Mg^{++}-dependent activity and control by F^- or Gpp (NH)p on the catalyst may be resolved from plasma membranes of tissues or cultured cells by genetic, chemical, or physical techniques (JBC 252:6966). Addition of detergent extracts of normal plasma membranes to membranes of AC-deficient S49 cells reconstitutes hormone-stimulated AC activity (PNAS 74: 3715). Inactivation of the catalyst in such extracts does not destroy the reconstitution of hormone-sensitive activity, suggesting that the process reflects the interaction of added regulatory protein with catalyst and hormone receptor already in the AC-deficient membrane. Stable binding of regulatory protein is time- and temperature-dependent and minimally requires a nucleotide, divalent cation, and regulatory ligand (hormone, F^-, Gpp(NH)p).

Cholera Toxin Action and Transport

267 MECHANISM OF ADENYLATE CYCLASE ACTIVATION BY CHOLERAGEN, Martha Vaughan and Joel Moss, Laboratory of Cellular Metabolism, National Heart, Lung, and Blood Institute, Bethesda, MD 20014.

Choleragen initially interacts with cells by binding to specific receptor molecules on the external surface membrane. Work in several laboratories is consistent with the view that the toxin receptor is the monosialoganglioside G_{M1}. Using cultured cells that lack G_{M1} and are resistant to the toxin, we have shown that exogenous G_{M1} can be introduced into the cell membrane and can serve as a functional choleragen receptor. In these studies the specificity of G_{M1} as the choleragen receptor and the quantitative relationship between G_{M1} content and cell responses to choleragen were demonstrated directly. The interaction between choleragen and G_{M1} was investigated further using liposomal model membranes. Choleragen was bound to and increased the release of trapped glucose from liposomes containing G_{M1} but did not interact significantly with liposomes lacking gangliosides or those containing G_{M2} or G_{D1a}. The B protomer of choleragen which is the portion of the molecule that binds to the cellular receptor was as effective as the holotoxin, whereas the A protomer was inactive. Presumably interaction of the ganglioside binding site on the B protomer with G_{M1} in the liposomes was responsible for perturbations of the model membranes that led to glucose release. Using anticholeragen antibodies and complement to increase the sensitivity of the assay, it was shown that the A protomer but not the B protomer or the holotoxin was bound to ganglioside-free liposomes. It appears that the A protomer must be freed of constraints present in the intact choleragen in order to interact with the lipid model membranes. Others have shown that the A_1 subunit after release from the intact toxin activates adenylate cyclase in cell homogenates in an NAD-dependent reaction (1). We have demonstrated that choleragen and the A protomer catalyze the hydrolysis of NAD to nicotinamide and ADP-ribose. Arginine and related compounds containing a guanidino group, but not other basic amino acids, markedly increased nicotinamide formation from NAD in the presence of choleragen. After incubation of the toxin with NAD and arginine, a compound tentatively identified as ADP-ribosyl-arginine was isolated. Based on the assumption that the ADP-ribose transferase reaction is a model for the NAD-dependent activation of adenylate cyclase by choleragen, we have proposed that the active A_1 subunit of choleragen catalyzes the ADP-ribosylation of an arginine or related amino acid residue in a protein which is the cyclase itself or is critical for its regulation.

1) Gill, D. M. (1975) Proc. Natl. Acad. Sci. USA 72, 2064-2068.

268 STRUCTURE, FUNCTION, AND EVOLUTION OF CHOLERA ENTEROTOXIN,

Alex Kurosky, Daiel E. Markel, Kelly E. Hejtmancik, and Johnny W. Peterson, Department of Human Biological Chemistry and Genetics and Department of Microbiology, University of Texas Medical Branch, Galveston, TX 77550.

Completion of the primary structure of the β chain of cholera toxin has provided some interesting insights concerning its possible function. The chemical similarity of the amino-terminal portion of the β chain of the toxin to a segment of the β chain of the glycoprotein hormones has led to new research directions. To further examine the function of the toxin we have undertaken chemical modification studies to probe the toxin's mechanism of action in specific detail. Initially we have examined the ability of modified B subunit and β chain fragments to bind to CHO cells. Chemically modified proteins were assessed for CHO cell-binding by their ability to inhibit adenylate cyclase stimulation by intact cholera toxin. Modification experiments included reactions with iodoacetamide, tetranitromethane, succinic anhydride, and cyanogen bromide. Immunological studies employing a radioimmunoassay revealed some cross-reactivity between the A and B subunits suggesting possible chemical similarities in these proteins. Cross-reactivity experiments were also conducted with glycoprotein hormone subunits. Inactivation of Escherichia coli and Salmonella typhimurium toxins by cholera antitoxin suggests that a family of homologous enterotoxins occurs within certain members of the Enterobacteriaceae.

Sequence, compositional, and carboxyl-terminal analysis have also been initiated on isolated α and γ chains of the A subunit. Hydrolysis of α and γ chains with carboxypeptidase A indicated serine to be the carboxyl-terminal residue for both chains.

This work was supported by grants HD 03321 from NICHD, AI 08518 from NIAID and by the J.W. McLaughlin Fellowship fund.

269 INTRACELLULAR MODE OF ACTION OF CHOLERA TOXIN,

D. Michael Gill, Department of Biology, Harvard university, Cambridge, Mass. 02138.

Cholera toxin necessarily interacts first with the outside of a target cell but the kinetics of adenylate cyclase activation in intact and broken cells indicate that the crucial event is intracellular. A piece of the toxin, fragment A_1, must cross the plasma membrane and catalyze a cytosolic reaction that changes the adenylate cyclase system permanently. This reaction requires a nucleoside triphosphate, NAD, and a small cytosolic protein. The requirement for NAD is highly specific. One possible interpretation is that NAD is one substrate in a toxin-catalyzed ADP-ribosyl transfer reaction while the nucleoside triphosphate plays an allosteric, not a substrate, role.
To detect any toxin-specific incorporation of ADP-ribose from exogenous ^{32}P-NAD into membrane proteins of lysed cells it was necessary to reduce by about one hundred fold the background incorporation of ADP-ribose and yet maintain the ability to activate adenylate cyclase. This has been achieved, using lysed pigeon erythrocytes, by reducing the endogenous NAD level and by adding suitable inhibitors of the background incorporations. It is now possible to demonstrate toxin-specific labeling of several membrane proteins and of some soluble proteins. In each case the ADP-ribose transfer is catalysed by fragment A_1 which may be isolated in advance or generated in situ by treating the holotoxin with SDS and DTT. Transfer of ADP-ribose to the membrane proteins occurs only under conditions that lead to adenylate cyclase activation and in many respects the two activities occur in parallel. Both require ATP and the cytosolic macromolecule. However, many more copies of the membrane proteins can be modified than could be relevant to the cyclase activation. We may suppose, therefore, that cyclase activation results from the modification of a certain protein, but not necessarily every copy of that protein. In addition the modification of other proteins may have physiological consequences unrelated to the adenylate cyclase system.

D.M. Gill. Multiple roles of erythrocyte supernatant in the activation of adenylate cyclase by Vibrio cholerae toxin in vitro. J. Infect. Dis. 133, S55-S63 (1976).
\- Mechanism of action of cholera toxin, in Advances in cyclic nucleotide research, (edited by P. Greengard and G.A. Robison) Raven Press, New York, 8, 85-118 (1977).

270 CHOLERA-TOXIN ACTIVATION OF NEUROBLASTOMA ADENYLATE CYCLASE, Arthur J. Blume and Susan L. Levinson, Department of Physiological Chemistry and Pharmacology, Roche Institute of Molecular Biology, Nutley, NJ 07110.

The adenylate cyclase of mouse neuroblastoma cells, clone NS20, is activated in situ by cholera toxin addition to intact cells or in vitro by addition of toxin to preparations of cell membranes. The in vitro activation process requires NAD and nucleotide triphosphates in addition to toxin. The activity of the NS20 adenylate cyclase before treatment with toxin (normal enzyme) is low when assayed at $MgCl_2$ concentrations of 10 mM in assay systems containing ATP and a nucleotide-triphosphate regenerating system. The V_{max} of the normal enzyme is increased when 2-chloroadenosine, PGE_1, NaF or GMP-P(NH)P is present. After incubation of the normal enzyme with GMP-P(NH)P and subsequent washing, the enzyme still exhibits maximum activity when assayed in the absence of GMP-P(NH)P and is no longer effected by addition of 2-chloroadenosine, PGE_1, NaF or GMP-P(NH)P. The in situ and in vitro cholera toxin-activated enzyme exhibit identical properties. When assayed with 10 mM $MgCl_2$, ATP and a nucleotide-regenerating system, toxin-activated enzyme has a very high activity, which is similar to that found with the normal enzyme when PGE_1, NaF or GMP-P(NH)P are present and this activity is not significantly elevated by addition of 2-chloroadenosine, PGE_1, NaF or GMP-P(NH)P. Although the toxin-activated and GMP-P(NH)P-activated enzymes appear similar at 10 mM $MgCl_2$, they are differently affected by concentrations of $MgCl_2$ in excess of 20 mM. At these elevated $MgCl_2$ concentrations, the toxin-activated enzyme, but not the GMP-P(NH)P-activated enzyme, again requires added PGE_1, NaF or GMP-P(NH)P for maximum activity. A guanine nucleotide requirement for expression of maximal activity by the cholera-toxin-activated enzyme could also be demonstrated at $\leq$ 10 mM $MgCl_2$, when AMP-P(NH)P was used as substrate in assay systems devoid of a nucleotide-triphosphate regenerating system; GTP as well as GMP-P(NH)P fulfills this requirement. Under these assay conditions, the normal enzyme is not activated by 2-chloroadenosine, PGE_1 or GTP, but is activated by GMP-P(NH)P or NaF. Addition of a regenerating system to these assays returns the ability of 2-chloroadenosine and PGE_1 to activate the normal enzyme. PGE_1 and 2-chloroadenosine are found to increase the amount of GDP required to inhibit activation by GMP-P(NH)P of the normal and cholera-toxin-activated enzyme. We have hypothesized that at physiological concentrations of $MgCl_2$, the toxin-activated enzyme is "fixed" in its highly active state (E-GTP); in contrast, the normal enzyme decays rapidly from such a state. The normal decay process is proposed to be related to a Mg^{++}-dependent GTPase activity, and activation of the enzyme by cholera toxin to be due to a reduction or prevention of GTP to GDP hydrolysis.

Processing, Storage and Release of Hormones

271 RECONSTITUTION OF FUNCTIONAL ROUGH MICROSOMES FROM HETEROLOGOUS COMPONENTS, Günter Blobel, Department of Cell Biology, The Rockefeller University, New York, N.Y. 10021

Previously (1,2) we have used cell-free protein synthesizing systems supplemented with microsomal membranes to reconstruct in vitro the early events in the intracellular pathway of secretory proteins. Translation of mRNA's for various secretory proteins (3,4) yielded cleavage of the signal peptide portion of nascent presecretory proteins at the correct site and segregation of the processed nascent secretory proteins within the lumen of the microsomal vesicles. Translation of mRNA's for cytosol proteins, such as the α and β chains of rabbit globin, did not result in segregation (1). Furthermore cleavage and segregation of nascent presecretory proteins occurred only when microsomal membranes were present during translation, and not when added after completion of translation (1, 3, 4). These results provided strong evidence for the signal hypothesis (5). Recently we found that the cell-free systems supplemented with microsomal membranes have additional capacities. Translation of a mRNA for an integral membrane glycoprotein (G) of vesicular stomatitis virus (VSV) yielded correct insertion of the newly synthesized G protein into the microsomal membrane (6). Furthermore the newly synthesized G protein was found to be core-glycosylated (6). Both insertion and core-glycosylation occurred only when microsomal membranes were present during translation and not when added after translation (6). The observation that removal of the signal peptide region, glycosylation, and segregation occur during translation suggests that these reactions proceed in an interlocking cascade wherein the product of one co-translational event is the substrate for another.

(1) Blobel, G., and Dobberstein, B. (1975) J. Cell Biol. 67:852-862. (2) Dobberstein, B., and Blobel, G. (1977) Biochem. Biophys. Res. Commun. 74:1675-1682. (3) Shields, D., and Blobel, G. (1977) Proc. Natl. Acad. Sci. U.S.A. 74:2059-2063. (4) Lingappa, V.R., Devillers-Thiery, A., and Blobel, G. (1977) Proc. Natl. Acad. Sci. U.S.A. 74:2432-2436. (5) Blobel, G., and Dobberstein, B. (1975) J. Cell Biol. 67:837-851. (6) Katz, F.N., Rothman, J.E., Lingappa, V.R., Blobel, G., and Lodish, H.F. (1977) Proc. Natl. Acad. Sci. U.S.A. 74:3278-3282.

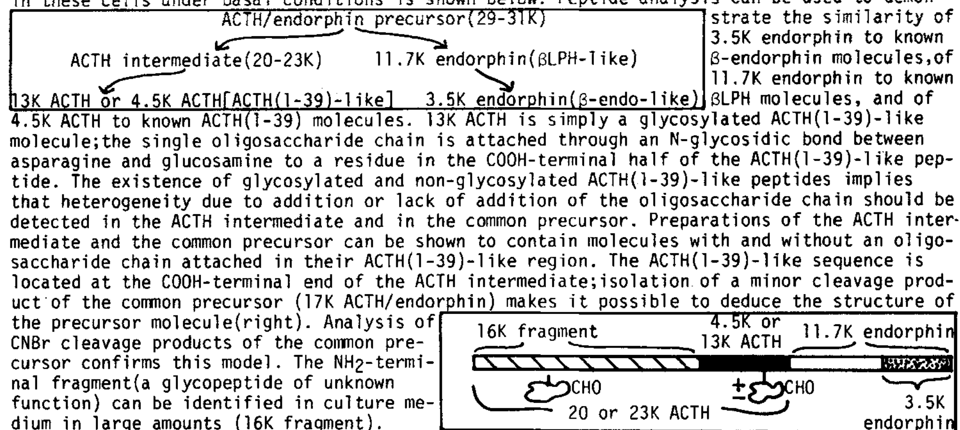
272 SECRETION MECHANISMS AND INSULIN RELEASE P. J. Randle, Dept. Clinical Biochemistry, Radcliffe Infirmary, Oxford OX2 6HE, U.K.

Current evidence implicates Ca^{2+} and cyclic AMP as potential intracellular messengers for secretion by exocytosis. There are a number of unanswered questions about the mechanism of exocytosis in general. Is Ca^{2+} the primary initiator of exocytosis and what is its site of action? Is cyclic AMP a primary initiator of exocytosis; or does it facilitate the action of Ca^{2+}? What is the site of action of cyclic AMP and does it involve protein phosphorylation? What is the basis of motility and membrane associated events in exocytosis? In the specific case of insulin release from adult β-cells, cyclic AMP appears to act permissively and not as an initiating messenger. A controversial question specific for insulin release is whether glucose and other substrate initiators of insulin release act via a direct receptor or through a metabolite intermediate. In attempting to answer some of these questions studies have been carried out with parotid acinar cells (Dr. P. Kanagasuntheram) and with hepatocytes (Sue Foden) as model systems. In parotid acinar cells stimulation of amylase release by α-adrenergic agonists, or A23187 depended on external calcium. These agents stimulated calcium uptake. Agents which elevated perotid cell cyclic AMP (β-adrenergic agonists, dibutyryl cyclic AMP, methyl xanthines) stimulated amylase release in the absence of external calcium. These agents did not stimulate calcium uptake but they decreased the ^{45}Ca exchangeable pool in mitochondrial and microsomal fractions (1,2). In hepatocytes α-adrenergic agonists stimulate cell uptake of calcium and activate phosphorylase (depends on extracellular calcium). Agents which elevate hepatocyte cyclic AMP (glucagon, dibutyryl cyclic AMP, methylxanthines) did not stimulate cell uptake of calcium but they decreased the ^{45}Ca exchangeable pool in mitochondria. The effects of glucagon were blocked by insulin (3, 4). These data support the concept that agents which elevate cell cyclic AMP can release calcium from intracellular organelles. Attempts have been made to identify troponin in secretory cells. Affinity columns of troponin-C linked to Sepharose 4B gave evidence for the presence of troponin I in parotid and pancreatic acinar cells. Exocrine pancreas on fractionation can yield peptide fractions which inhibit actomyosin ATPase and restore Ca^{2+} sensitivity (Kanagasuntheram, P., Randle, P. J. unpublished work). The presence of troponin-like complex in brain (5) and of troponin-C like peptide in adrenal medulla (6) has been described.

1. Dormer,R.L., Ashcroft,S.J.H.,1974, Biochem. J. 144, 543; 2. Kanagasuntheram, P., Randle, P.J.,1976, Biochem. J. 160, 547; 3. Foden,S.,Randle,P.J.,1978, Biochem. J. In press; 4. Assimocopoulos-Jeannet,P.D. et al., 1977, J. Biol. Chem. 252, 2662; 5. Mahendran,C., Berl,S.,1977, Biochemistry, 74, 2273; 6 Kuo,I.C.Y.,Coffe,C.J., 1976, J. Biol. Chem. 251, 6315.

273 BIOSYNTHESIS OF ACTH AND ENDORPHIN: STRUCTURE OF THE COMMON PRECURSOR, Betty A. Eipper and Richard E. Mains, Dept. Physiol., C-240, Univ. Colo. Med. Center, Denver, Colorado 80262

A mouse pituitary tumor cell line(AtT-20/D-16v) has been used to study the biosynthesis of two pituitary peptide hormones:adrenocorticotropic hormone(ACTH) and endorphin. Cells are incubated with a labeled amino acid or sugar;a double antibody immunoprecipitation procedure using antisera to ACTH or endorphin is used to separate labeled ACTH- or endorphin-containing molecules from other labeled cell products. The AtT-20 cells secrete several forms of ACTH (31K,23K,13K,4.5K) and endorphin(31K,11.7K,3.5K);molecules are named according to their apparent molecular weight in a particular SDS gel system and the antiserum used to immunoprecipitate them. The 31K molecule has antigenic determinants for both ACTH and endorphin, and the tryptic and chymotryptic peptides of 31K ACTH and 31K endorphin are identical;thus 31K ACTH/endorphin can serve as the biosynthetic precursor to both ACTH and endorphin. Analysis of the kinetics of labeling in pulse-chase experiments indicates that the major biosynthetic pathway in these cells under basal conditions is shown below. Peptide analysis can be used to demonstrate the similarity of 3.5K endorphin to known β-endorphin molecules,of 11.7K endorphin to known βLPH molecules, and of 4.5K ACTH to known ACTH(1-39) molecules.



13K ACTH is simply a glycosylated ACTH(1-39)-like molecule;the single oligosaccharide chain is attached through an N-glycosidic bond between asparagine and glucosamine to a residue in the COOH-terminal half of the ACTH(1-39)-like peptide. The existence of glycosylated and non-glycosylated ACTH(1-39)-like peptides implies that heterogeneity due to addition or lack of addition of the oligosaccharide chain should be detected in the ACTH intermediate and in the common precursor. Preparations of the ACTH intermediate and the common precursor can be shown to contain molecules with and without an oligosaccharide chain attached in their ACTH(1-39)-like region. The ACTH(1-39)-like sequence is located at the COOH-terminal end of the ACTH intermediate;isolation of a minor cleavage product of the common precursor (17K ACTH/endorphin) makes it possible to deduce the structure of the precursor molecule(right). Analysis of CNBr cleavage products of the common precursor confirms this model. The NH_2-terminal fragment(a glycopeptide of unknown function) can be identified in culture medium in large amounts (16K fragment). Based on the molecular weights of the fragments (as determined by gel filtration in 6M GdnHCl) there is little additional peptide at either end of the molecule or separating the fragments.

Nervous System Receptors

274 ENDORPHIN SYNTHESIS BY A CLONAL CELL LINE

Steven L. Sabol, Laboratory of Biochemical Genetics, NHLBI, National Institutes of Health, Bethesda, Maryland, 20014

Endorphins, a class of peptides found in nervous tissue and the pituitary gland, are thought to be endogenous ligands for the opiate receptor. Cultured clonal mouse AtT-20 pituitary tumor cells, which are known to produce corticotropin, have recently been shown also to produce endorphins (1). Opiate activity of AtT-20 extracts was routinely assayed by the opiate-receptor mediated, i.e. naloxone-reversed, inhibition of adenylate cyclase activity of homogenates of NG108-15 neuroblastoma x glioma hybrid cells. Acetic acid (2 M) extracts of AtT-20 cells contained opiate activity equivalent to that of 0.85-1.2 nmol synthetic β-endorphin [β-lipotropin-(61-91)] per mg of original cell protein. The activity was separated by Bio-Gel P4 column chromatography into two fractions, the major one (70% of the activity) having a mobility very similar to that of β-endorphin, and the minor one (30% of the activity) having the mobility of α- or γ-endorphin [β-lipotropin-(61-76) or -(61-77), respectively]. The major fraction was further purified by CM-Sephadex column chromatography to yield material which was comparable to synthetic β-endorphin with respect to specific activity and chromatographic properties.

Cultivation of AtT-20 cells in the presence of glucocorticoids for 2 days or longer, a treatment known to reduce the amount of intracellular and secreted corticotropin, resulted in a reduction of intracellular opiate activity. Treatment with 0.1 μM dexamethasone for 2 or 4 days resulted in decreases of 53% and 65%, respectively, of opiate activity; the half-maximally effective dexamethasone concentration was 0.6 nM. Similar suppression of opiate activity was obtained with 0.01-1 μM cortisol or corticosterone, while 1 μM deoxycorticosterone and aldosterone were less effective, and 1 μM testosterone, estradiol, and progesterone were ineffective. These results suggest that physiological concentrations of glucocorticoids, presumably interacting with intracellular glucocorticoid receptors, regulate the synthesis and/or secretion of endorphin from AtT-20 cells, possibly by altering the synthesis or processing of the recently reported (2) common precursor peptide for corticotropin and β-endorphin. Studies on clonal AtT-20 cells may help to illuminate the mechanisms regulating the biosynthesis and secretion of endorphins of the pituitary gland and perhaps of the nervous system.

1) Giagnoni, G., Sabol, S.L., and Nirenberg, M. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 2259-2263.
2) Mains, R.E., Eipper, B.A., and Ling, N. (1977) Ibid. 74, 3014-3018.

275 SYNTHESIS AND PROCESSING OF A COMMON PRECURSOR TO ACTH AND β-ENDORPHIN IN MOUSE PITUITARY CELLS, Edward Herbert, Marjorie Phillips, Patricia A. Rosa, Marcia L. Budarf, and Richard G. Allen, Department of Chemistry, University of Oregon, Eugene, OR 97403, and James L. Roberts, University of California Medical Center, San Francisco, CA 94143.

Radioactive proteins synthesized in a cell-free system under the direction of mRNA from mouse pituitary tumor cells (AtT-20/D_{16V} cells) were isolated by immunoprecipitation with antiserum to ACTH or β-endorphin. Each immunoprecipitate was fractionated by SDS gel-electrophoresis and shown to contain only one labeled protein with an apparent molecular weight of 28,500 (28.5 K). The ACTH and β-endorphin proteins had the same tryptic peptide maps and contained a single copy of ACTH and β-lipotropin (β-LPH). β-LPH was shown to be located carboxy terminal to ACTH. ACTH-LPH messenger RNA was purified from AtT-20/D_{16V} cells and used to make complementary DNA (cDNA). The cDNA was digested with specific restriction endonucleases and the restriction fragments were purified by gel-electrophoresis for nucleotide sequence analysis and for cloning experiments and results will be reported. SDS gel-electrophoresis of tumor cell extracts immunoprecipitated with antiserum to ACTH or β-endorphin revealed the presence of 3 high molecular weight proteins (29 K, 32 K and 34 K) which reacted with both antibodies and lower molecular weight forms which reacted with only one antibody. The 29-34 K ACTH-LPH forms were all glycosylated. Other labeling experiments showed that the 32 K and 34 K forms contained more carbohydrate than the 29 K form, suggesting that they are derived from the 29 K form by further glycosylation. Glycopeptide analysis showed that the α(22-39) sequence of ACTH is glycosylated in 32 K but not in 29 K ACTH-LPH, suggesting that the 32 K form may be the precursor of the 13 K ACTH and the 29 K form may be the precursor of 4.5 K ACTH. The distribution of ACTH and β-endorphin in anterior and intermediate-posterior lobes of mouse pituitary was determined by SDS gel-electrophoresis. All of the forms of ACTH and β-endorphin detected in the tumor cell culture were observed in the pituitary including 29, 32 and 34 K ACTH-LPH. Tumor cell cultures were pretreated with dexamethasone (dex) for 48 hrs and then labeled with radioactive amino acids. Incorporation of label into ACTH-LPH proteins after a 10 min pulse was reduced by the dex treatment but the distribution of label in different forms of ACTH and β-LPH after longer labeling periods was not affected by dex. The effect of dex on mRNA levels in the tumor cells will be reported.

Steroid and Thyroid Hormones

276 INTERACTION OF STEROID HORMONES WITH TARGET CELLS. Elwood V. Jensen, Geoffrey L. Greene and Eugene R. DeSombre, Ben May Laboratory for Cancer Research, University of Chicago, Chicago, IL 60637.

In contrast to peptide hormones, which interact with receptors in the plasma membrane, steroid hormones enter the target cell and bind to extranuclear receptor proteins, inducing their conversion to an activated form. The activated steroid-receptor complex serves as the carrier of regulatory information, entering the nucleus to bind in the chromatin and modulate the synthesis of RNA. In the stimulation of uterine growth by estrogens, physiologic amounts of hormone cause the translocation of several thousand steroid-receptor complexes in each target cell; full hormonal action requires the continued presence of estrogen-receptor complex in the nucleus for a prolonged period of time. In the case of estrogenic and androgenic hormones, the effect of the hormone given in vivo on the RNA synthesizing capacity of isolated target cell nuclei can be mimicked by exposure of the nuclei to activated but not to native hormone-receptor complex in vitro.

Although the overall pattern of the interaction of steroid hormones with target cells in now established, detailed understanding of the molecular basis of receptor activation, translocation and nuclear interaction is still far from complete. In the hope that the techniques of immunochemistry might provide new insight into these phenomena, we have prepared specific antibodies to a highly purified preparation of the nuclear form of the estrogen receptor protein (estrophilin) of calf uterus. Immunoglobulin obtained from the serum of rabbits immunized with this protein has been shown to contain specific antibodies to estrophilin by four criteria: (a) precipitation of the radioactive steroid marker upon addition of goat anti-rabbit-γ-globulin to a mixture of the tritiated estradiol-receptor complex and the immunoglobulin, (b) adsorption of the estradiol-receptor complex by the immunoglobulin linked to Sepharose, (c) adsorption of the estradiol-receptor complex in the presence of the immunoglobulin by Staphylococcus aureus protein-A linked to Sepharose, and (d) the ability of the immunoglobulin to increase the sedimentation rate of the estradiol-receptor complex. Antibodies to calf nuclear estrophilin cross-react with that of rat uterine nuclei, as well as with the extranuclear receptor of calf, rat, mouse and guinea pig uterus and of human breast cancer. The antibodies do not react with either the nuclear or extranuclear dihydrotestosterone-receptor complexes of rat prostate or with the extranuclear progesterone-receptor complex of chick oviduct. These findings indicate immunochemical similarity among estrophilins from several mammalian species, as well as between extranuclear and nuclear forms of the receptor, but not among receptor proteins for different steroid hormones. They provide additional support for the two-step interaction mechanism in which the nuclear receptor is derived from the hormone-induced translocation of the extranuclear receptor protein and open the way for precise localization of intranuclear receptor-binding sites through electron microscopy.

277 REGULATION OF GENE EXPRESSION BY STEROID HORMONE RECEPTORS, Bert W. O'Malley and William T. Schrader, Dept. of Cell Biology, Baylor Coll. Med., Houston, TX 77030

The chick oviduct progesterone receptor system has been studied extensively as a general model for steroid hormone action. We have succeeded in demonstrating a direct stimulatory effect of homogeneous progesterone-receptor complexes upon RNA transcription _in vitro_. The native _in vivo_ form of the cytoplasmic receptor protein is an asymmetric 6S molecule (MW = 225,000). It is a dimer of two non-identical subunits (MW = 80,000 and 117,000), each with one progesterone binding site. The dimer and both subunits have been purified to homogeneity. Recent studies with reversible crosslinking reagents have confirmed the existence of an aporeceptor dimer, containing one A and one B subunit. All three preparations have been tested for their binding affinity _in vitro_ to target cell nuclear constituents. Dimers are taken up by nuclei only if complexed with hormone. The isolated subunits have distinctly separate nuclear affinities: the smaller subunit (A) binds preferentially to free DNA; the other (B) binds only to target cell chromatin. The intact A-B dimers bind to chromatin but have no detectable DNA binding activity. Thus in the dimer the chromatin site on the B subunit functions but the A subunit's DNA site appears to be blocked. Chromatin RNA chain initiation sites were measured using a rifampicin challenge assay. Intact 6S progesterone receptor complexes added to oviduct chromatin _in vitro_ stimulate production of new RNA chain initiation sites. At half maximal stimulation (5×10^{-9}M receptor) about 40% more sites are measured by the rifampicin challenge assay than are seen in untreated chromatin. Only the intact 6S receptor dimers complexed with progesterone are active. Subunit B alone binds to the chromatin but does not stimulate in the assay. Subunit A alone stimulates but only at 10 to 50-fold higher concentration than the intact 6S dimer. We have recently been able to add the purified hormone-receptor complex (6S) to interphase chromosomes _in vitro_ and "turn-on" transcription of the ovalbumin gene. These observations suggest a _novel model_ of steroid hormone action. It requires two receptor-bound hormone molecules per functional gene site, one on the B "specifier" subunit and one on the A "regulatory" subunit of an A-B dimer. The B subunit confers binding specificity at nuclear "acceptor sites" and thereby carries the A subunit to the neighborhood of responsive genes. The metastable dimer may then dissociate liberating the A subunit which, after binding to sites on DNA, may promote the initiation of synthesis of specific new species of RNA. Recently we have completed the _in vitro_ synthesis of the full ovalbumin structural gene. From whole chick DNA we have _succeeded_ recently in isolating the "natural" ovalbumin gene presumably with attached regulatory sequences. Following amplification of these sequences in bacterial plasmids and confirmation of its "novel" structure, this DNA should prove quite valuable for understanding the interactions of RNA polymerase, nonhistone chromatin proteins and hormone receptors during regulation of eucaryotic gene expression.

278 INTERACTION OF THYROID HORMONES WITH TARGET CELLS, Jack H. Oppenheimer, Harold L. Schwartz, Wolfgang H. Dillmann and Howard C. Towle, Section of Endocrinology and Metabolism, Department of Medicine, University of Minnesota, Minneapolis, MN 55455.
Although the basis for the protean manifestation of thyroid hormone effects on mammalian tissues remains obscure, a growing body of evidence has suggested that triiodothyronine (T_3) action is initiated at a set of specific nuclear receptor sites. These appear to be nonhistone proteins of approximate M.W. 50,000. Unlike the situation for steroid hormones, cytoplasmic binding is not required for translocation to the nucleus. Receptors have been identified in all T_3-responsive tissues examined to date. Although the sites obtained from various tissues appear similar by physico-chemical criteria, their concentration varies greatly from one tissue type to another. A rapid exchange of T_3 between nuclear, cytoplasmic and plasma T_3 pools has also been demonstrated. The percent of total available sites occupied in each tissue under physiological conditions appears the same, approximately 40-50%. Occupancy by T_3 is associated both with a highly generalized increase in transcriptional activity as determined by total content and sequence complexity analysis of liver poly(A)-mRNA as well as with increases of certain specific products such as α_{2u} globulin, malic enzyme (ME), mitochondrial α-glycerophosphate dehydrogenase (α-GPD) in the liver and growth hormone (GH) in the pituitary. The relationship between nuclear occupancy and the rate of induction of new protein appears linear in the case of pituitary growth hormone, but for α-GPD and ME in liver the relationship is highly nonlinear with increasing amplification of the signal for new protein formation as the sites are progressively occupied. At full saturation, the rate of induction of these enzymes is approximately 10-19 fold that which characterizes the euthyroid state when only half of the sites are occupied. Amplified responses may have special significance in understanding the toxic manifestation of thyroid hormone excess. The response to T_3 is also determined by the level of other hormones as well as by tissue-specific and metabolic factors. Thus, neither adult nor neonatal brain responds to T_3 with increased oxygen consumption even though the concentration of T_3 receptors per g tissue in neonatal brain approximates that of responsive adult liver. Of interest is the recent finding that the receptor concentration in liver is reduced by partial hepatectomy and starvation, a reduction which results in the anticipated decrease in ME response to T_3 but leaves unimpaired the α-GPD response. This dissociation is compatible with the hypothesis that metabolic factors may bring about a selective reduction in receptor sites associated with specific genes such as the gene for ME. Recent studies have also indicated that glucagon, which is known to be elevated during starvation and after partial hepatectomy, results in a decrease in hepatic receptor sites. An understanding of the factors controlling receptor concentration and post-receptor mechanisms appears essential in understanding the diversity of thyroid hormone effects at the tissue level.

279 EFFECT OF THYROID HORMONES ON BIOCHEMICAL PROCESSES, Isidore S. Edelman, Cardiovascular Research Institute, University of California, San Francisco, Ca. 94143.
Thyroid thermogenesis appears to result from a complex series of events, including an expanded mitochondrial capacity for oxidative phosphorylation, an increase in energy expenditure in active Na^+ transport and probably a simultaneous increase in Na^+ (perhaps K^+ as well) permeability. To elucidate the nature of thyroidal stimulation of the Na^+ pump, kinetic and Na^+ pump labeling studies were carried out in collaboration with Chu-Shek Lo. Administration of T3 to hypothyroid rats elicited 50-70% increases in V_{max} for ATP, Na^+ and K^+, respectively in rat renal cortex. There were no changes in the apparent K_m's for ATP, Na^+ and K^+. Moreover, P^{32} incorporation from $ATP^{32}(\gamma)$ and ^3H-ouabain binding in rat renal cortex confirmed that the increase in the number of pump sites accounted quantitatively for the thyroid-dependent increase in NaK-ATPase activity. Double isotope incorporation studies (^3H & ^{35}S-methionine) revealed that thyroid hormone induced the synthesis of the large sub-unit of the Na^+ pump with no change in the rate of degradation of this sub-unit. Since T3 receptors have been identified in nuclei of target cells, and the hormone is an inducer of RNA and protein synthesis, it is probable that the thermogenic and Na^+ pump effects are also a consequence of the direct action of the hormone on the genome. To be able to test for this possibility, an isolated cell system thermogenically responsive to T3 was developed, in collaboration with F. Ismail-Beigi and D. M. Bissell. Primary hepatocyte cultures prepared from hypothyroid rat liver by collagenase perfusion were maintained in a Medium with 2% hypothyroid rat serum. Addition of T3 to the cultures at six hours after cell plating increased Q_{O_2}, NaK-ATPase and α-glycerophosphate dehydrogenase (GPD). These increases were detectable at 24 hours and maximal at 72-96 hours. Seventy-two hours after addition of T3 to the medium, Q_{O_2} increased from 25 to 38 μL O2/mg protein per hour. Concomitantly, NaK-ATPase and GPD activities increased 40% and 61%, respectively, while Mg-ATPase remained unchanged. T3 at concentrations of 4×10^{-11} M, 4×10^{-10} M and 4×10^{-9} M elicited approximately 0%, 25% and 75% (respectively) of the maximal change in Q_{O_2}. Stimulation of NaK-ATPase and GPD by T3 occurred over the same dose range. ATP content remained invariant at about 10 nmole/mg protein after 72 hours of exposure to T3 at concentrations up to 2×10^{-7} M. We conclude that primary hepatocyte cultures provide, for the first time, a valid model for the study of the biochemical basis of thyroid thermogenesis *in vitro*. The data indicate that T3 exerts a direct and co-ordinate stimulatory effect on Q_{O_2} and on NaK-ATPase in this experimental system.

Technology for the Identification of Cell Surface Receptors

280 TARGET SIZE ANALYSIS OF ADENYLATE CYCLASE, Werner Schlegel, National Institutes of Health, Bethesda, Maryland 20014

Irradiation inactivation analysis (12 MeV electrons) of the functional target size of adenylate cyclase in hepatic plasma membranes has been carried out under a variety of conditions with several effector ligands (glucagon, Gpp(NH)p, GTP, fluoride ion). Under non-cyclase conditions (non-treated membranes), the enzyme displays two characteristic sizes, a large size (about 1.4×10^6) and a smaller size (3×10^5). The actions of the various effectors differentially affect the distribution of these sizes. Pretreatment of the enzyme under assay conditions with Gpp(NH)p (or GTP) leads to "dispersion" of the large size to the small size of the enzyme system. Pretreatment with glucagon (+GTP) also leads to dispersion; however, the size of the dispersed state is about 5×10^5 in contrast to 3×10^5 given with the nucleotides (or fluoride ion). Gpp(NH)p ($\pm$ glucagon) gives a persistently active state with molecular weights in the range of 3×10^5. The glucagon receptor, in its unoccupied state, has a molecular weight of 6×10^5, which is higher than that of the enzyme system activated by the nucleotides and/or hormone. The data suggest an important role for aggregation and dispersion mechanisms in the regulation of adenylate cyclase activity by hormones and guanine nucleotides.

281 INTERACTION OF IODINATED ENKEPHALIN WITH OPIATE RECEPTORS IN CELLS, Kwen-Jen Chang, Richard J. Miller and Pedro Cuatrecasas, Wellcome Research Laboratories, Research Triangle Park, N.C. 27709

High specific activity ^{125}I-labeled derivatives of the metabolically stable enkephalin analogs, [D-ala^2, D-Leu^5] and [D-ala^2, leu^5]-enkephalin, have been prepared. These derivatives show stereospecific binding to morphine receptors in brain particulates and in many cell lines. Levorphanol is considerably more potent in displacing specifically bound enkephalin than dextrorphan. The binding is specifically inhibited by low concentrations of the natural leu- and met-enkephalins, and β-endorphin but not β-lipotropin. The association and dissociation rate constants of binding to cells are 3×10^7 liters mol^{-1} min^{-1} and 3.5×10^{-2} min^{-1}, respectively, at 24°C. In cells, [^{125}I] [D-ala^2-D-leu^5]-enkephalin binding is saturated with 30 fmoles bound per 10^6 cells. The K_d is 1 to 2 nM. Scatchard and Hill plots reveal the presence of one binding site without cooperative interaction. The receptors in intact cells are very resistant to proteases and phospholipase A digestion, in contrast to those in membrane preparations which are very sensitive. This resistance apparently is not due to intracellular localization since the cell homogenate has the same number of receptor sites as intact cells. In cells the affinity of the receptor for enkephalin is very sensitive to the cationic composition of the medium. Removal of the divalent cations, Mg^{2+} and Ca^{2+}, reduces the affinity by about 3.5 fold. The biological potencies of series of drugs and enkephalin anologs are compared to the potency in displacing [^{3}H] Naloxone and [^{125}I]-enkephalin binding in both cells and brain membrane preparation.

282 A CROSSLINKING APPROACH TO THE ORGANIZATION OF H-2 IN THE EL-4 CELL, John W. Huggins and C. Fred Fox, The University of California, Los Angeles, Los Angeles, CA 90024

The murine histocompatability antigen H-2 is an intrinsic cell surface glycoprotein. Evidence derived from extraction and co-purification has led to models for its organization on the cell surface. The question is clouded, however, by the problems of association induced by the extraction and purification procedures. Chemical crosslinking offers an approach to nearest neighbor interactions since cells can be crosslinked and any unreacted crosslinker destroyed before cells are disrupted. Current crosslinking approaches are not, however, applicable to proteins present as minor components since they are not easily detectable. We therefore, chose to purify the crosslinked H-2 complex and look at those proteins which are associated with H-2. Murine lymphosarcoma EL-4 cells were surface labeled with ^{125}I by lactoperoxidase, crosslinked with DTBP, a cleavable imidate crosslinker at temperatures from 4-37°C. the reaction quenched, extracted with 0.5% NP-40 and H-2 purified by immunoprecipitation and analyzed by two-dimensional off-diagional electrophoresis. The major association seen was between proteins of 46,000 and 12,000 daltons identified as H-2 and Beta$_2$ microglobulin, which accounted for up to 40% of the total extracted H-2. The association was not due to native or induced disulfide since iodoacetamide treated cells did not show the complex in noncrosslinked cells. A second crosslinked complex corresponding to a H-2 dimer accounted for 1-3% of the total extracted H-2. A minor association of H-2 with certain other proteins could be seen.

283 GAP JUNCTIONS AND ADRENOCORTICOTROPIC HORMONE (ACTH) SENSITIVITY IN Y-1 TUMOR CELLS, Sandra A. Murray, William J. Larsen and Sam T. Donta, University of Iowa, Iowa City, Iowa 52242.

ACTH-insensitive and ACTH-sensitive Y-1 tumor cells each respond to the addition of cholera toxin to their medium by rounding and producing steroid hormone. The ACTH sensitive cells also round and produce steroids after addition of ACTH but the kinetics of both responses are different from those of cholera toxin stimulated responses. In the case of both rounding and steroid production, the lag before response and the duration of the response are significantly longer in cholera toxin treated cells over a wide range of toxin concentration. In addition, scanning electron microscopy reveals that dense areas of ACTH sensitive cultures round in response to cholera toxin but not in response to ACTH.

Freeze fracture electron microscopy reveals numerous gap junctional particle aggregates within ACTH sensitive Y-1 membrane after treatment with ACTH while none are observed in the membrane of untreated or cholera toxin treated ACTH-insensitive Y-1 tumor cells.

Since it seems likely that the behavior of gap junctions in a variety of tissues, including adrenal cortical cells, is related to peptide hormone action (Larsen, W.J. Tiss. Cell, 9:373), we are investigating further, effects of ACTH, ACTH analogs, cholera toxin and other steroidogenic agents on gap junctions in Y-1 tumor cells.

284 SPECIFIC BINDING OF ENKEPHALIN TO NEURONAL CELLS AND MEMBRANES, Edward J. McMurchie and C. Fred Fox, Molecular Biology Institute, University of Calif., Los Angeles, CA 90024

Methionine enkephalin, Tyr-Gly-Gly-Phe-Met (met^5-enkephalin) is one of class of mammalian brain peptides which may function as endogenous neurotransmitters which interact with the opiate receptor. Hybrid neuronal cells (rat glioma x mouse neuroblastoma), which possess opiate receptors, have been shown to respond to opiates or enkephalins by a lowering of 3',5'-cAMP levels and by inhibition of adenylate cyclase. However, only limited specific binding of (dihydro)morphine has been demonstrated in such cells.

We are using a photoactivable cross-linking reagent (an azido met^5-enkephalin) as a probe for the opiate receptor in brain and hybrid neuronal cells. In hybrid neuronal cells, we find that underivatized ^{3}H-met^5-enkephalin binds most specifically at 2°, where 70% or greater competition is observed with excess enkephalin or morphine. At higher temperatures and with increased time of incubation, nonspecific binding is increased. At 37°, almost all binding is non-competible with excess unlabeled enkephalin or morphine. The majority of radioactivity bound at 37° is non-displaceable and remains cell-associated. Inclusion of bacitracin or excess tyrosine in the medium does not affect the level of cell-associated radioactivity, while the opiate antagonist naloxone significantly elevates this level. The plasma membrane fraction from hybrid neuronal cells specifically binds ^{3}H-met^5-enkephalin at 2°, 22°, and 37° (70% or greater at 22°). These data suggest that in hybrid neuronal cells at least, the specific binding of enkephalin to the opiate receptor may be coupled to processes which internalize part or all of the enkephalin molecule at 37°. These processes may or may not relate to the molecular action of enkephalins.

285 MECHANISM OF Mg·ATP REGULATION OF MEMBRANE BOUND TYPE 1 cAMP ACTIVATED PROTEIN KINASE, James R. Owens and Boyd E. Haley, Dept Biochem, Univ of Wyoming, Laramie, WY 82071

The transmembrane signaling elicited by some hormones binding to outer cell surfaces may result in the intracellular production of cAMP and subsequent activation of specific protein kinases which catalyze the phosphorylation of specific cellular proteins. The phosphorylation of these proteins may cause a change in cellular properties such as increased membrane permeability or activation of specific enzymes or metabolic pathways. Using isotopically labeled, photoaffinity analogs of cAMP and ATP, [^{32}P]8-N_3cAMP and [β,γ^{32}P]8-N_3ATP respectively, we have investigated nucleotide involvement in the regulation of the membrane bound, cAMP activated, protein kinase of human erythrocytes. 8-N_3cAMP is an excellent biological mimic of cAMP and a proven photoprobe for cAMP binding proteins (1,2). Both Mg·ATP and Mg·8-N_3ATP prevent photoincorporation of [^{32}P]8-N_3cAMP into the regulatory subunit(R) of this kinase. [β,γ ^{32}P]8-N_3ATP is also photoincorporated into R at concentrations consistent with those that decrease both [^{3}H]cAMP & [^{32}P]8-N_3cAMP reversible binding to R. The presence of catalytic subunit (C) is not required for MgATP or Mg8-N_3ATP reversal of cyclic nucleotide binding. However, presence of C does decrease the concentration of MgATP required for inhibition of photoincorporation of [^{32}P]8-N_3cAMP. Mg8-N_3ATP is not a substrate for the kinase eliminating autophosphorylation from the regulatory mechanism. The data support a mechanism in which MgATP has a regulatory site on R that is different from the cAMP site. Binding of MgATP decreases affinity for cAMP & increases it for C. The increased affinity of R for C in the presence of MgATP is proposed to be the "turn off" mechanism for some cAMP activated protein kinases.

1)Haley,B.,(1975) Biochemistry 14 3852. 2)Owens, J. & Haley,B.,(1976)J.Supra Mole Structure 5 91

286

CHARACTERIZATION OF GLUCAGON RECEPTORS ON HEPATOCYTE AND HEPATOMA PLASMA MEMBRANES, Marvin Bregman and Daniel Levy. Univ. of Southern California, School of Medicine, Dept. of Biochemistry, Los Angeles, Calif. A photosensitive derivative of glucagon, ^{125}I-N^{ϵ}-4-azido-2-nitrophenyl-glucagon (NAP-glucagon) has been synthesized and used to specifically label glucagon binding proteins in hepatocyte plasma membranes. Photolysis of this derivative in the presence of intact hepatocytes or hepatocyte plasma membranes results in the incorporation of radioactivity primarily into membrane components with a molecular weight range of 23,000-25,000. The degree of labeling was significantly increased by Mg^{2+} and PP_i. The binding properties were also effected by Mg^{2+}. The binding and covalent labeling was inhibited by greater than 90% in the presence of excess glucagon. Binding and labeling studies suggest that the labeled membrane proteins may be components of the glucagon receptor. Hepatoma tissue culture cells were shown to bind less than 2% of glucagon as well as NAP-glucagon when compared to normal isolated hepatocytes. Similar results were obtained from the corresponding purified plasma membranes. Photolysis of NAP-glucagon in the presence of the hepatoma membrane system did not result in the labeling of any membrane components, further supporting the specificity of the receptor labeling reaction with normal hepatocytes.

287 RECONSTITUTION OF NEUTRAL AMINO ACID TRANSPORT ACTIVITY FROM CHOLATE EXTRACTS OF EHRLICH ASCITES TUMOR CELLS, Gary Cecchini and Dale L. Oxender, Department of Biological Chemistry, The University of Michigan, Ann Arbor 48109

The understanding of the molecular basis of amino acid transport will require the isolation and characterization of transport components from the membranes of cells. Therefore, plasma membrane vesicles from Ehrlich ascites tumor cells were solubilized with 2% cholate, 0.5 mM EDTA, 1 mM dithiothreitol, in 0.1 M potassium phosphate buffer, pH 7.4. The extract was centrifuged at 100,000 X g for 1 hr and the supernatant usually contained 40-60% of the protein found in the membrane vesicles. The solubilized activity was partially purified by a combination of ammonium sulfate precipitation and column chromatography. The partially purified protein fraction could be reconstituted into artificial lipid vesicles prepared from soybean phospholipids. The soybean phospholipid vesicles were prepared by the cholate dialysis method of Racker (J. Biol. Chem. 247:8198, 1972). The solubilized protein was then added to the liposomes in a ratio of 20:1 (phospholipid to protein). Liposomes could also be reconstituted by sonication and a freeze-thaw step as shown by Kasahara and Hinkle (J. Biol. Chem. 252:7384, 1977). Active transport of alanine or leucine could be demonstrated into the reconstitued soybean phospholipid vesicles. The active accumulation of these amino acids was sodium ion dependent. Transport was assayed by filtration through 0.45 μ millipore filters or by utilizing small Sephadex G-50 columns for separation of free and bound ligand as described by Penefsky (J. Biol. Chem. 252:2891, 1977). The transport properties of the partially purified extract are similar to those found for Ehrlich ascites plasma membrane vesicles.

(Supported by NIH Grant GM 20737.)

288 HYDRODYNAMIC PROPERTIES OF THE INSULIN RECEPTOR FROM TURKEY ERYTHROCYTES, Ramani A. Aiyer, The Biological Laboratories, Harvard University 16 Divinity Avenue, Cambridge, Mass. 02138

The insulin receptor from turkey erythrocyte plasma membranes has been solubilised with Triton X-100 and its hydrodynamic properties investigated using the techniques of density gradient ultracentrifugation and gel filtration. Sedimentation was carried out in linear 5-20% sucrose gradients in the presence of 0.1% Triton X-100. A major peak of insulin binding activity moves between catalase ($s_{20,w}$ 11.3S) and fumarase ($s_{20,w}$ 8.9S). The position of this peak in D_2O is shifted nearer to fumarase relative to its position in H_2O. This result allows one to calculate the partial specific volume of the protein-detergent complex and to estimate the amount of detergent bound. Gel filtration was performed on Sepharose CL-6B. The insulin-receptor complex elutes ahead of β-galactosidase ($s_{20,w}$ 15.9 S, Stokes' radius, 69.1 A). This indicates that the insulin receptor is a large, asymmetric molecule.

289 RECEPTOR-BOUND CONFORMATION ON ENKEPHALIN, Garland R. Marshall, T.M. Balasubramanian and Frederic A. Gorin, Washington University School of Medicine, St. Louis, MO 63110

The initial event in receptor activation is recognition of the effector resulting in binding and subsequent activation. Enkephalin, Tyr-Gly-Gly-Phe-(Leu, Met), is recognized by the same receptor which recognizes opiates. A model for the recognition requirements for this receptor has been postulated based on structure-activity relations in analgesics in *in vitro* systems. A systematic search of conformational space led to one conformer of enkephalin which presented the correct spatial orientation of groups required for recognition. Based on this conformer (Gorin and Marshall, Proc. Natl. Acad. Sci. U.S., Nov. 1977), the activity of twenty analogs of enkephalin with modification which effect the possible backbone conformations were predicted. Thirteen of these analogs have subsequently been reported and are in agreement with the predictions. Further synthetic efforts to eliminate conformational variables have resulted in an analog, Tyr-D-Ala-Gly-Ind (Ind = 1-aminoindane), in which two degrees of freedom have been removed and high activity is retained. Comparison of these results with physical studies by others conclusively demonstrates that the analgesic receptor either induces the conformation required for recognition and activation or selects a minor conformer from the ensemble available in solution.

(This work supported in part by Grant RR-00396 from the National Institutes of Health. F.G. is a Graduate Fellow, Medical Scientist Training Program (GM-02016).

290 ISOLATION OF CELL SURFACE PROTEINS THAT BIND TO CHROMAFFIN GRANULES, David I. Meyer and Max M. Burger, Biocenter, 4056 Basel, Switzerland.
We have previously postulated that recognition and binding precedes fusion of secretory granules with the cell membrane in exocytosis. Central to this hypothesis is the notion that a receptor molecule exists on the inner surface of the cell membrane whose function is to recognize and bind complementary sites on the secretory granule. In an effort to ascertain the presence of such a substance, we have developed an affinity chromatography system based on immobilized, fixed secretory granules.

To date, using this method, we have been able to isolate two major peptides from solubilized fractions enriched in plasma membranes. The amount of this material derived is directly proportional to the amount of plasma membrane (based on enzyme activity) present in solubilized fractions. Low pH (1.5) has been used most successfully as the elution medium. The molecular weights of these peptides in SDS are ca. 15,000 and 50,000.

291 SYNTHESIS AND CHARACTERIZATION OF A BIOTINYL DERIVATIVE OF PROPRANOLOL. Kathryn E. Meier and Arnold E. Ruoho, Dept. Pharmacology, Univ. of Wisconsin, Madison, WI 53706

We have synthesized a biotinyl derivative of the beta-adrenergic antagonist propranolol for use in the localization and purification of the beta-receptor. Biotinyl-N-hydroxy-succinimide was reacted with the amino group of hexaglycine to give biotinyl-hexaglycine. The carboxyl group of biotinyl-hexaglycine was then reacted with the amino group of 1-(1-naphthoxy)-3-N-(ethylamino)propan-2-ol (abbreviation: NEDA) to give biotinyl-hexaglycyl-NEDA (abbreviation: BGN). This bifunctional molecule binds with high affinity to the biotin-binding protein avidin. The half-time for dissociation of BGN from avidin is 7 hours at 37° and 16 hours at 25°. We have used the duck erythrocyte, which possesses a catecholamine-activated adenylate cyclase, as a model beta-receptor system. The avidin-BGN complex inhibits isoproterenol activation of adenylate cyclase in duck erythrocytes (K_D=1400 nM) and in duck erythrocyte ghosts (K_D=3000 nM). The complex also displaces [^{3}H]-dihydroalprenolol from the digitonin-solubilized beta-receptor (K_D=110 nM). The dissociation constants of BGN alone in these assays were 900 nM, 800 nM, and 70 nM, respectively. These data indicate that BGN is capable of binding simultaneously to the beta-receptor and to avidin. By utilizing fluorescein- or ferritin-labelled avidin, we may be able to visualize catecholamine binding sites on cell surfaces. In addition, BGN may be bound to avidin-Sepharose and used for affinity chromatography of the solubilized beta-receptor. (Supported by NIH Grant # NS 12392).

292 A POSSIBLE MECHANISM FOR IMMUNE COMPLEX/Fc RECEPTOR TRANSDUCTION IN ALVEOLAR MACROPHAGES, Sorell L. Schwartz, Mark W. Cowden and Po-Shun Wu, Georgetown University, Washington, D.C. 20007
Previous studies from this laboratory indicated that immune complexes cause an increase in fluidity of lipid-protein monolayers prepared from plasma membrane of rabbit alveolar macrophages. These studies were extended to compare the thermodynamics of monolayer expansion with the thermodynamics of endocytosis by intact cells. Lyophilized membrane from normal (N-M) and BCG-induced (BCG-M) RAM were placed on an automated Langmuir trough and the monolayer allowed to form. Experiments were done in which the surface pressure was constant and the area allowed to change. An immune complex prepared from egg albumin and rabbit anti-egg albumin expanded the mambrane a low concentrations (5×10^{-8} for N-M; 5×10^{-9} for BCG-M). This expansion was apparently specific based on less or no response with other substances and with non-RES cell membranes (i.e. NRK cell line). Arrhenius plots revealed a Tc of 22 °C for N-M monolayers and 28° C for BCG-M monolayers. Pinocytic activity of BCG-induced RAM was enhanced by the immune complex to a greater degree than the enhancement of the pinocytic activity of normal RAM by the immune complex. Arrhenius plot of pinocytic activity showed a Tc of 23.5° C for normal RAM and 28 ° C for BCG-induced RAM. This comparison of the thermodynamic properties of the monolayer and pinocytic responses suggests that the immune complex may initiate macrophage endocytic response via a viscotropic activation of effector substances (ATPase?) within the membrane.

293 AUTOANTIBODIES INHIBIT INSULIN BINDING AND IMMUNOPRECIPITATE THE SOLUBLE INSULIN RECEPTOR, L.C. Harrison, J.S. Flier, C.R. Kahn, and J. Roth, NIH, Bethesda, MD 20014.
We have previously shown that serum from patients with an unusual form of diabetes contains cell membrane autoantibodies which inhibit the binding of insulin to intact membrane receptors and elicit insulin-like biological responses. We have now studied the effect of these anti-receptor sera on the binding of ^{125}I-insulin to the Triton-solubilized human placental insulin receptor. The anti-receptor sera inhibit insulin binding to the soluble receptor at titers comparable to those for receptors on intact cells. Inhibition of binding to soluble receptors appears to be due to a reduction in the number of available binding sites, whereas with intact membrane receptors the predominant effect of the anti-receptor sera is to decrease receptor affinity. All the anti-receptor sera tested were able to quantitatively immunoprecipitate the ^{125}I-insulin-labeled soluble receptor after addition of anti-human IgG, antiserum against either κ or λ light chains, or Staphyloccal protein A covalently bound to agarose. Immunoprecipitation titers ranged from 1/50 to 1/6400, and in most cases were higher than the corresponding binding inhibition titers. IgG prepared from the serum with the highest titer had a precipitating capacity of 24 mmole insulin binding sites per mole of IgG. Other ligands (EGF, hGH, oPRL) which were specifically bound to the soluble placental membrane were not precipitated by these sera. Together, these findings further demonstrate the direct interaction of these anti-receptor antibodies with the insulin receptor. They also indicate that immunoprecipitation is a sensitive assay for insulin receptor antibodies and that receptor purification is feasible with this approach.

294 FURTHER PURIFICATION AND RECONSTITUTION OF THE INSULIN-SENSITIVE D-GLUCOSE TRANSPORT ACTIVITY FROM RAT ADIPOCYTE PLASMA MEMBRANES. Michael F. Shanahan and Michael P.Czech, Brown University, Providence, Rhode Island 02912.
We have reported partial purification of the rat adipocyte hexose transport system using dimethylmaleic anhydride extraction (J. Biol. Chem. 252: 6554-6555, 1977). More recently we have reported additional purification and reconstitution of this transport system into phospholipid vesicles (J. Biol. Chem. 252: 8341, 1977). Although this cholate-solubilized, reconstituted membrane fraction contained predominantely 94,000 dalton glycoproteins, trace amounts of both 78,000 and 56,000 dalton protein fractions were detected on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). We therefore initiated procedures to isolate these various fractions in order to determine which was associated with transport activity. Attempts to reconstitute hexose transport from membranes solubilized with other detergents, i.e. Triton-X-100 and SDS, resulted in complete loss of activity. Cholate-solubilized membranes were therefore subjected to column gel chromatography in the presence of 1% sodium cholate using either Sephadex G-200 or Sepharose 6B. The majority of protein applied to both columns eluted in the same volume as Dextran Blue (M.W. ∿ 2,000,000) indicating the presence of large protein aggregates. When this fraction was applied to SDS-PAGE, all three protein bands were present. However, when reductant (5 mM dithiothreitol) was included in the eluant, in addition to the large multimeric complexes, small amounts of 78,000 and 56,000 dalton proteins eluted as separate and distinct peaks which appeared as single bands on SDS-PAGE. Experiments are currently in progress to test reconstitution of these column fractions in order to further resolve the membrane protein components of this transport system.

295 PREPARATION OF ANTIBODIES TO A PARTIALLY PURIFIED PROTEIN FRACTION FROM RAT ADIPOCYTE PLASMA MEMBRANES CONTAINING HEXOSE TRANSPORT ACTIVITY. Dennis J. Pillion and Michael P. Czech, Div. of Biology and Medicine, Brown University, Providence, RI 02912.

Rat adipocytes were isolated and plasma membrane vesicles were prepared according to established procedures and extrinsic proteins were removed from the vesicles by treatment with dimethylmaleic anhydride (DMMA) (Shanahan, M.F. and Czech, M.P.,J.Biol.Chem. 252 (1977) 6554-61). The residual membrane material, which consisted mainly of 2 glycoprotein bands on SDS-PAGE, was administered to rabbits in a 1:1 mixture with Freund's complete adjuvant. Serum was collected and the Ig fraction was partially purified by treatment of the whole serum with $(NH_4)_2SO_4$. DMMA-treated membranes were incubated with the Ig fraction or whole serum from untreated rabbits or from rabbits which had been injected with DMMA-treated membranes; the membranes were then centrifuged, washed, and solubilized in one of the following solutions: 0.1% Triton X-100, 1% Triton X-100 or 0.1% Triton X-100 plus 2% cholate. An aliquot of each solution was then placed on an immunodiffusion plate which contained the same amount of detergent as the sample, and goat anti-rabbit Ig conjugated with fluoroscein was then added to each plate. DMMA-treated membranes formed a precipitin line in all of the different detergent combinations when the Ig fraction from treated rabbits was used; no precipitin line formed when the Ig fraction from untreated rabbits was used. Studies are currently in progress to determine the effects of the Ig fraction from treated rabbits on insulin-receptor interaction and hexose and anion transport.

296 SPECIFIC INTERACTION OF THE THIRD COMPONENT OF HUMAN COMPLEMENT (C3) WITH LEUKOCYTES AND ERYTHROCYTES, Brian F. Tack,David M. Segal and Alan N. Schechter, Lab. Chem. Biol, NIAMDD and Immunol. Branch, NCI, NIH, Bethesda, Md. 20014

The ability of phagocytic and lymphoid cells, including the macrophage, polymorphonuclear leukocyte, and B-lymphocyte, to recognize and bind to immune complexes (IC) is dependent in part on the interaction between an IC bound form of C3 and cell surface receptors specific for this protein.We are attempting to measure directly the equilibrium and kinetic parameters of binding between activation products of human C3 and their receptors on human blood cells. The activation fragments C3b, C3c, and C3d have been generated by digestion with porcine elastase and subsequently purified.Each fragment has been radiolabeled using chloramine-T and $Na^{125}I$ to a specific activity of 1-2 x 10^6 cpm/ug. The interaction with erythrocytes and lymphocytes has been measured using a direct binding assay where equilibrium saturation, time course, and competition experiments have served to characterize the specific nature of these interactions. The separation of bound and free ligand was obtained by layering the cell suspension on a column of synthetic oil (density = 1.033 gm/cc) and pelleting the cells by spinning in a rapidly accelerating centrifuge. The nonspecific cell associated component was determined by measuring the cell associated counts in the presence of a 40-fold molar excess of nonlabeled protein. Initial studies have indicated that the binding of C3 and C3b to human red cells is a saturable reaction with an association constant (K_a) of 1-4 x 10^7 M^{-1}. The number of receptors/cell is about 1 x 10^3. Similiar studies with an established lymphoblastoid cell line have again indicated a saturable reaction with a K_a of 0.7-1.0 x 10^7 M^{-1}. The number of receptors/cell is estimated to be 1.5-2.0 x 10^4.

297 A SERIOLOGICAL ASSAY FOR THE DETECTION OF CELL SURFACE RECEPTORS FOR NERVE GROWTH FACTOR, Arne Sutter, John Samuelson, Astrid Zimmermann, and Eric M. Shooter, Dept. Neurobiol., Stanford Univ. Sch. Med., Stanford, CA 94305

We have developed a hormone, anti-hormone-antiserum and complement dependent cytotoxicity assay which can be used as an alternative to hormone receptor binding assays for the study of Nerve Growth Factor (NGF) receptors. In receptor binding assays using radiolabeled NGF (^{125}I-NGF) two specific cell surface receptors with different affinities (K_d(I):2.3x10^{-11}M; K_d(II):1.7x10^{-9}M) can be detected on sensory ganglia cells of chick embryos. The analysis of the NGF concentration dependence of cytotoxicity and the dissociation behaviour of NGF as determined in binding assays indicates that cytotoxicity is mediated by NGF binding to its high affinity receptor (I) alone. When single cell suspensions prepared from 8 day old chick embryos (E8) are incubated with NGF, anti-NGF-antiserum and complement 20(±3)% of the cells can be killed. After separation of the non neuronal cells (comprising 50% of the cells in the dissociate) the percentage of cells killed in the assay increases from 20 to 40%. The killed cells are neurons. These results are in concordance with binding assay data using ^{125}I-NGF which show a doubling in the amount of high affinity receptor sites on the neuronal enriched cell population. Using this cytotoxicity assay no high affinity NGF receptors could be detected on sensory ganglia cells of early developmental stages (E4) or on non neural tissues like liver and heart (E8) which again is in accordance with binding assay results. We propose that this NGF dependent cytotoxicity assay can be used as a fast and easy test for the mapping of high affinity NGF receptors in the peripheral as well as central nervous system during ontogeny of the chick.

Receptor Mutants: Cell Lines Altered in Ligand Interactions

298 LIPOPOLYSACCHARIDE (LPS) EFFECTS ON CHINESE HAMSTER OVARY CELLS AND DERIVATION OF AN LPS-RESISTANT VARIANT LINE, Kenneth W. Brunson and Garth L. Nicolson, Department of Developmental and Cell Biology, University of California, Irvine, Calif. 92717
The *in vitro* growth of line CHO·K1·PRO was inhibited by gram-endotoxin (lipopolysaccharide [LPS] from *Salmonella typhosa* ST0901). Bizarre cell shapes, vacuolization and apparent PM defects at the EM level occurred concomitantly with growth inhibition. Growth was not inhibited until 3-4 days in LPS-DMEM+10% FCS. Inhibition was dose-dependent up to maximal at 500 μg LPS/ml (~10X fewer cells at day 7). Vacuolization and cell shape changes were observed by light and EM, began at 3-4 days incubation, and were most pronounced at day 7. Evidence indicated that the vacuoles contained lipid. The parental CHO line, upon repeated culturing with high LPS-DMEM+10% FCS yielded a stable LPS-resistant variant not inhibitable at high LPS. The variant cells or their clones displayed altered properties compared to the parental line including changes in morphology, adhesion and endocytosis. Variant cells showed an elongated morphology and grew mostly in swirls. The parental cells were markedly density-inhibited, whereas variant cells exhibited considerable vertical growth (into "mounds") at confluency. When sparse cultures were removed from TC dishes by 2 mM EDTA, no significant differences were seen between rates of detachment in parental or variant cells. However, at confluency, ~100% of the variant cells vs 35% parental cells were removed in 1 hr by EDTA. Measurements of ^{125}I-ferritin uptake by variant cells showed a 2X increase in uptake in LPS, while the parental cell line showed as much as a 20X increase of ^{125}I-ferritin uptake in LPS. These studies indicate that LPS can have profound cellular effects on CHO cells *in vitro*. Supported by NCI contract CB-74153 and ACS grant BC-211A (to GLN).

299 COLCHICINE POTENTIATES ISOPROTERENOL-STIMULATED CYCLIC AMP ACCUMULATION IN S49 CELLS. Michael S. Kennedy, Paul A. Insel, Depts. of Med. and Pharm., U. of Cal., S.F. 94143
Cell surface receptors for several agents including immunoglobulins, lectins, and insulin can be regulated by the cellular cytoskeleton(microtubules and microfilaments). Recent evidence indicates that the microtubular inhibitor colchicine(Col)augments hormone-stimulated increases in cAMP in leukocytes and macrophages. In order to examine the mechanism of this augmentation, we have studied Col's effect on S49 lymphoma cells, a cultured cell line in which variant clones have been isolated with lesions at individual steps in the pathway of cAMP generation and function. In wild-type(WT)S49 cells incubated with 5 x 10^{-5}M Col the beta-adrenergic agonist isoproterenol(Iso)stimulates cellular accumulation 2-5 fold more than in control cells. Col does not affect the K_a for Iso but instead increases maximal response. Ro 20-1724, a potent phosphodiesterase(PDE)inhibitor, potentiates the response to Col suggesting that Col's effect is not simply inhibition of PDE. In parallel studies using an S49 mutant clone with absent cAMP dependent protein kinase activity, Col potentiates the Iso response as in wild-type cells. In contrast Col does not stimulate response to Iso in a mutant clone that lacks 'coupling' between beta-adrenergic receptors and adenylate cyclase(AC). In purified plasma membranes from WT cells Col has no effect on basal, Iso or NaF-stimulated activity. Direct receptor binding studies using ^{3}H-dihydroalprenolol(DHA)and intact cells indicate that Col's potentiation of Iso cannot be explained by changes in the number or affinity of DHA binding sites. We conclude: 1) Col potentiates Iso-stimulated cAMP accumulation in S49 cells; 2) this potentiation results from altered receptor-AC interaction or an action on AC not detectable in plasma membranes; and 3) this effect does not require cAMP-dependent protein kinase.

300 RECONSTITUTION OF CHOLERA TOXIN-ACTIVATED ADENYLATE CYCLASE. Gary L. Johnson, Harvey R. Kaslow and Henry R. Bourne, University of California, San Francisco, CA 94143
Membranes of S49 cells have an absolute requirement for GTP or guanylyl imidodiphosphate (Gpp(NH)p for activation of adenylate cyclase (AC) by hormones or cholera toxin (CT). In the absence of hormone, Gpp(NH)p but not GTP increases AC activity, probably due to its resistance to hydrolysis. After CT treatment of membranes, GTP activates AC as well as Gpp(NH)p. If membranes from wild type (WT) cells are solubilized with Lubrol 12A9 after CT treatment, AC activity is low in response to GTP or Gpp(NH)p. When this extract is incubated with membranes of an S49 variant not regulated by guanine nucleotides (cyc$^-$) regulation is reconstituted. The GTP/Gpp(NH)p ratio of the reconstituted AC is similar to that observed with intact WT membranes (GTP/Gpp(NH)p = 1.0 with CT and 0.1 without). The reconstitution is dependent on both the amount of donor extract and recipient membranes. N-ethylmaleimide treatment of cyc$^-$ membranes abolishes the reconstitution indicating that functional cyc$^-$ membranes are required. CT treatment of cyc$^-$ membranes before reconstitution with control WT Lubrol extracts has no effect on the GTP/Gpp(NH)p ratio. Likewise, addition of Lubrol extracts of CT-treated cyc$^-$ membranes does not alter the GTP/Gpp(NH)p ratio of control WT membranes. cyc$^-$ membranes have a Mn^{2+}-stimulated AC which is not regulated by GTP or Gpp(NH)p and is unaffected by CT. These findings suggest that an AC regulatory component of WT membranes and not the catalytic component of AC is required for reconstitution with cyc$^-$ membranes. From these results we predict: 1) WT extracts donate the guanine nucleotide regulatory component of AC; 2) this component is the substrate for CT; and 3) is altered in cyc$^-$, leading to loss of regulation by guanine nucleotides and a response to CT.

301 CONVERSION BY DETERGENTS OF RAT LIVER ADENYLATE CYCLASE TO A FORM REQUIRING MnATP AS SUBSTRATE, C. Londos, P.M. Lad, T.B. Nielsen, and M. Rodbell, NIH, Bethesda, MD 20014
A general feature of membrane-bound adenylate cyclase systems is the "lability" of the basal enzyme to dispersion by detergents. A stable form of the detergent-solubilized enzyme is obtained only if the membrane-bound enzyme is first pretreated with fluoride or Gpp(NH)p. However, we have found with the basal hepatic enzyme that the lability is evident only when MgATP is used as substrate; substitution of MnATP for MgATP reveals that substantial basal activity survives detergent treatment. This effect is independent of the detergent; it is seen with either Lubrol PX or with deoxycholate. In addition to the altered substrate requirement, the membrane-bound and solubilized forms of the basal enzyme exhibit other differences. In contrast to the membrane-bound form, the solubilized enzyme shows 1) weak stimulation by Gpp(NH)p; 2) little inhibition by adenosine; 3) strong inhibition by P_i or PP_i; and 4) an apparent loss of the Me^{2+}-reactive regulatory site (C. Londos and M.S. Preston; J. Biol. Chem. 252, 5957, 1977). Such dissimilarities between membrane-bound and solubilized cyclase are not seen if the membranes are pretreated with Gpp(NH)p prior to exposure to detergents. The characteristics of the solubilized basal hepatic enzyme are remarkably similar to those of the naturally occurring soluble adenylate cyclase found in mature rat testes. It would appear that separation of adenylate cyclase from components that confer regulation by divalent cations and guanine nucleotides produces a form of the enzyme that will turnover only MnATP; this may represent the free catalytic moiety. Such preparations could be useful in reconstructing some of the regulatory functions of adenylate cyclase seen in its membrane-bound form.

302 STUDIES OF THE DIPHTHERIA TOXIN RECEPTOR, Rockford K. Draper, Daniel Chin and Melvin I. Simon, Department of Biology, University of California, San Diego, La Jolla, California 92093.
The interaction of diphtheria toxin (DT) with a receptor is required for specific translocation of the toxin to the cell interior where cytotoxic activity is expressed. Very little is known about this receptor. We have found that concanavalin A (ConA) inhibits the cytotoxic effect of DT on cultured hamster cells by competing with the toxin for a surface receptor. The apparent dissociation constant of ConA for this site is about 10^{-8}M. We have also found that the mannose and N-acetylglucosamine containing glycopeptide from ovalabumin inhibits the cytotoxic effect of DT. It is likely that this oligosaccharide competes with the toxin receptor for DT. Our results suggest that the DT receptor is either an oligosaccharide containing molecule which binds ConA or that it is so close to such a site that the binding of ConA prevents the approach of DT.

We have isolated mutants with different phenotypes of DT resistance to study the relationship between the DT and ConA binding sites. Preliminary genetic analysis indicates the presence of several complementation groups. In one group, a 20-fold resistance to DT is accompanied by a decrease in the ability of ConA to inhibit DT. This further suggests the possibility of a structural relationship between the DT and the ConA binding sites.

303 HORMONE SENSITIVITY OF AVIAN CELLS IS ALTERED BY RSV TRANSFORMATION, J.R. Sheppard and R.R. Friis, University of Minnesota and Institute for Virologie, Geissen, Germany.

Catecholamine (β-adrenergic) hormonal responsiveness was determined in avian cells infected by RSV, RAV and several temperature sensitive variants of the RSV. Cellular cyclic AMP, which mediates the physiological effect of β-adrenergic hormones, was assayed as a measure of the cell's response. Normal, uninfected or RAV infected cells have a greater responsiveness to the catecholamine isoproterenol than the RSV infected cell. Likewise, the non-permissive grown (41°C) ts-RSV infected cells have a greatly increased hormonal responsiveness in comparison to the cells grown at 35°C, a condition which is permissive for transformation. Normal, RAV infected or wild type RSV infected cells do not change their hormonal responsiveness as a function of growth temperature. We also tested the hormonal responsiveness of ts virus mutants called "partially transformation-defective" (PTD) because they behave like ts mutants for some transformation characteristics but act like the wild type virus for others. One PTD mutant (#251) which is constitutive for high plasminogen activator activity was found to be super sensitive (even more than the uninfected cell) to β-adrenergic hormones at the non-permissive temperature but was essentially unresponsive (like the wild type RSV infected cell) at 35°. These data indicate that β-adrenergic hormone responsiveness is associated with the expression of growth regulated phenotype in this avian cell culture system. The PTD mutant study suggests that a membrane associated protease may participate in the activation of the adenylate cyclase enzyme. (This research was supported by an IUCC grant, the Leukemia Task Force and the Deutsche Forschurgsgemeinschaft)

304 UNRESPONSIVENESS OF C57BL/10ScN MICE TO MITOGENESIS INDUCED BY LIPOPOLYSACCHARIDE.
Kenneth B. Von Eschen, NIAID, Rocky Mountain Laboratory, Hamilton, MT 59840
Lipopolysaccharides (LPS) extracted from gram-negative bacteria are potent mitogens for bursa-equivalent lymphocytes obtained from many strains of mice. To date, only one mouse strain (C3H/HeJ) has been reported to be unresponsive to mitogenesis induced by LPS. The present report describes some characteristics of C57BL/10ScN (BL-10) mice which were also found to be unresponsive to LPS. The BL-10 mice used in these experiments were from a colony maintained at the Rocky Mountain Laboratory and derived from breeding pairs obtained from the Veterinary Resources Branch of the National Institutes of Health. Different LPS preparations were unable to trigger mitogenic responses in spleen cells from BL-10 mice. These same preparations were highly mitogenic for spleen cells from C57BL/6, BDF_1, and RML Swiss mice. BL-10 spleen cells made typical responses to other lymphocyte mitogens including concanavalin A, phytohemagglutinin, purified protein derivative and endotoxin protein. Unresponsiveness of the BL-10 spleen cells to LPS was not overcome by using different doses of LPS or by changing the day of assay. Also, unresponsiveness was not the result of a nonspecific toxic effect of LPS on cultured BL-10 lymphocytes. Further studies showed that lymphocytes from BL-10 and LPS responder mice bound similar amounts of radiolabeled LPS. Apparently, BL-10 lymphocytes can bind LPS but this binding does not result in mitogenic activation of the cells. Therefore, comparative studies of the events following binding of LPS by responsive and unresponsive lymphocytes may help to elucidate the mechanisms by which LPS induces mitogenesis.

Sialogangliosides as Cell Surface Receptors

305 EVIDENCE FOR A ROLE OF MEMBRANE CARBOHYDRATE IN THE FUNCTION OF THE INSULIN RECEPTOR: INSULIN ANTAGONISTIC AND INSULIN-LIKE PROPERTIES OF SACCHARIDE INHIBITORS OF CONCANAVALIN A.
Howard M. Katzen, Merck Institute for Therapeutic Res., Rahway, N.J. 07065
It is well known that concanavalin A (Con A) possesses a variety of insulin-like properties. In the present study, various carbohydrates that reflect the carbohydrate binding specificity of Con A also inhibit the binding of insulin-Sepharose to insulin receptors on fat cells in the affinity-binding buoyant density assay. Moreover, the carbohydrate inhibitory specificities for insulin-Sepharose and Con A bindings appear strikingly similar to each other. The glycosides also either antagonize or antagonize and mimic insulin's ability to stimulate glucose utilization by these cells. In equilibrium dialysis experiments, radio-labeled saccharides readily bind to Con A but have no affinity for insulin, indicating that the carbohydrates act by binding to the cell rather than to the insulin molecule. This is consistent with the abilities of the "insulin-like" glycosides to act in the absence of insulin. We previously observed an important contribution for the carbohydrate moiety in the insulin-like activities attributed to a degradation product of some aminoalkylglycosides (Cascieri et al and Durette et al, 1977). The present studies of a pattern of insulin antagonistic and insulin-like effects of various carbohydrates indicate a role for membrane carbohydrate in the action of insulin. A working hypothesis is presented which proposes that the exogenously-added saccharides act by mimicking or competitively inhibiting the interaction of a native, Con A-sensitive, carbohydrate on the membrane with a lectin-like saccharide binding site functionally linked to the insulin receptor.

306 LATERAL MOBILITY OF A FLUORESCENT GANGLIOSIDE GM_1 ANALOG IN CELL MEMBRANES.
J. Reidler*, C. Eldridge*†, Y. Schlessinger*†, E. Elson*, H. Wiegandt**, Depts. Chem.* and Appl. & Engr. Phys.†, Cornell U., Ithaca, N.Y. 14853 and Physiol.-Chem. Inst., Philipps-Uni., D-355 Marburg/L., BRD**.
Recent recognition of the role of gangliosides as membrane receptors has increased interest in the interactions of membrane-bound gangliosides with ligands and with other cell membrane constituents. To gain further insight into mechanisms of binding and entry of hormones and toxins into cells, a Fluorescence Photobleaching Recovery technique was used to study the lateral mobility of a fluorescein-labelled "lyso" ganglioside GM_1 analog (fl-GM_1) and its interactions with Cholera Toxin on 3T3 cell membranes. The behavior of the fl-GM_1 was similar to that of an artificial lipid probe, 3,3'-dioctadecylindocarbocyanine (diI). The fl-GM_1 ranged in the membrane over areas greater than $5 \mu m^2$ with diffusion coefficient, $D=(5.5 \pm 1.1) \times 10^{-9}$ cm^2/sec @ 23C. This diffusion coefficient is two-fold smaller than that of diI but is approximately twenty-five-fold greater than that of typical membrane proteins. As with diI, the mobility of the fl-GM_1 was independent of cell type and was not affected by drugs which interfere with metabolism or cytoskeletal structure. When cells or lipid bilayers were treated with Cholera Toxin, approximately one half of the fl-GM_1 was immobilized while the diffusion coefficient of the still mobile molecules was reduced two-fold. These results indicate that free fl-GM_1 does not exhibit strong, long-lived interactions with slowly moving or stationary membrane structures and that Cholera Toxin at high dose can impair the mobility of fl-GM_1. The extent to which these results are influenced by the lack in fl-GM_1 of one of the two gangliosides hydrocarbon chains or by presence of the fluorescein is now being tested using other types of ganglioside analogs.

307 GLYCOLIPIDS AS INDICATORS OF THE TUMORIGENIC TRANSFORMATION, D. James Morré, T. M. Kloppel, W. D. Merritt and T. W. Keenan, Purdue University, West Lafayette, IN 47907
Glycolipids, and especially gangliosides (sialoglycolipids), emerge as useful models for the study of cell surface heteroglycans. They are enriched at the surface of the cell and function as specific membrane receptors where their biological role is just beginning to be identified. Yet, there has appeared no simple correlation between ganglioside composition and the oncogenic transformation. As part of an investigation into the early biochemical events of tumorigenesis, we have studied ganglioside composition and biosynthesis during normal liver development and in experimental liver tumors induced by the carcinogen N-2-fluorenylacetamide in the rat. Total ganglioside sialic acid as well as individual gangliosides are elevated in fetal liver, drop precipitously at birth, and remain low during adult development. Following administration of carcinogen, both gangliosides and total sialic acid increase dramatically in small hyperplastic nodules and reach a maximum with small hepatomas. Thereafter, total ganglioside sialic acid decreases, falling below normal liver levels in certain hepatoma lines. Ganglioside patterns show a progressive simplification from well differentiated through poorly differentiated invasive hepatomas. These and other findings indicate that ganglioside deletions are a late event in the tumorigenic transformation the onset of which may correlate with the onset of malignancy. Elevated glycolipid levels are also reflected in the sera of tumor bearers. In rats bearing transplantable hepatomas, serum levels of sialic acid are elevated 2.5-fold. Similar results were obtained with sera of mice bearing transplantable mammary carcinomas and of carcinoma patients (PNAS 74:3011, 1977). These findings provide new emphasis for gangliosides in both cancer detection and as regulatory signals for growth and multiplication of cells. Supported by NIH CA 21958.

308 CHOLERA TOXIN IS A MITOGEN FOR SWISS 3T3 CELLS. Rebecca M. Pruss and Harvey R. Herschman, UCLA, Los Angeles, CA 90024
Swiss 3T3 cells can be stimulated to divide by low concentrations (10^{-9}M) of epidermal growth factor (EGF), a 6,045 dalton polypeptide purified from the submaxillary glands of adult male mice. Cholera toxin, an 84,000 dalton protein isolated from Vibrio cholerae, was tested for its ability to block EGF induced mitogenesis in this cell line. In the course of these studies we discovered that cholera toxin at low concentration (10^{-10}M) could 1) elevate cyclic AMP levels more than 40 fold, 2) stimulate [^{3}H]thymidine incorporation and cell division, and 3) enhance the mitogenic action of EGF. Elevation of cyclic AMP in 3T3 cells is measurable only after an hour of exposure to cholera toxin. Addition of EGF has no effect on cyclic AMP levels produced by cholera toxin. Choleragenoid, the binding but inactive subunit of cholera toxin, is unable to mimic the mitogenic action of cholera toxin. Likewise, elevation of cyclic AMP by other agents does not stimulate cell division, indicating that the A subunit of cholera toxin produces its mitogenic effect through some other mechanism than simple elevation of cyclic AMP levels. These data provide additional evidence that cyclic AMP levels alone do not determine a cell's ability to traverse the cell cycle and become committed to divide. (Supported by NIH Grants T32-CA-09056 (RMP) and CA 15276 (HRH) and ERDA Contract EY-76-C-03-0012 (HRH).)

309 SPECIFIC RECOGNITION OF GLYCOPROTEINS BY RAT ALVEOLAR MACROPHAGES, P. Stahl, R. Townsend, J.S. Rodman, J. Miller and P. Schlesinger, Department of Physiology, Washington University Medical School, St. Louis, MO 63110
Alveolar macrophages have been shown to possess a cell surface recognition system which mediates binding of glycoproteins (viz., β-glucuronidase, ribonuclease B, agalacto-orosomucoid) and synthetic glycoconjugates (neoglycoproteins) which have mannose, N-acetylglucosamine or glucose in the exposed, non-reducing position. Galactose-terminal glycoproteins or glycoconjugates are not bound. Binding of radiolabelled ligands to cells is nearly completely impaired by the presence of an excess of yeast mannan. Binding is temperature sensitive and proceeds optimally at pH 7.0. Prior treatment of the cells with trypsin severely reduces their capacity to bind ligands.
An inhibition assay has been developed using radioiodinated glucose-albumin conjugate β-glucuronidase and ribonuclease B as ligands. Various glycoproteins have been shown to be effective inhibitors of ligand binding including ovalbumin, agalacto-fetuin and ribonuclease B. Ribonuclease A and asialo-fetuin were ineffective as inhibitors. When the inhibitors were ranked for their ability to produce 50% inhibition of binding, the order was independent of the ligand employed. These and other data suggest the presence of a cell surface receptor on alveolar macrophages which binds glycoproteins having terminal sugars with the mannose and glucose configuration.

310 INHIBITION OF EGF-INDUCED DNA SYNTHESIS IN CULTURES OF 3T3 CELLS BY ISOLATED SURFACE MEMBRANES. S.W. Peterson, R. Vale, M. Das and C.F. Fox, Molecular Biology Institute, University of California, Los Angeles, CA 90024.
Whittenberger and Glaser (Proc. Natl. Acad. Sci. USA 74: 2251, 1977) have recently reported that serum-stimulated DNA synthesis in cultures of subconfluent murine 3T3 cells can be inhibited by isolated surface membranes from confluent 3T3 cells. Membranes derived from SV-40 transformed 3T3 cells did not inhibit DNA synthesis even when added at 10-fold higher concentrations than that of 3T3 surface membranes which produced maximal inhibition. We have developed a more defined system in which the mitogen epidermal growth factor (EGF) has been used as the primary agent to stimulate mitogenesis of a 3T3 cell line. We have examined the action of this mitogenic hormone on cell lines in the presence of membranes from transformed cells and membranes from a 3T3 variant, NR6, which demonstrates density-dependent inhibition of mitogenesis but does not bind EGF. These experiments show that EGF-stimulated thymidine incorporation is inhibited in the 3T3 system when NR6 plasma membranes are present, but is not inhibited by SV3T3 plasma membranes. This inhibition in the presence of NR6 membranes is not reversed even in the presence of a large excess of EGF. We are now examining 1) the binding of EGF to receptors and down regulation of receptors in the EGF-3T3 system in the presence and absence of NR6 and SV3T3 membranes, and 2) the stage of cell cycle in which the inhibiting membranes exert their influence on cell proliferation.

Receptors in Developing Systems

311 ALKALINE PHOSPHATASE INDUCTION IN CULTURED HUMAN FIBROBLASTS, John R. Riordan, Lynda Wijcik and Manuel Buchwald, Research Institute, The Hospital for Sick Children, Toronto, Canada M5G 1X8
Alkaline phosphatase of cultured human fibroblasts was induced by dibutyryl cyclic AMP, sodium butyrate or the serum glycoprotein, fetuin. Both the first step of the induction mechanism and the final insertion into plasma membrane of increased amounts of this glycoprotein ecto-enzyme are transmembrane events. The basal activity of the enzyme increased at later stages of growth and sensitivity to induction by fetuin concomitantly decreased. Effective doses of dibutyryl cAMP or sodium butyrate totally inhibited cell growth, consistent with the correlation, in a number of other cell types, between inhibition of DNA synthesis and enhanced expression of cell surface components. Fetuin was without influence on cell growth and hence acts by a different mechanism, possibly causing a concerted induction of lysosomal hydrolases and other catabolic enzymes. Experiments in which the combined effects of these three agents were tested, in fact showed additivity in all cases, indicating different modes of action. The results suggest that the differences in response to these agents reflect the different signals which they generate rather than altered insertion of the enzyme into the plasma membrane. The generation of or response to these signals varies considerably among different cell strains. No consistent differences were detected in responses between four carefully matched pairs of cell stains from normal individuals and patients with cystic fibrosis. (Supported by the Canadian Cystic Fibrosis Foundation.)

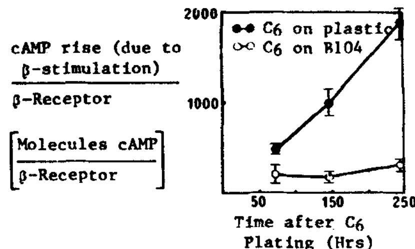
312 REGULATION OF STEROID HORMONE-RECEPTOR BINDING BY PROTEASE INHIBITORS AND SUBSTRATES, M.E. Baker, D.A. Vaughn, N.S. Anderson and D.D. Fanestil, Dept. Med. Univ. CA, San Diego, La Jolla, CA 92093
Steroid hormones induce developmental changes by a process that first involves binding of the hormone to specific receptors inside target cells. We find that protease inhibitors (PMSD, TPCK, TLCK) and substrates (TAME, TME) can inhibit the binding of steroid hormones to their specific cytoplasmic receptors in the following systems:

Dexomethasone HTC cells, rat thymus; Aldosterone rat kidney; Progesterone chick oviduct; Estradiol rat alpha-fetoprotein, rat kidney; Testosterone S115 cells

These findings may be relevant to work on regulation of growth and development in at least two ways. First, it may be erroneous to ascribe the actions of protease inhibitors on cell growth and development solely to the inhibition of proteolytic activity, for effects could be due to reaction with steroid receptors. Second, understanding the mechanism of protease/inhibitor substrate interaction with steroid receptors may suggest exciting techniques for regulation of action of steroid hormones on development.

313 GLYCOCALYX PERTURBATION AND ITS POTENTIAL FOR TRANSMEMBRANE SIGNALING, Chris W.M. Grant, Dwight G. Barratt, John MacLeod, Frances J. Sharom, Janice L. Hazlett and Jackilynn D. Rogers, Dept Biochem. The University of Western Ontario, London, Ontario, Canada, N6A 5C1
Several methods have been devised for employing gangliosides and a transmembrane glycoprotein (glycophorin) to modify or perturb the surface carbohydrate layer of rat myoblasts in culture. Lines studied were the 'normal' L6 and a non-differentiating mutant, B3 (it has been suggested that lack of the ganglioside, GD_{1A}, may be involved in the mutant's failure to fuse to form myotubes (1)). Glycolipids and glycoprotein have been physically associated with the surface of cultured cells either by adding them directly to the culture medium, or by fusion of cells with lipid bilayer structures whose walls contain them. The potential for affecting cells in this way has been followed initially by freeze-fracture EM of cells in situ and by scanning EM. In order to be able to visually identify morphological features associated with external oligosaccharide contacts, the sialoglycoprotein, glycophorin, has been covalently coupled to solid glass supports of various types which can be added to cell cultures. These external, oligosaccharide-based modifications or perturbations of the glycocalyx seem capable of affecting cell morphology, intramembranous particle distribution and cell division.
(1) *Whatley, R, Ng, S.K.-C., Rogers, J., McMurray, W.C. and Sanwal, B.D. (1976) Biochem. Biophys. Res. Commun. 70, 180-185.*
(Supported by the Medical Research Council of Canada and the National Cancer Institute of Canada.)

314 CELLULAR INTERACTIONS CAUSE β-ADRENERGIC RECEPTOR UNCOUPLING FROM ADENYLATE CYCLASE Gary Ciment[o] and Jean S. de Vellis[o], Departments of Neuroscience[o] and Anatomy[o], UCLA, Los Angeles, CA 90024
C6 glioma cells show marked differences in the expression of differentiated functions when grown on either plastic or a bed of B104 neuroblastoma cells (Both of these cloned cell lines were derived from chemically-induced rat CNS tumors). When grown by themselves, C6 cells accumulate large amounts of cAMP upon stimulation with β-agonists. However, when grown on a bed of B104 cells, the β-stimulation of cAMP is greatly attenuated, while the numbers and affinities of L-^{3}H-Alprenolol specific binding sites are relatively unaffected. This trophic interaction is not due to a non-specific toxicity because C6 growth continues and basal levels of Glycerol Phosphate Dehydrogenase (a differentiated function of C6) are unaffected by the presence of the neuroblastoma cells. Alternative causes of this loss in β-responsiveness, including changes in the activities of Phosphodiesterase and Adenylate Cyclase, as well as changes in the rate of cAMP release by the glial cells, have been explored.



315 A GLIOMA CELL PRODUCED GROWTHFACTOR STIMULATES DNA SYNTHESIS IN RESTING NEURONAL CELL CULTURES, Wilfried Seifert and Jim Morgan, Max-Planck-Institute, F.Miescher-Laboratory, Tubingen, W-Germany
Clonal cell lines derived from chemically induced rat brain tumors are used in our laboratory to study the interaction between glial and neuronal cells. Neuronal cell lines were found to be more serum-dependent in growth than glial cell lines. One of these lines (B12) grows even in the absence of serum, while a typical neuronal cell line (B104) can be arrested in the G_1-phase of the cell cycle by serum starvation, as shown by FMF analysis and by the kinetics of DNA synthesis after serum induction.
Such resting neuronal cell cultures are induced for DNA synthesis by addition of B12 conditioned medium. (W.Seifert, Meeting Ges.Biol.Chemie, Regensburg,1976) We have further characterized this growthfactor activity and the properties of this glioma-produced growthfactor (GGF), will be discussed. Experiments are in progress to further purify this factor, to check for its occurrence in brain extracts and to test its effect on growth or survival of primary nerve cells in culture.

316 THYROID HORMONES: MODULATORS OF THE "COUPLING" BETWEEN FAT CELL ADENYLATE CYCLASE (AC) AND BOTH THE BETA-ADRENERGIC RECEPTOR (βAR) AND THE GUANINE NUCLEOTIDE REGULATORY SITE (GR), Craig C. Malbon and John N. Fain, Div. Biol. & Med., Brown University, Prov.,RI 02912

Fat cells obtained from hypothyroid (HT) rats display a severely blunted lipolytic sensitivity to epinephrine (EPI), although 0.1 mM EPI stimulates lipolysis in these cells to the same level demonstrated by fat cells from euthyroid (ET) rats. The lipolytic log-dose response curve to EPI is shifted to the right by a factor of 10 by hypothyroidism. EPI produced no detectable increase in cyclic AMP accumulation in fat cells from HT rats. Fat cell ghosts prepared from HT rats display reduced catecholamine-stimulated AC activity, although the catalytic activity of the ghosts measured in the presence of 10 mM NaF is the same as that of the ET. Fat cell ghosts from HT rats were incubated with the guanine nucleotide analog, guanyl-5'-yl imidodiphosphate (GMPPNP), in an attempt to restore the reduced catecholamine-stimulated AC activity. GMPPNP activated the AC and increased catecholamine-stimulated AC activity of ghosts prepared from fat cells of both ET and HT rats, but did so to a much greater extent in the ghosts from the ET rats. Fat cell ghosts from HT rats incubated with 0.1 mM GMPPNP displayed 37% less AC activity than the ghosts from the ET rats. βARs were measured in membranes prepared from fat cells obtained from ET and HT rats with the use of the potent β-adrenergic antagonist, (-)[^{3}H] dihydroalprenolol. The number, affinity and character of βARs are identical in both ET and HT rat fat cell membrane preparations. [^{3}H]GMPPNP binding was also examined and the number and affinity of binding sites were identical in both groups. Thyroid hormones, thus, may influence fat cell cyclic AMP metabolism via regulation of signal transduction from βAR and GR to the AC.

317 A POSSIBLE MODULATION OF THE ACETYLCHOLINE RECEPTOR ON EMBRYONIC MUSCLE CELLS BY THE ANTAGONIST ALPHA-BUNGAROTOXIN, Hannah Friedman Elson, Department of Biology, UCSD, La Jolla, CA 92093

The acetylcholine receptors of skeletal muscle cells can be titrated with a tightly binding antagonist isolated from snake venom, α-bungarotoxin (α-BT). A tritiated preparation of α-BT was found to bind specifically to embryonic chick skeletal muscle cells growing in primary cell culture. It does not bind to primary fibroblasts obtained from skin or muscle tissue, or to chick red blood cells, as expected. When a time course of α-BT binding to receptors is followed, it appears that binding increases for thirty minutes but then the amount bound drops, so that only about two-thirds of the toxin bound at thirty minutes remains at sixty minutes. This decline in bound toxin molecules occurs only when the binding is performed at 37°C. If toxin is bound at room temperature, the binding saturates and does not decline. The decline is also seen only when binding is done in full growth medium and not when the cells are maintained in phosphate-buffered saline.

It is possible that a component of the growth medium is inactivating toxin molecules at 37°C, possibly by protease action on α-BT or the α-BT-receptor complex. This possibility was tested by transferring the toxin-containing medium, after binding to one culture, onto a fresh culture. The second culture bound α-BT as well as the first. After binding to the second culture, the same medium was then transferred to yet a third culture. There was still as much binding in the third culture as was found in the second culture. Thus it is not likely that a component of the medium is inactivating α-BT molecules.

318 DISTRIBUTION OF GM1 GANGLIOSIDE IN DEVELOPING CEREBELLUM, Marian Willinger and Melitta Schachner, Dept. Neuropath.,Harvard Medical School, Boston, Mass. 02115

It is generally accepted that GM1 ganglioside is the receptor on the external cell surface for the B subunit of cholera toxin. We have raised an antiserum against cholera toxin and have employed immunofluorescence and immunoperoxidase techniques to study the distribution of GM1 ganglioside in the developing cerebellum. The developing cerebellar cortex consists of a limited number of cell types. Most of these are generated, migrate and make synaptic contacts during the first fifteen postnatal days. From postnatal day 4, granule cell neurons are the predominant cell type which binds cholera toxin or choleragenoid. This has been determined by testing frozen brain sections, dissociated cells, and cerebellar cells cultured in vitro, using mice of various postnatal ages. The number and intensity of positive granule cells increases with maturation. In vivo, the cell bodies bind cholera toxin and the molecular layer made of fibers derived from the granule cells do not. In monolayer culture, granule cell bodies as well as processes contain GM1. Cholera toxin binding sites are not present on fibroblasts and astroglial cells. Preliminary evidence indicates that a small percentage of embryonic cerebellar cells contain GM1. At birth almost 70% of the cells bind toxin. The percentage of positive cells steadily declines until granule cell generation and maturation peaks after day 4. It is not known why an increase in plasma membrane GM1 occurs at birth and what types of cells contain it. Experiments are in progress to separate GM1 positive and negative cells by immunoselection in order to study their interactions in vitro. Choleragenoid was a generous gift of Dr. R.A. Finkelstein.

319 ENHANCEMENT OF AUTOAGGRESSIVE IMMUNE REACTIONS AGAINST ENDOCRINE HORMONES, RECEPTORS, AND TISSUES BY THYMECTOMY. H.L. Lipscomb, S.A. Bohbrink, and J.G. Sharp, Dept. of Anat., Univ. of Nebr. Med. Ctr., Omaha, Nebr.

Allison (NEJM, 295:821, 1976) has hypothesized that some B-lymphocytes possess the ability to manufacture antibodies directed against various components of the thyroid gland, but such autoaggressive cells are normally controlled by thymic-derived suppressor T cells. There are, however, conflicting reports of the effects of thymectomy on the development of autoaggressive diseases. This may be related to the timing of thymectomy, since we have shown that inbred Fischer 344 rats thymectomized at 5 days, but not at 1 day, of age demonstrated reduced thyroid, ovary, adrenal, kidney, spleen, and lymph node weights, as well as decreased serum thyroxine levels and histological signs of thyroid follicular destruction. Our results also show that these effects are best observed in inbred as compared to outbred rats, and further, even minor variations in genetic background of the inbred rats can alter the severity and/or target organs of the disease process. The affected animals may be reconstituted by cells from thymus, spleen, and lymph nodes, indicating that cells having a protective function exist in these tissues. This data is compatible with the hypothesis that there exists a "window" in effective suppressor cell function shortly after birth during which nonspecific suppressor cell function (T1 in the mouse) is declining, and specific suppressor cell function (T2 in the mouse) is not yet fully effective. During this period, establishment of antigen recognition function preceeds development of specific suppressor cell function by several days, and thus, thymectomy at this time may lead to enhanced autoaggressive disease. (Supported by NIH Grant #AM 21137, NSF RIAS #SER 77-06922, and the Morsman Foundation).

320 RELATIONSHIP BETWEEN MITOGENIC RESPONSE TO EPIDERMAL GROWTH FACTOR, RECEPTOR BINDING AND RECEPTOR DOWN REGULATION IN THREE MURINE EMBRYO CELL LINES, Aharon Aharonov and Harvey R. Herschman, Dept. of Biol. Chem. and Lab. Nuc. Med. and Rad. Biol., Univ. of California at Los Angeles, Los Angeles, Ca. 90024

Epidermal growth factor (EGF) is a 6,045 dalton polypeptide which stimulates growth of a variety of cells. We have previously shown that EGF binds to a specific cell surface receptor on 3T3 cells. Exposure of confluent 3T3 cells to 10^{-8}M EGF at 37°C leads to a 70-85% loss of EGF-receptors due to "down regulation", with maximal effect at 4 hr incubation. In this study we used three different murine embryo cell lines: Swiss/3T3, C3H/M2 and C3H/10T1/2 in order to compare their mitogenic response to EGF, EGF-receptor binding properties and EGF-receptor down regulation. The three cell lines respond differently to EGF. In terms of increase in cell number, 3T3 cells response is 3 fold greater than that of 10T1/2 using 10 ng/ml of EGF. The growth response of M2 cells is intermediate. In contrast, 3T3 (the line with the best growth response) have 60,000 $\pm$ 1,300 EGF binding sites per cell while 10T1/2 and M2 cells have 84,000 $\pm$ 2,000 and 120,000 $\pm$ 4,000 binding sites respectively. Using Scatchard analysis we found that the affinity of the receptor for EGF is the same in all three cell lines with a Kd of 3.4 X 10^{-9}M. EGF in concentration of 10^{-8}M caused down regulation of 60-75% of the receptors in all three cell lines, regardless of the initial number of receptors per cell. We suggest that there is no direct correlation between the number of receptors per cell, the number of receptors remaining on the cell surface after down regulation and the mitogenic response to EGF.

321 STEROID HORMONE RECEPTOR CHARACTERIZATION OF SEVERAL HISTOLOGICAL VARIANTS OF A RAT PROSTATIC ADENOCARCINOMA, Francis S. Markland, Richard T. Chopp, Malcolm D. Cosgrove, and Edwin B. Howard, USC School of Medicine, Los Angeles, CA 90033

We have characterized several histological variants of the transplantable R-3327 prostatic adenocarcinoma, and correlated these histological types with steroid hormone receptor content. One type is clearly an adenocarcinoma; this tumor is hormonally responsive and contains substantial amounts of both androgen and estrogen receptors. In contrast another histological type, a fibrosarcoma, is hormonally non-responsive and does not contain either receptor. There is also a third histologic variant which is classified as a carcinosarcoma. These tumors contain histological elements of both adenocarcinoma and fibrosarcoma and are also hormonally responsive; they contain lower receptor levels than the adenocarcinomas but more than the fibrosarcomas. The androgen receptor found in the different histological forms of the tumor has a sedimentation coefficient of 7.8S and the dissociation constant for methyltrienolone is 4 ($\pm$3) x 10^{-9}M. The estrogen receptor has a sedimentation coefficient of 8.3S and the dissociation constant for estradiol is 9 ($\pm$4) x 10^{-10}M. Rat plasma was shown to be devoid of androgen or estrogen receptors and the prostate from normal rats was shown to contain low levels of an androgen receptor but essentially no estrogen receptor. It is possible that during growth and/or passage of R-3327 tumor, the hormonally responsive adenocarcinoma cells do not survive and there is a gradual emergence of the non-responsive fibrosarcoma. Clearly, if as we suspect the receptors are found in the epithelial cells and not the stromal cells, there would be considerable variation of receptor content in the different intermediary histological forms of the tumor. Research supported in part by NIH Grant No. CA 14089.

Cell Surface Receptor Display

322 ACCUMULATION OF A SLOWLY DISSOCIABLE PEPTIDE HORMONE BINDING COMPONENT BY ISOLATED TARGET CELLS, David B. Donner, Dwight W. Martin and Martin Sonenberg, Memorial Sloan-Kettering Cancer Center, New York, NY 10021.

Mechanistic descriptions of the regulation of peptide hormone-receptor site interactions have assumed the presence of a rapidly reversible association between cell surface binding sites and hormone. This assumption has been tested in a study of the interaction of labelled human growth hormone and hepatocytes isolated from hypophysectomized rats. The overall rate and fraction of bound radioiodinated human growth hormone which dissociated from hepatocytes varied with time of association. A smaller fraction of bound hormone was dissociable from isolated target cells with increased receptor occupancy and increased incubation time prior to the onset of dissociation. The inability of bound label to completely re-equilibrate with the medium was demonstrated further by pre-incubating cell populations with labelled hormone prior to the initiation of saturation experiments where time dependent changes in the binding properties of bound label were observed in Scatchard plots. These data suggest that bound hormone may be distributed between at least two kinetic compartments. This phenomenon could be interpreted in terms of heterogeneity of sites, a slow conformational change in the receptor, or by a model incorporating spatial compartmentalization of sites. The presence of a slowly dissociating hormone binding component must be considered in any mechanistic description of hormone-cell interaction. This work was supported by grants CA-08748 and CA-15773 from the N.I.H. and by a grant from the N.Y. Diabetes Association.

323 ELECTROKINETIC EVIDENCE FOR CELL RECOGNITION AS A MEMBRANE CHARGE PHENOMENON. Philip N. Sawyer, Walenty Wrezlewicz, Boguslaw Stanczewski, George P. Hoskin, Electrochemical and Biophysical Labs., Downstate Medical Center, Brooklyn, New York.

Work in our laboratory has revealed that cellular non-self recognition is reflected in altered electrokinetic characteristics of the cells. Specifically, the average electrophoretic mobility of lymphocytes is reduced when lymphocytes from known incompatible strains of rats and mice are mixed, although virtually no change in electrophoretic mobility is observed when lymphocytes from compatible strains are mixed. This phenomenon of alterable cell surface charge is related to the occurance of known histocompatibility gene loci and is independent of cell surface neuraminic acid. In human studies there is a strong correlation between altered electrophoretic mobility of recipient-donor mixed lymphocytes in cases of kidney transplant rejection but little or no change in cases of successful tranplants. The role of calcium ion in cell interaction of incompatible lymphocytes leading to altered surface charge is discussed. Comparative data between lymphocytes and macrophages are presented. The technique provides a new approach to understanding cell-cell recognition.

324 IDENTIFICATION AND ISOLATION OF CELL SURFACE ANTIGENS SHED FROM TUMOR CELLS *IN VIVO*, Harry G. Rittenhouse, Diane Ar, Matthew D. Lynn, and David K. Denholm, The University of Michigan, Ann Arbor, MI 48109

The spontaneous release of cell surface antigens from tumor cells is thought to be an important mechanism whereby tumors avoid immune recognition and destruction by the host. Recently, we have shown that Ehrlich carcinoma cells rapidly shed their cell coat during growth *in vivo* (Rittenhouse, et al., Biochemistry, in press). In the present investigation, immunochemical studies were employed to demonstrate the association of immunoglobulin G (IgG) with Ehrlich tumor cells grown *in vivo*. Subcellular fractionation studies revealed that this tumor associated immunoglobulin (TaIg) was localized in the cell coat fraction. Indirect membrane immunofluorescence of cells grown *in vivo* showed a highly irregular fluorescent pattern (fluorescent aggregates in patches and polar caps) at the cell surface using either antisera directed toward the cell coat fraction or to mouse IgG. In both cases fluorescent debris was present in the media consistent with the shedding of this material from the cell surface. Transfer of Ehrlich tumor cells from *in vivo* to *in vitro* conditions resulted in the loss of IgG from the cell surface into the media. Tumor cell antigens recognized by TAIG were identified by an immune precipitation-detergent gel electrophoresis-autoradiography method and isolated by immunoaffinity chromatography. These methods revealed that the Ehrlich tumor-bearing host (mouse) recognized multiple antigens ranging from 45,000 to over 200,000 MW located in the cell coat. Analysis of serum and ascites fluid from tumor-bearing hosts indicates that these antigens are shed from tumor cells in the form of immune complexes.

325 ANTIBODIES TO PURIFIED INSULIN RECEPTOR HAVING INSULIN-LIKE ACTIVITY, Steven Jacobs, Kwen-Jen Chang, and Pedro Cuatrecasas, Wellcome Research Laboratories, Research Triangle Park, NC 27709

A highly purified preparation of insulin receptor, which was obtained by subjecting Triton X-100 solubilized liver membranes to sequential chromatography on DEAE cellulose, insulin sepharose, and Conconavalin A sepharose, was used to immunize a rabbit. The resulting anti-receptor serum does not inhibit insulin binding to liver membranes or solubilized liver membranes. However, when this anti-receptor serum is incubated with solubilized insulin receptor, which has been labeled with [^{125}I] insulin, the labeled receptor-antibody complex is precipitated with anti-rabbit serum, indicating that it directly interacts with the insulin receptor at sites not involved in insulin binding. The anti-receptor serum also has insulin-like activity which is capable of stimulating glucose conversion to CO_2 in isolated rat fat cells. A 1:20,000-fold dilution of the antiserum results in half-maximal stimulation. This insulin-like activity is precipitated with anti-rabbit serum, clearly indicating it is an immunoglobulin. These findings have important implications with regard to the mechanism of action of insulin.

326 THE EFFECT OF THYMUS-LEUKEMIA (TL) ANTIBODIES ON THE DENSITY OF TL ANTIGENS FORMED BY SOMATIC HYBRID AND PARENTAL MOUSE CELLS AND THEIR SUSCEPTIBILITY TO SPECIFIC ANTISERUM AND COMPLEMENT, Edward P. Cohen and Weitze Liang, La Rabida-University of Chicago Inst, Chicago, IL 60649

Membrane-associated proteins of the TL system found with murine leukemia cells reversibly disappear from the surfaces of cells exposed to TL antisera; the cells acquire resistance to fresh TL antiserum and complement (antigenic modulation). The cellular density of TL protein-antigens fails to correlate with resistance to specific antiserum. Based upon the results of three independent methods of detecting "exposed" TL antigens, cells converted to antibody and complement (C) resistance express TL antigens, in some instances in greater density than sensitive cells. Cells converted to antibody and C resistance reduce known titers of TL antiserum by absorption; they stain positively in immunofluorescence studies, and TL proteins, labeled previously with ^{125}I, may be recovered by immunoprecipitation from nonidet P-40 extracts of resistant cells. Continued exposure of the cells to TL antibodies leads to a complete disappearance of TL antigens from the cell membranes. Thymus cells of F_1 hybrids of TL(+) and TL(-) cell lines form TL antigens and are susceptible to TL antibodies and C. The relative density of TL antigens of such cells is less than leukemia cells converted to TL antibody and C resistance. Somatic hybrids of TL(+) and TL(-) cell lines form TL antigens but fail to undergo modulation, even under more "stringent" conditions. The relative density of TL antigens of such cells is significantly less than that of resistant parental cells.

327 MODULATION OF TRANSPORT BY SERUM, Ross Allen, R. T. Hamilton, Marit Nilsen-Hamilton and Debra Braun, The Salk Institute, San Diego, CA 92112

The removal of serum from ovarian granulosa cells in exponential or confluent stages of the growth results in a rapid and pronounced decrease in the rate of transport of the non-metabolizable analogues γ-aminoisobutyric acid (AIB) and 3-O-methylglucose (3OMG). Initial rates of transport decrease by 50% within 60 min and remain at that level for at least one hour. The decrease of AIB transport is independent of RNA or protein synthesis and is not affected by protease action. The decrease is rapidly and completely reversed, without detectable lag, by the addition of 10% serum or G-50 serum. The reversal is insensitive to inhibitors of RNA or protein synthesis. Fibroblast growth factor (FGF) and epidermal growth factor (EGF) can, after a lag period, reverse the decrease in 3OMG uptake. The kinetics of uptake of 3OMG indicate that removal of serum increases both K_m and V_{max}. Analogous kinetics are seen with AIB.

328 DEXAMETHASONE MODULATES THE BINDING AND ACTION OF GROWTH FACTORS. J.B. Baker, G.S. Barsh, D.H. Carney, and D.D. Cunningham, University of California, Irvine 92717

The findings below suggest that glucocorticoids cause some of their many effects on cell proliferation by altering cell surface growth factor receptors. In human diploid foreskin (HF) cell cultures without serum or growth factors, the synthetic glucocorticoid, dexamethasone (dex) did not affect cell division. However, dex enhanced the mitogenic response of these cells to epidermal growth factor (EGF) by 50% at all EGF concentrations. Dex coordinately increased the capacity of HF cells to bind ^{125}I-EGF. The effect on binding was specific for glucocorticoids and required protein synthesis. Dex appeared to cause a qualitative change in the EGF receptors, since the increased ^{125}I-EGF binding capacity of dex-treated cells, which was twofold at physiological ^{125}I-EGF concentrations, diminished at higher concentrations of ^{125}I-EGF. Dex also modulated the binding of other peptide factors to HF cells, increasing ^{125}I insulin binding, and decreasing ^{125}I-thrombin binding. In studies with cell types mitogenically sensitive to insulin or thrombin, binding of these proteins could be examined only at submitogenic concentrations, because at higher concentrations a large fraction of their binding was nonspecific. Although dex increased insulin and thrombin initiation of growth in chick embryo cells, and inhibited thrombin growth stimulation in mouse embryo cells, binding analyses with submitogenic levels of ^{125}I-insulin and ^{125}I-thrombin did not detect dex-mediated binding changes in these cells. 3T3 cells, which dex sensitized to insulin stimulation of thymidine incorporation, exhibited enhanced binding of ^{125}I-insulin when treated with dex. (Supported by grant CA-12306 from the USPHS)

329 THE DISTRIBUTION OF LECTIN-BINDING GLYCOPROTEINS ON NEUROBLASTOMA CELLS AS VISUALIZED BY FLUORESCENT LIGHT AND SCANNING ELECTRON MICROSCOPY, Pamela Maher and Robert S. Molday, University of British Columbia, Vancouver, B.C., Canada V6T 1W5

Concanavalin A (Con A), Ricinus communis agglutinin I (RCA I), and wheat germ agglutinin (WGA) conjugated to either fluorescent dyes or fluorescent microspheres were used to study the distribution of glycoproteins on murine neuroblastoma cells by fluorescent light and scanning electron microscopy (SEM). Undifferentiated and differentiated cells labeled at 4°C or prefixed with glutaraldehyde displayed a dense, uniform distribution of Con A, RCA I and WGA markers on their cell surfaces and neurite projections. When unfixed cells were labeled at 37°C, a redistribution and energy-dependent endocytosis of the lectin markers was observed. Colchicine and cytochalasin B inhibited this labeling-induced movement of cell surface lectin receptors. The mode of redistribution of Con A receptors was different than that of WGA or RCA I receptors as revealed in double labeling experiments. Whereas, unlabeled Con A receptors were coordinately removed from the cell surface along with the labeled Con A receptors, labeled WGA and RCA I receptors redistributed independently of their unlabeled counterpart. This difference is common to a number of cell types. It may reflect differences in transmembrane signaling and result in differential effects on the morphological and functional properties of cells. The spatial distribution of specific lectin receptors was studied using microspheres of various sizes in conjunction with double labeling experiments. These studies indicate that most, if not all, WGA receptors are located on different membrane glycoproteins than Con A receptors, but are associated with a class of RCA I receptors.

330 INTERACTIONS BETWEEN CELLULAR MEMBRANE RECEPTORS AND ONCOVIRUS ENVELOPE GLYCOPROTEIN, Subal C. Bishayee[1], Mette Strand[2] & J. Thomas August[2]
[1]Indian Institute of Experimental Medicine,Calcutta-700032,India
[2]Department of Pharmacology and Experimental Therapeutics,The Johns Hopkins University School of Medicine,Baltimore,Md 21205,U.S.A.

Binding of purified envelope glycoprotein(gp$^{69/71}$) of Rauscher murine type-C oncovirus to cellular membrane receptors has been analyzed with reaction systems using intact cells or membranes of disrupted cells. The reaction was highly specific; only cells permissive to infection by Rauscher virus bound the ^{125}I-labeled viral glycoprotein. The specificity of binding was also demonstrated in respect to virus interference; cells productively infected with murine ecotropic type-C virus failed to bind the virus envelope glycoprotein whereas permissive cells infected with murine xenotropic virus continued to bind the Rauscher ecotropic virus glycoprotein. Studies of the chemical basis of the receptor binding property of the glycoprotein showed that treatment with urea or guanidine-HCl abolished the activity; modification of tyrosyl, histidyl or tryptophanyl residues and the reduction of disulfide bonds also blocked the reaction.Sialic acid of the glycoprotein appeared not to be required. The specific binding of ^{125}I-labeled-gp$^{69/71}$ to membrane receptors was abolished by treatment of the membranes with trypsin,chymotrypsin and phospholipase-C.

331 CHARACTERIZATION OF A RECEPTOR FOR INSULIN-LIKE GROWTH FACTORS ON CHICK EMBRYO FIBROBLASTS, James F. Perdue, Lady Davis Institute-Jewish General Hospital, Montreal, Que.
Insulin-like activity (ILAs) with a pI of 6.4-6.7 and molecular weight of 9-10,000 daltons was prepared from an acid-ethanol extract of human plasma by sephadex and carboxymethyl cellulose (CMC) chromatography (Guyda, H.J., Posner, B.I. and Rappaport, R. (1975) Clin. Res. 23, 635A). At a concentration of 100 μU/ml, ILAs stimulated TdR incorporation 2.0 and 1.5 fold into confluent cultures of chick embryo fibroblasts (CEF) and 3T3 cells, respectively; 10% fetal calf serum increased TdR incorporation 4 and 2.5 fold. A 44% and 112% stimulation of 2-deoxyglucose uptake and a 56 and 25% stimulation of α-aminoisobutyric acid uptake was observed 10 min after adding 10 μU/ml of ILAs to CEF and 3T3 cells, respectively. The CMC-purified material was iodinated with chloramine-T and purified by binding and elution from placenta membrane. At 24°C, ILAs binding to CEF was proportional to protein concentration and was maximum by 120 min. Bound ILAs dissociated completely from CEF during 120 min of incubation at 24°C; dissociation was not accelerated following the addition of unlabeled ILAs. ILAs binds specifically to CEF. Fifty μU/ml of unlabeled ILA inhibited the binding of 200 pg of ^{125}I-ILA by 50%. The apparent K_d was calculated to be 0.4×10^{-9} M. Insulin's inhibition of ^{125}I-ILAs binding was 1-2,000 fold less potent. ^{125}I-Insulin binding to CEF (Raizada and Perdue, J. Biol. Chem. 251, 6445, 1976) was inhibited by ILAs and at a concentration that was 1/1000 the quantity of insulin required to achieve a similar level of inhibition. These results are consistent with the hypothesis that a surface receptor reversibly binds ILAs-like peptides with high affinity and that the occupancy of this receptor initiates the pleotypic responses associated with replication.

332 NEURAL UPTAKE OF CATECHOLAMINES AND THEIR MOLECULAR STRUCTURES, Hideo Uno, Oregon Regional Primate Research Center, Beaverton, Oregon 97005, and Jack H. Fellman, University of Oregon Health Sciences Center, Portland, Oregon 97201.
Using 6-hydroxydopamine (6-OHDA) for an ultrastructural marker, we studied the uptake mechanism of catecholamines in the sympathetic nerve terminals of Macaca arctoides. An intradermal injection of 6-OHDA (1 mg per 1 cm^2 skin surface) causes ultrastructural degeneration of the terminal axons in the piloarrector muscles, changes that result in depletion of endogenous catecholamines in the nerve terminals. These changes are inhibited by the simultaneous injection of other catecholamines or their metabolites with 6-OHDA; the degree of inhibition depends upon the dose. We utilized this in vivo approach to examine the competitive uptake between 6-OHDA and these substances by the terminals and to study axonal membrane transport of catecholamines. The results indicated that catechol acid (3,4-dihydroxymandelic acid and 2,4,5-trihydroxyphenylacetate) did not inhibit 6-OHDA uptake. Catecholamines (norepinephrine, epinephrine, and dopamine), 5-hydroxydopamine, and catechol-O-methylated derivatives (normetanephrine and metanephrine) did inhibit 6-OHDA disruption of nerve terminal uptake. However, only the catecholamines intensified the electron-dense granules in the vesicles (electron microscopy studies) or fluorescence in the terminals (histofluorescence studies). Fluorescence in the nerve terminals when metanephrine or normetanephrine with 6-OHDA was employed indicated the presence of endogenous catecholamine in the terminals. From these results we conclude that catechol-O-methylated compounds inhibit 6-OHDA uptake in the membrane site but they are not transported into the axons. (Supported in part by NIH Grants RR00163, RR05694, and NS01572 and Grant 7624 from the Medical Research Foundation of Oregon.)

333 NERVE GROWTH FACTOR MEDIATED INDUCTION OF TYROSINE HYDROXYLASE IN A CLONAL CELL LINE. Rosaane Goodman and Harvey Herschman, UCLA, Los Angeles, Ca. 90024.
Nerve growth factor (NGF) causes hypertrophy and hyperplasia of sympathetic ganglia neurons when injected into neonatal rats. This response is accompanied by a 5-fold increase in the specific activities of tyrosine hydroxylase and dopamine β-hydroxylase (H. Thoenen, P.U. Angeletti, R. Levi-Montalcini, and R. Kettler, PNAS 68:1598-1602, 1971). Hill and Hendry (Neuroscience 1:489-496, 1976) and Otten and Thoenen (J. Neurochem. 29:69-75, 1977) have shown that under various experimental conditions, the superior cervical ganglia of older rats also exhibit NGF-mediated induction of tyrosine hydroxylase. Otten and Thoenen have also shown that the time of exposure to NGF necessary to elicite this effect is markedly shortened by preincubation with the glucocorticoid dexamethasone.

We have developed a clonal cell line in which NGF-mediated induction of tyrosine hydroxylase occurs. The clonal line G-100-NGF, was originally derived from a rat pheochromocytoma. It was isolated by culturing the cells in a selective tyrosine-free medium containing the inducer, NGF. Incubation of the G-100-NGF clone with 0.5 ug/ml NGF for four days elicits a 4-6 fold induction in the tyrosine hydroxylase specific activity. Addition of the glucocorticoid, Dexamethasone (10^{-6}M), causes a further two fold increase in the tyrosine hydroxylase specific activity. The time course dose dependence and specificity of this response will be shown.

334 PURIFICATION AND STRUCTURAL RELATIONS OF MURINE THY-1, Robert K. Zwerner, Larry D. McClain, Paul A. Barstad and Ronald T. Acton, University of Alabama in Birmingham, Birmingham, Alabama 35294.
The Thy-1 molecule has been purified from lymphoblastoid cells, Thy-1.1 from BW5147 and Thy-1.2 from EL-4. In addition, Thy-1.2 has been purified from C57BL/6J mouse brain. The purification procedures are essentially the same for Thy-1 from all three sources. In brief, a membrane preparation of brain or lymphoblastoid cells are acetone treated and the resulting pellet solubilized by deoxycholate. The 100,000xg supernatant is passed through a Lens culinaris lectin affinity column and the bound glycoprotein fraction eluted with alpha-methyl-glucopyranoside. The Thy-1 activity was detected in the glycoprotein fraction and concentrated by alcohol precipitation. Thy Thy-1 was then purified by AcA gel filtration chromatography. The Thy-1 active fractions are pooled and concentrated. This final preparation gives only a single band when assayed by SDS-polyacrylamide gel electrophoresis with an apparent molecular weight of 24,000. The amino acid and carbohydrate compositions of the three Thy-1 molecules did not demonstrate any significant variations. The N-terminal amino acids of all three molecules are 'blocked' leading to problems in determining the amino acid sequence of Thy-1. (Supported by grants from the NIH, ACS, AHA and NSF.)

335 INITIATION OF CELL DIVISION BY THROMBIN ACTION AT THE CELL SURFACE, D.H. Carney and D.D. Cunningham, University of California, Irvine, California 92717
The site of action of most peptide hormones and growth factors is currently unclear. Previous studies utilizing Sepharose-immobilized peptides to show cell surface action have been compromised by demonstrations that large amounts of soluble material can be released from these beads and that some of this material can be "superactive". To determine if thrombin initiates cell division by action at the cell surface we linked ^{125}I-thrombin to carboxylate-modified polystyrene beads by carbodiimide condensation. Addition of optimal amounts of these beads to quiescent chick embryo cells resulted in 30 to 40% increases in cell number over controls by 24 hours. The total amount of acid-precipitable material released from these beads into the medium during this period was from 15 to 60-fold less than the amount of soluble thrombin necessary to initiate the same amount of cell division. Although a few beads were endocytosed by the cells, they were completely surrounded by an intact membrane. We could not detect any release of material from these beads directly into the cytoplasm. Together these studies show that thrombin action at the cell surface is sufficient to initiate cell division. In addition, we have demonstrated specific saturable binding of thrombin to receptors on the surface of mouse embryo cells. Small amounts of calf serum completely inhibited this specific binding. These concentrations of serum also inhibited the ability of thrombin to initiate cell division, but had no effect on non-specific binding or on the proteolytic activity of thrombin. Thus, binding to specific receptors appears to be a necessary cell surface event in the initiation of cell division by thrombin. (Supported by Grant CA 12306 from U.S.P.H.S.

336 METABOLIC STABILITY OF THE NUCLEOSIDE TRANSPORT SYSTEM OF NOVIKOFF RAT HEPATOMA CELLS, Richard Marz, Robert M. Wohlhueter and Peter G. W. Plagemann, University of Minnesota, Dept. of Microbiology, Minneapolis, Minnesota 55455.
Generally, rates of thymidine (dThd) incorporation into mammalian cells have been construed as rates of dThd transport. This conclusion is wrong, as we have shown using a recently developed, rapid sampling technique (Wohlhueter et al., J. Cell. Physiol. 89,605, 1976). With this technique we have reexamined the metabolic stability of the nucleoside carrier. An asynchronous culture of Novikoff rat hepatoma cells was monitored from early log to stationary phase for dThd transport, dThd incorporation into the acid soluble pool, DNA synthesis, and dThd kinase activity. While dThd incorporation, DNA synthesis, and dThd kinase showed the familiar peak in early log phase, and then declined sharply, dThd transport remained constant regardless of the age of culture. Upon treatment with cycloheximide (25 µg/ml) dThd kinase activity and dThd incorporation dropped to 40% of control after 1 hr, and decreased to 20% after 6 hrs. Actinomycin D treatment (1.25 µg/ml) also caused a strong, but more gradual decrease in dThd incorporation, but had little effect on dThd kinase. dThd transport was unaffected by either treatment. Experiments with uridine gave similar results: transport rates were unaffected by the age of culture and did not decay during 6 hrs of exposure to inhibitors of RNA or protein synthesis. We conclude that the nucleoside transport system shows great metabolic stability and is thus not suited to regulation by mechanisms involving carrier turnover.

337 EFFECTS OF CYTOSKELETAL PERTURBANTS ON THE COOPERATIVITY IN THE INHIBITION OF INTACT ASCITES CELL 5'-NUCLEOTIDASE BY CONCANAVALIN A. Carraway, C. A. C., Doss, R. C. and Carraway, K. L., Dept. of Biochemistry, Oklahoma State University, Stillwater, OK 74074.

Cell surface receptor mobility in many cell types is thought to be regulated via cytoskeletal elements attached to the cytoplasmic surface of the plasma membrane. The plasma membrane ectoenzyme 5'-nucleotidase of a number of cell types is reversibly and specifically inhibited by Concanavalin A (Con A), indicating that this enzyme is a Con A receptor. Studies on the 13762 rat mammary ascites carcinoma have shown that the Con A-enzyme interaction is not cooperative in intact cells and Zn^{++}-stabilized cell surface envelopes, which retain attached cytoskeletal elements. On the other hand pronounced cooperativity was exhibited by solubilized membranes and EDTA-extracted envelopes, which have fragmented and lost substantial amounts of their cytoskeletal components (Carraway et al. (1976) J. Biol. Chem. 251, 6173-6178). It was suggested that the altered cooperativity of the Con A-enzyme interaction in the intact cell may be due to cytoskeletal restrictions of the mobility of the enzyme in the membrane. Therefore we have investigated the effects in intact cells of agents which are known to perturb cytoskeletal structure. Colchicine, a microtubule effector, and cytochalasins B and D, microfilament perturbants, cause the Hill coefficient for the Con A-nucleotidase interaction to go from 1 in the untreated intact cells to about 2 in the treated cells. These results support the previous suggestion that the induction of cooperativity results from increased mobility of the enzyme when freed from cytoskeletal constraints. However, the possibility that these agents interact with the plasma membrane to promote cooperativity of the Con A-enzyme interaction must also be considered in interpreting such effects.

338 LECTIN RECEPTOR MOBILITY, MORPHOLOGY AND THE EXPRESSION OF CELL SURFACE CARBOHYDRATE IN TWO MAMARY ADENOCARCINOMA SUBLINES. K.L. Carraway, R.W. Chesnut, R.L. Buck, A.P. Sherblom, J.W. Huggins, C.L. Ownby, and T.P. Trenbeath, Dept. of Biochem., Physiology & Microbiology, Oklahoma State Univ., Stillwater, OK 74074

Two sublines (MAT-B and MAT-C) of the 13762 ascites mamary adenocarcinoma exhibit grossly different responses to lectins. MAT-B cells are strongly agglutinated by Concanavalin A(Con A), whereas MAT-C cells are not agglutinated. Subline B cells readily patch and cap with fluorescent Con A, but C cells show no evidence of receptor mobility. Treatment of the C cells with trypsin, neuraminidase, colchicine or cytochalasin B does not enhance agglutination, patching or capping by Con A. Agglutinability differences are not due to differences in lectin binding since MAT-C cells bind about 40% more Con A than MAT-B cells. Scanning electron microscopy shows that the surfaces of MAT-C cells exhibit a highly irregular structure, covered with microvilli extending as branched structures from the cell body. MAT-B cells have a more normal appearance, with fewer microvilli which are long and unbranched. These membrane differences correlate with an enhanced carbohydrate expression at the cell surface of the C cells. Although MAT-C cells have only 40% more total sialic acid than MAT-B cells, neuraminidase or trypsin releases 5-6 fold more sialic acid from C cells. Cell surface labeling with lactoperoxidase or periodate-borohydride indicates one major surface glycoprotein for both cell types. It migrates slowly on dodecyl sulfate acrylamide gels and is present to a greater extent on MAT-C cells and on their isolated membranes. The results suggest that the expression of sialoglycoprotein may be coordinated with organization of the internal cytoskeleton exhibited in the microvilli.

339 CYTOSKELETON-MEMBRANE INTERACTIONS IN ASCITES TUMOR CELLS. Pamela B. Moore, Charlotte L. Ownby & Kermit L. Carraway, Departments of Biochemistry and Physiology, Oklahoma State Univ., Stillwater, OK 74074.

Control of cell surface receptor topography is widely believed to be exerted by a submembrane cytoskeletal assembly. Membrane envelopes from ascites tumor cells have significant quantities of associated cytoskeletal proteins, most notably actin, myosin, α-actinin and actin-binding protein (ABP). Their association with the membrane and organization have been investigated by a series of extraction, proteolysis and crosslinking techniques. Actin, α-actinin and ABP are released concomitantly with envelope fragmentation under alkaline conditions at low ionic strength. Actin-binding protein is preferentially released by distilled water. Triton X-100 removes 60% of membrane lipid, 70-80% of labeled cell surface proteins and eliminates the trilaminar membrane appearance, but does not destroy the envelope shape or remove significant amounts of the cytoskeletal proteins. Proteolysis of envelopes with papain cleaves proteins in the order ABP > myosin. Envelope fragmentation does not require myosin cleavage. α-Actinin and actin are not cleaved substantially when associated with the membranes. Crosslinking studies over a range of reagent concentrations showed the formation of very high molecular weight complexes containing all of the cytoskeletal components but no significant amounts of intermediates, suggesting close and extensive associations between the components. A model is presented in which short actin microfilaments are attached to the membrane interior surface through α-actinin linkages and further associated by crosslinks involving ABP or myosin to form a coherent network under the cell membrane, stabilizing the envelopes.

340 STIMULATION OF QUIESCENT 3T3 FIBROBLASTS BY SERUM OR INSULIN IS ASSOCIATED WITH AN INCREASED AFFINITY OF THE URIDINE PHOSPHORYLATING SYSTEM FOR ATP.
W.D. Stein and G.J. Goldenberg, Hebrew University, Jerusalem, Israel.

Serum or insulin added to quiescent 3T3 cells activates within minutes the uptake of labelled uridine. This activation of uptake is due to an increased trapping of the uridine in phosphorylated derivatives (Rozengurt, Stein and Wigglesworth. Nature, 267, 442, 1977). We have studied how the rate of uptake in both activated and quiescent cells varies as a function of the concentration of the two substrates of the trapping reaction, ATP and uridine.

The activation of trapping is associated with a thirty-fold decrease in the apparent Km for ATP (Km is some o.1mM in serum-stimulated cells, some 2.9mM in quiescent cells), with little change in the Vmax, measured at saturating levels of uridine. The apparent increase in the Vmax for uptake, when uridine is the variable substrate, is wholly accounted for by this decrease in Km for the second substrate, ATP.

This difference in behaviour of the uridine phosphorylating system in intact cells disappears when extracts are made of activated or quiescent cells, the extracts in both cases displaying the kinetics of the intact quiescent cells.

Transmembrane Protein Transport

341 EFFECTS OF GTP AND A SOLUBLE MACROMOLECULE ON THE ACTIVATION OF ADENYLATE CYCLASE BY CHOLERA ENTEROTOXIN,
Kei-ichi Enomoto, Dale E. Yelton and D. Michael Gill
Dept. of Biology, Harvard University, Cambridge, Mass. 02138

The activation of adenylate cyclase in broken cells by cholera toxin is greatly stimulated by NAD, a particular unidentified cytosolic macromolecule, and a nucleoside triphosphate. After depletion of endogenous ATP, the last requirement is usually met by many nucleotides, including AMPP(NH)P, but best by ATP. However, it now seems that ATP may act merely by maintaining the GTP level.

The macromolecular fraction of pigeon erythrocyte cytosol prepared by G25 gel filtration is hardly able to support intoxication, even with ATP and NAD, but is restored to its original activity (allowing for dilution) by 1 mM GTP. Under these conditions ATP is not needed. GTP is required during the activation step (with toxin) and not only during the subsequent assay of adenylate cyclase activity (conducted without toxin). GDP or GMP replace GTP if ATP is present but not with AMPP(NH)P. CTP, TTP, UTP, and cyclic GMP are much less effective. The enhanced cyclase activity is stabilized by GTP. The cytosolic factor is being purified using an assay that includes GTP. It elutes from a G75 column after hemoglobin corresponding to about 20,000 daltons.

Cholera toxin also catalyzes the transfer of ADP-ribose from NAD to certain membrane and soluble proteins. In the absence of membaranes transfer to soluble proteins is stimulated several-fold by GTP. Thus the effects of GTP are not confined to its interaction with adenylate cyclase.

342 PROCESSING OF A PRECURSOR TO ACTH AND LPH IN MOUSE PITUITARY CELLS,
Marjorie Phillips, Richard Allen, James L. Roberts, and Edward Herbert,
Depts of Biol and Chem, University of Oregon, Eugene, Or 97403

When mRNA extracted from mouse pituitary tumor cells is translated in a reticulocyte cell-free synthesizing system, immunoprecipitated with antiserum to either ACTH or LPH, and analyzed by SDS-PAGE, a single protein of 28.5 K molecular weight is observed. SDS gels of cell extracts immunoprecipitated with antiserum to ACTH or LPH reveal 3 high molecular weight forms (34 K, 32 K, and 29 K ACTH/LPH) which react with both antibodies and lower molecular weight forms which react with only one antibody. All three high molecular weight forms are glycosylated; the 28.5 K cell-free product is not. Pulse chase and continuous labeling experiments suggest that 29 K ACTH-LPH is a precursor to 32 K and 34 K. Double label experiments with ^{35}S-methionine and ^{3}H-glucosamine or ^{3}H-mannose show that 32 K and 34 K contain more carbohydrate than 29 K, suggesting that they are derived from 29 K by further glycosylation. Analysis of ^{3}H-glucosamine labeled tryptic peptides show that the tryptic peptide containing the α(22-39) sequence of ACTH is glycosylated in 32 K but not in 29 K, suggesting that 32 K may be a precursor to 13 K ACTH and 29 K may be a precursor to 4.5 K. Processing of the 29-34 K class of molecules proceeds by cleavage to yield the 20-26 K class of ACTH and 11K LPH. The cleavage of the 20-26 K molecules proceeds rapidly to yield 13-15 K ACTH and 4.5 K ACTH and cleavage of LPH proceeds with a longer time course to give endorphin.

343 RELATIONSHIP OF RICIN AND RICINUS communis AGGLUTININ AND THEIR ACTION ON PLANT AND ANIMAL RIBOSOMES. L. L. Houston, Mary L. Hedblom and Daniel B. Cawley, University of Kansas, Lawrence, Kansas 66045

Ricin, a toxic glycoprotein from castor beans, inhibits protein synthesis by rat liver ribosomes, but not by wheat germ ribosomes. Wheat germ S-100 contains no factor which protects rat liver ribosomes and rat liver S-100 does not sensitize wheat germ ribosomes to ricin. Only the 60S rat liver subunit was sensitive when the 16 possible hybrids of ricin-treated and control 40S and 60S subunits were tested for inhibition of poly(U) translation. These data indicate that ricin sensitivity does not segregate strictly along eucaryotic-procaryotic lines as previously believed. Even though ricin acts as an enzyme, stoichiometric binding of ricin, the A chain and the B chain could be demonstrated. Both ricin and the B chain bind to a single site on the ribosome with a dissociation constant of 2 μM. Lactose prevents this binding. The A chain binds specifically to the 60S subunit of rat liver and is unaffected by lactose. No binding to E. coli ribosomes or to 40S subunits was observed.

We have demonstrated three different forms of ricin and two forms of a related protein, Ricinus communis agglutinin (RCA), and the subunits have been purified. In collaboration with Dr. Kenneth A. Walsh, the sequence was determined for 19 residues for both the A and B chain of RCA and for 7 and 17 residues of the A and B chain, respectively, of ricin. For at least 7 residues on the A chain and 17 residues on the B chain, ricin and RCA have identical sequences. Little or no homology was apparent between the A and B chains. This work supports the hypothesis that a precursor-product relationship exists between RCA and ricin.

344 COORDINATE CONTROL OF CORTICOTROPIN AND ENDORPHIN RELEASE BY HYPOTHALAMIC EXTRACT AND DEXAMETHASONE IN A MOUSE PITUITARY TUMOR CELL LINE, Richard G. Allen, Michael Hinman, Edward Herbert, and Candace Pert, Depts of Chem and Biol, University of Oregon, Eugene, OR 97403.

High molecular weight forms of ACTH exist in normal pituitary tissue and in an ACTH secreting tumor designated AtT-20/$D_{16}v$. The largest form of ACTH is glycosylated and has an apparent molecular weight of 30 K. Recent work has shown that the 30 K form of ACTH contains the sequence of β-lipotropin (β-LPH). It is known that the C-terminal region of β-LPH (61-91) is the opiate peptide β-endorphin. Exposure of the tumor cells to acid extracts of hypothalami (HE) results in stimulated release of ACTH, β-LPH, and β-endorphin. 48 hr treatment of the tumor cells with 10^{-6} M dexamethasone causes a decrease in intracellular content, basal secretion, and stimulated release of these pituitary hormones. Radioimmunoassay and/or immunoprecipitation with antisera specific for ACTH and β-endorphin were used to measure the amounts of hormone releases. $NaDodSO_4$ polyacrylamide gel electrophoresis was performed on 30 min, 1.5 hr, and 3 hr stimulated culture medium. It was found that at 30 min and 3 hrs (measured by radioimmunoassay and immunoprecipitation) two forms of ACTH (13 K, 4.5 K) are released. At 1.5 hr (measured by RIA) only the 30 K precursor and β-LPH appear in the culture medium. At 3.0 hrs β-endorphin appears in the culture medium in a 1:5 ratio to β-LPH. Further incubation of the stimulated culture medium (in the absence of cells) at 37° for 1.5 hr shows no change in this ratio.

345 STUDIES ON THE ACTION OF TRINITROBENZENE-SULFONATE MODIFIED RICIN, W. Kapmeyer, J. Beglau, W. MacConnell and N. O. Kaplan, Chem. Dept., UCSD, La Jolla, CA 92093

Ricin is a plant toxin which consists of two protein chains, linked together by a disulfide bond. The B chain recognizes the receptor site at the membrane. The A chain acts catalytically at the 60 S subunit of the ribosome and inhibits protein synthesis in vitro. To investigate the mechanism of internalisation of the A chain, we performed a chemical modification of the lysines of ricin with trinitrobenzene-sulfonate (TNBS). After the modification of 3 lysines in the B chain and 1.5 lysines in the A chain, an almost complete loss of toxicity is observed in Hela cells as well as in mice. We assayed the A chain of the TNBS-ricin for its inhibition of protein synthesis in vitro and found no significant loss of activity.
To investigate the reason for the loss of toxicity of TNBS-modified ricin we determined the association constant for the reversible binding to Hela cells at 0°C and for the irreversible uptake of the toxin by Hela cells at 37°C. We found a mainly unchanged association constant and the same rate of irreversible binding for the modified ricin. We conclude therefore that the modification of ricin with TNBS interferes with its internalisation. This still unknown step between the irreversible binding of the toxin to the cell membrane and the catalytic action of the A chain on the ribosome is partially altered after the modification of one or more lysines of the whole toxin. (Supported by USPHS Grant CA 11683 and Amer. Can. Soc. Grant BC-60).

346 ANALYSIS OF TRANSMEMBRANE DYNAMICS OF CHOLERA TOXIN USING PHOTOREACTIVE PROBES, M. Shiflett, J. Mekalanos, G. Gilliland, J. Bramhall, J. Collier and B. Wisnieski, Department of Bacteriology and the Molecular Biology Institute, University of California, Los Angeles, California 90024
Using SDS-PAGE and autoradiography, we have shown that ^{125}I-labeled cholera toxin binds to Newcastle disease virus (NDV). Pretreatment of NDV with "cold" toxin (37°C, 30') inhibits the binding of ^{125}I-labeled toxin in a subsequent incubation (37°C, 30'). These results suggest that cholera toxin binds to NDV in a specific manner. The precise receptor for toxin is unknown in NDV but is presumed to be the ganglioside GM_1. We have previously shown that the photoreactive probe 12-(4-azido-2-nitrophenoxy)stearoylglucosamine-1-^{14}C labels only the membrane proteins of NDV. Since the reactive group of the probe resides within the membrane bilayer, studies are underway to determine which, if any, of the subunits of cholera toxin cross the membrane of NDV and become radioactively labeled upon photoactivation of the probe at 360nm. Photoactivation will be effected at various times after the addition of toxin or specific subunits so that the dynamics of the process can be elucidated. The results of these studies will be presented. [Supported by USPHS Grants GM22240 (BW), AI07877 and AI08024 (JC), GM07104 (JM), GM07185 (GG), the Calif. Institute for Cancer Research, and the UCLA Academic Senate. JB is a Fulbright-Hayes Scholar; BW is the recipient of USPHS Career Development Award GM00228. Funds from NSF RIAS Grant SER76-18070 stimulated this collaborative research effort.]

347 REAGENTS FOR THE INVESTIGATION OF TRANSMEMBRANE SIGNALING EVENTS, Rebecca Kerr, Brian Ishida, John Bramhall and Bernadine Wisnieski, Department of Bacteriology and the Molecular Biology Institute, University of California, Los Angeles, California 90024
Mitogens, toxins, antigens and ionophores are all groups of cell effectors whose common mode of action is thought to involve transmembrane signaling events. Work currently in progress in this laboratory is directed at the elucidation of the temporal sequences of some of these signaling events. Our approach has been to insert photoreactive derivatives of fatty acids into a defined monolayer of model membrane systems. A tyrosyl derivative of stearic acid has been synthesized and labeled with ^{125}I to very high specific radioactivity. We have shown that dipeptide derivatives of stearic acid bearing an ESR spin label are restricted to one monolayer of the envelope of Newcastle disease virus. The photoreactive counterparts of the ESR probes are thought to assume similar dispositions within the membrane. Use of monolayer restricted photoreactive probes permits the ready detection of proteins which reside within or cross a specific monolayer of sealed membrane systems; sensitivity of detection is directly proportional to the specific radioactivity of the probe used. [Supported by USPHS Grant GM22240, the Calif. Inst. for Cancer Research, and the UCLA Academic Senate. JB is a Fulbright-Hayes Scholar. BW is the recipient of the USPHS Career Development Award GM00228.]

348 SYNTHESIS OF HYBRID MOLECULES CONTAINING FRAGMENT A OF DIPHTHERIA TOXIN AND PROTEIN MOIETIES WHICH BIND CELL SURFACES. Gary Gilliland, R. J. Collier, Dept. of Bacteriology, UCLA, Los Angeles, CA 90024
Simple methodology has been developed for crosslinking Fragment A of diphtheria toxin through a disulfide bridge to protein moieties which bind to cell surfaces. The cell surface binding protein is modified by crosslinking cystamine to intrinsic carboxyl groups in the presence of the water-soluble carbodiimide EDAC. The hybrid molecule is subsequently formed by a disulfide exchange reaction between reduced Fragment A and the cystamine-modified protein. This general technique has been applied in the construction of hybrid molecules containing Concanavalin A and ovalbumin. Fragment A-SS-Concanavalin A has been purified by affinity chromatography on NAD-Sepharose and Sephadex G-100 chromatography. The hybrid molecule is active in ADP-ribosylation of EF-2 under reducing conditions; Concanavalin A in hybrid form retains its ability to bind sugar residues as determined using conjugates prepared with I^{125}-labeled Fragment A. Fragment A-SS-ovalbumin has been prepared using ovalbumin which has been extensively derivatized with cystamine. Ovalbumin thus modified binds strongly to phosphocellulose and presumably will adhere to cell surfaces by virtue of its cationic nature. Toxicity and in vivo binding properties of these hybrids has been investigated.

349 CHOLERA TOXIN CRYSTALS SUITABLE FOR X-RAY DIFFRACTION, Paul B. Sigler*, Mary Ellen Druyan,† John Zelano,* Ada Yonath*, Helen C. Kiefer‡ and Richard A. Finkelstein.§ *The University of Chicago, Chicago, Il., 60637, †Dept. of Biochem., Loyola University School of Dentistry, Maywood, Il. 60153, ‡Dept. of Biochem., Northwestern University Medical and Dental Schools, Chicago, Il. 60611, §Dept. of Microbiology, University of Texas Southwestern Medical School, Dallas, Texas 75235.

Large crystals of the cholera toxin were grown. Their dimensions, symmetry ($P2_1$), order (better than 2.5 Å resolution), and unusual resistance to radiation make them ideally suited for a high-resolution X-ray structure determination. There is one molecule (approximately 84,000 daltons) per asymmetric unit, and therefore the lattice reveals no molecular symmetry. Two distinct bioassays indicate that the protein from dissolved crystals retains its full capacity to activate adenylate cyclase in the rabbit ileal epithelium and induce morphologic changes in mouse adrenal cells. Preliminary experiments indicate that the molecules in the lattice react with the oligosaccharide component[1] of the specific receptor ganglioside, G_{M1}, (but not with the control oligosaccharide) and in doing so stress the lattice. Current efforts are focused on: (1) establishing the general features of the molecular aggregate at low resolution - in particular the degree of symmetry exhibited by the five B subunits, (2) the structural changes induced by ligand binding, (3) preparation of heavy-atom derivatives suitable for a complete high resolution structure determination. Promising crystals of the B subunits (choleragenoid) have been grown and are now being characterized.

[1]Gift from Prof. H. Wiegandt, Marburg.

350 ENZYMICALLY ACTIVE FRAGMENT OF PSEUDOMONAS AERUGINOSA EXOTOXIN A. Stephen Lory, R. J. Collier, Department of Bacteriology, University of California, Los Angeles, Ca. 90024

Exotoxin A from *Pseudomonas aeruginosa* is secreted as a single enzymically inactive polypeptide chain of molecular weight 66,000 daltons. Partial reduction of the toxin yields an active enzyme, capable of hydrolyzing NAD and ADP-ribosylating elongation factor 2. The toxin may also be activated by limited protelysis. Although the enzymic activity of the toxin is destroyed by incubation with chymotrypsin, the presence of NAD completely protects against chymotryptic inactivation. Structural analogs of NAD (adenine, nicotinamide, ADPR) offer limited protection. SDS polyacrylamide gel electrophoresis of toxin treated with chymotrypsin in the presence of NAD revealed a single major band of molecular weight around 26,000 daltons. This peptide has been purified by DEAE-cellulose chromatography and shown to contain both NAD-glycohydrolase and ADP ribosylation activities. However, it lacks toxicity in cell culture. These properties are identical to those of a naturally occurring, enzymically active peptide found in culture fluids from *Pseudomonas aeruginosa* PA 103, and are similar to those of fragment A from diphtheria toxin.

351 AFFINITY FILTERS, A NEW APPROACH TO THE ISOLATION OF TOX MUTANTS OF VIBRIO CHOLERAE, John J. Mekalanos, R. John Collier, and William R. Romig, University of California, Los Angeles, Ca. 90024

We have devised a novel plate assay method for detecting mutants of *Vibrio cholerae* altered in the production of cholera toxin (*tox* mutants). Colonies replicated from a master plate are grown on the surface of a cellulose filter disc to which ganglioside-albumin conjugates have been attached. The toxin which the colonies excrete is tightly bound to the ganglioside filters. After removal of the cells by washing the bound toxin may be detected by treating the filters with radioactively labeled antibodies against either whole toxin or one of its constituent polypeptide chains, followed by autoradiography. Colonies producing significantly greater or lesser amounts of toxin than the parental type are easily recognized and can be shown in liquid culture to have the corresponding hyper-, hypo-, or non-toxinogenic phenotypes. This method, termed the ganglioside filter assay, is applicable to screening large numbers of colonies and should facilitate isolation of various specific classes of mutants in cholera toxin production. In modified form the method will be applicable to a variety of systems in which mutants in excreted proteins are sought.

Cyclic Nucleotide Directed Protein Phosphorylation

352 ENDOGENOUS PHOSPHORYLATION OF RAT SYNAPTIC MEMBRANE PROTEINS: INHIBITION BY MORPHINE IN VIVO, J.P. O'Callaghan and D.H. Clouet, Ofc. of Drug Abuse Services, Brooklyn, N.Y.

A synaptic plasma membrane (SPM) fraction was prepared from rat striata by differential centrifugation under hypotonic conditions and subfractionated on a discontinous sucrose gradient. The endogenous phosphorylation of SPM proteins was assayed in vitro for 30 seconds using $AT^{32}P$ as a phosphate donor. Following the separation of membrane proteins by SDS-polyacrylamide gel electrophoresis, the incorporation of phosphate into specific membrane proteins was analyzed by gel slicing and autoradiographic techniques. The electrophoretograms revealed at least six peaks of radioactivity associated with specific SPM proteins. As a consequence of the chronic administration of morphine, the phosphorylation of several proteins was reduced including protein II (MW 48-52K) and protein III (MW 11-18K), which were reduced by about 15% and 50%, respectively; the phosphorylation of protein II was regulated by cyclic AMP and the phosphorylation of protein III was not. The incorporation of phosphate from ATP into proteins II and III derived from rats undergoing naloxone-precipitated withdrawal was similar to the incorporation of phosphate into these same proteins derived from control subjects. The incorporation of phosphate into SPM proteins of intact synaptosomes incubated under physiological conditions was also assayed in vitro using $^{32}P_i$ as a source of phosphate. Under these conditions the phosphorylation of several proteins, including protein III, derived from morphine-tolerant rats was reduced compared to the phosphorylation of these proteins derived from non-tolerant rats. Changes in the phosphorylation of specific SPM proteins may be involved in the development of tolerance to and dependence on opiates. (supported by USPHS grant DA-00087).

353 THE EFFECTS OF GLUCAGON, α AGONISTS AND CALCIUM IONOPHORES ON THE PHOSPHORYLATION OF HEPATOCYTE CYTOSOLIC PROTEINS. J. C. Garrison, Dept of Pharmacology, Univ. of Virginia Charlottesville, Va. 22903.

Recent experiments show that stimulation of rat hepatocyte α-receptors alters the activity of enzymes known to be regulated by cycles of phosphorylation and dephosphorylation without increasing the activity of cyclic AMP dependent protein kinase. The present study compared the effects of glucagon and catecholamines on the incorporation of phosphate into cytosolic proteins obtained from intact rat hepatocytes. SDS-polyacrylamide slab gel electrophoresis resolved 27 phosphorylated bands in the molecular weight range 200,000 to 29,000. Treatment of the intact hepatocytes with glucagon or cyclic nucleotides increased the phosphate content of 12 of these phosphoproteins. Stimulation of the α-receptor with α agonists in the presence of propranolol increased the phosphorylation of at least 10 of the same 12 phosphorylated bands stimulated by glucagon. The increase in phosphorylation mediated by α receptors was only 50 to 60% of that observed with glucagon and occurred in the absence of any change in the level of cyclic AMP. The α-receptor stimulation was antagonized by ergotamine or phentolamine. Treatment of the cells with the Ca^{++} ionophore A23187 increased the phosphorylation of only 4 of the phosphoproteins altered by glucagon or catecholamines. The effects of the ionophore depended on the presence of extracellular Ca^{++} ion. It is concluded the α-receptor occupation alters the activity of a cyclic AMP independent protein kinase or phosphatase with a specificity similar to those affected by cyclic nucleotides. (Supported by AM-19952)

354 ACTIVATION OF CYCLIC-AMP-DEPENDENT PROTEIN KINASE AND STIMULATION OF PROTEIN PHOSPHORYLATION IN RESPONSE TO ADENOSINE IN C-1300 MURINE NEUROBLASTOMA. Richard D. Green and Thomas A. Noland. Department of Pharmacology, School of Basic Medical Sciences, University of Illinois, Chicago, Illinois 60614.

DEAE-cellulose chromatography of the 20,000 g supernatant fraction of homogenates of C-1300 murine neuroblastoma (clone N2a) yields 1 major and 2 minor peaks of cyclic AMP-dependent protein kinase activity. Assessment of the endogenous activation state of the enzyme(s) (proportion of the holoenzyme dissociated into free regulatory and catalytic subunits) reveals that the enzyme is fully dissociated (i.e., activated) by treatment with adenosine (10 μM) in the presence of the phosphodiesterase inhibitor Ro 20 1724 (0.7 mM). This treatment produces a large elevation in the cyclic AMP content of the cells. Treatments with adenosine alone (1-100 μM) or Ro 20 1724 alone (0.1-0.7 mM) produce minimal elevations in cyclic AMP but nevertheless cause significant activations of the enzyme. The autophosphorylation of whole homogenates of treated and untreated cells was studied using [γ-^{32}P] ATP, SDS-polyacrylamide gel electrophoresis and autoradiography. Treatments which activate cyclic AMP dependent protein kinase selectively stimulate the incorporation of ^{32}P into proteins with molecular weights of approximately 15,000 and 11,000. Similar results can be seen after the addition of cyclic AMP to phosphorylation reactions containing homogenate from untreated cells or in whole cell preparations permeabilized to cyclic AMP and ATP by sonication.

355 MEMBRANE SIGNALS CONTROLLING CARDIAC CONTRACTILITY, R. John Solaro, David A. P. Small and Elizabeth Howe, University of Cincinnati, Ohio 45267.
In isolated beating rabbit hearts, stimulation of cardiac surface membranes by adrenergic agonists has been shown to enhance the amount of covalent phosphate attached to troponin I, a thin filament regulatory protein (Solaro, Moir and Perry, Nature 262:615-617,1976). In vitro studies suggest that the signal flow between membrane and myofibril occurs via the cascade of reactions involving activation of adenyl cyclase, elevation of cAMP and activation of a "troponin kinase" probably identical to cyclic AMP dependent protein kinase. Although the phosphorylation signal is associated with enhanced contractility, in vitro phosphorylation of cardiac myofibrillar troponin I depressed myofibrillar ATPase between 0.01 and 10.0 uM calcium ion concentration. Moreover, our data shows that myofibrillar calcium binding is also depressed over this range of free calcium. These data thus suggest that this membrane signal may activate a feedback circuit controling transitions between states of contractility and/or act as part of the mechanism underlying the enhanced rate of cardiac relaxation during beta adrenergic stimulation. Since the change in calcium binding may reflect a change in myosin interaction with the thin filament (Solaro, Bruni and Gleason, Biochim. Biophys. Acta 449:304-309,1976), we are investigating whether the level of phosphorylation of myosin light chains (Frearson, Solaro and Perry, Nature 264:801-802,1976) is altered during our in vitro phosphorylation experiments or whether such changes in phosphate content of the light chains affects myofibrillar calcium binding. Supported by PHS Grant No. HL 00464 and American Heart Association Grant No. 74-865.

356 PHOSPHORYLATION OF MEMBRANE PROTEINS BY A cAMP-DEPENDENT KINASE INHIBITS TRANSPORT OF α-AMINOISOBUTYRIC ACID,
Marit Nilsen-Hamilton and Richard T. Hamilton, Cell Biology Lab, The Salk Institute, San Diego, CA 92112
Treatment of membrane vesicles isolated from 3T3 cells with a cAMP-dependent protein kinase plus cAMP and ATP results in approximately 60% inhibition of the initial rate of α-aminoisobutyric acid (AIB) uptake. The purified catalytic subunit of this enzyme also inhibits transport, and inhibition depends upon the presence of ATP. ATP alone has no effect on AIB transport. Transport by membranes isolated from SV3T3 cells is not affected by kinase treatment. The catalytic subunit phosphorylates several membrane proteins. We propose that the decrease in transport which occurs when 3T3 cells become quiescent is due to phosphorylation by a cAMP-dependent protein kinase.

357 INSULIN EFFECTS ON FAT CELL MEMBRANE PROTEIN PHOSPHORYLATION, William B. Benjamin, Nancy-Lee Clayton and Dominick Pucci, SUNY-Stony Brook, Stony Brook, N.Y., 11794
Molecular mechanisms of hormone action can be studied by determining their effects on target cell protein phosphorylations. Insulin action increased the phosphorylation of two fat cell phosphoproteins designated-2 and -6b (monomer M_r were 130,000 and 62,000 respectively). Epinephrine and isoproterenol action decreased phosphoprotein-6b phosphorylation and markedly increased phosphoprotein-5 phosphorylation (monomer M_r ranges from 65,000 to 70,000). Since insulin and epinephrine action affect the phosphorylations of fat cell phosphoproteins-5 and -6b reciprocally we determined the localization of radiolabeled phosphoproteins within fat cells. Subcellular fractionation of ^{32}P-labeled fat cells demonstrated that phosphoprotein-2 was found with the cytosol fraction while phosphoproteins-4 and -6a (monomer M_r was 87,000 and 65,000 respectively) were present in the "crude membrane" fraction. Phosphoprotein-6b was not extracted from fat cells by standard buffers but was found with the "fat cake". Non-ionic detergent extraction of the "fat cake" released phosphoprotein-6b which was now found with a new pellet. Electron microscopy of this pellet demonstrated it to be composed almost exclusively of filaments and short membrane segments consistent with its being part of fat cell cytoskeletal elements. N-ethylmaleimide and cytochalasin B, agents which inhibit insulin action, were found to affect phosphoprotein-6b phosphorylation. Since phosphoprotein-6b is both associated with membrane filaments and its phosphorylation is hormonally regulated, phosphoprotein-6b may be part of the mechansim for the transduction of the "insulin message".

358 CHARACTERIZATION OF PLATELET MEMBRANE PROTEINS WHOSE PHOSPHORYLATION IS cAMP-DEPENDENT BY A TWO-DIMENSIONAL ELECTROPHORESIS METHOD. D.M. Peterson, J.L. Moake, W.G. Hicks and S.H. Yu. Hematology Div, Dept Internal Med, U of Texas Med School, Houston, Texas.
Isolated platelet plasma membranes were incubated with Mg^{2+} and [γ-^{32}P]ATP in the presence or absence of cAMP. Membranes were then solubilized in NP-40 and 8 M urea, and separated by two-dimensional electrophoresis with isoelectric focusing in the first dimension and sodium dodecyl sulfate polyacrylamide gel electrophoresis in the second dimension. Proteins of 43,000 mw and isoelectric pH's of 6.2, 6.7 and 6.8 were phosphorylated only in the presence of cAMP. These phosphorylated proteins were distinctly separated from actin (isoelectric pH of 6.4)and are quantitatively minor membrane components since the radiolabeled gel positions in two-dimensional separations were not associated with detectable amounts of protein. When two-dimensional gels were stained for protein with Coomassie blue, four major membrane proteins with mw's of 45,000, 95,000, 115,000 and 150,000 and many minor proteins were observed. Iodination of washed, intact platelets resulted in intense labeling of 4 membrane proteins which are exposed on the external platelet surface. The most heavily iodinated surface protein has mw (95,000) and isoelectric pH (6.0) identical to one of the major membrane proteins. This protein was also phosphorylated in isolated membrane preparations, although cAMP-stimulation was not demonstrated. Iodination of isolated membranes and of membrane proteins which had been solubilized in 2% NP-40 was carried out by the lactoperoxidase method. There was one additional heavily iodinated protein (mw of 70,000 and isoelectric pH more basic than actin) in membrane samples solubilized before iodination as compared to samples iodinated before solubilization. This protein may be essentially unexposed on either membrane surface.

Ion Transport and Protein Phosphorylation

359 REGULATION OF PYRUVATE DEHYDROGENASE BY PHOSPHORYLATION/DEPHOSPHORYLATION, P.H.Sugden & P.J.Randle, Dept.Clinical Biochem.,Radcliffe Infirmary, Oxford OX2 6HE, U.K.

The pig heart pyruvate dehydrogenase multienzyme complex (PDH, Mr about 7 million) consists of three enzymes which catalyse the conversion of pyruvate to acetyl-CoA: pyruvate decarboxylase (Mr 151000), dihydrolipoyl acetyltransferase (Mr 76000) and dihydrolipoyl dehydrogenase (Mr 58000). The decarboxylase is a tetramer of two α (Mr 41000) and two β (Mr 35000) subunits. Using the radioamidination technique of Bates et al., 1975, (FEBS Lett., 60,427-430) with methyl [1-^{14}C] acetimidate, the molar ratio of subunits (α:β: acetyltransferase : dehydrogenase) in the complex as isolated was 4:4:2:1. PDH is regulated at least in part by an integral ATP-Mg^{2-} dependent PDH kinase which phosphorylates only the α subunit and hence inactivates the decarboxylase thus inhibiting the overall reaction. PDH is reactivated by PDHP phosphatase. Inactivation of PDH involves incorporation of 1 mol phosphate/mol $\alpha_2\beta_2$. This was shown by a dual-labelling technique in which PDH was titrated to inactivation using limiting amounts of [γ- ^{32}P] ATP and was then radioamidinated. Following separation of subunits by SDS-polyacrylamide disc gel electrophoresis and excision of the α subunit band, the molar ratio of ^{32}P:α chain was calculated. Further incorporation of phosphate into the α subunit can occur to the extent of 3 mol/mol $\alpha_2\beta_2$ without further change in enzyme activity. However, PDH containing $\alpha_2P.\beta_2$ is reactivated about three times more rapidly by PDHP phosphatase than that containing $\alpha_2P_3.\beta_2$. Hence regulation of PDH kinase (it is inhibited by pyruvate and activated by high concentration ratios of acetyl-CoA/CoA, NADH/NAD^+ and ATP/ADP) may indirectly regulate PDHP phosphatase activity and hence PDH reactivation.

360 THE STRUCTURE OF MAMMALIAN (Na-K)-ATPASE, Robert A. Farley and Jose Castro, Biol. Labs, Harvard University, Cambridge, Mass. 02138
Controlled enzymatic and chemical proteolysis has been used to fragment the catalytic subunit of the (Na-K)-ATPase isolated from canine renal medulla by the method of Jorgensen. The different fragments obtained by digestion under various conditions have been aligned from amino-terminus to carboxy-terminus to provide a frame of reference for distance along the polypeptide chain. Binding sites for analogs or derivatives of some substrates or ligands, and the sites of modification by some chemical modification reagents have been mapped. The site of phosphorylation by ATP during the catalytic cycle has been localized to a small region about 15,000 daltons in length, approximately 75% of the distance from the N-terminus to the C-terminus.

Calcium as A Cellular Effector

361 CALCIUM INHIBITION OF RENAL CORTICAL ADENYLATE CYCLASE (AC) ACTIVITY.
Susan T. Nielsen and W. F. Neuman, U. of Rochester, Rochester, NY 14642
Effects of calcium (Ca^{++}), parathyroid hormone (PTH), and guanylylimidodiphosphate (Gpp(NH)p) on AC activity were characterized to see how interactions of stimulatory and inhibitory agents alter the activity in vitro. Rat renal cortical basal lateral plasma membranes were isolated from a cortical tissue homogenate by centrifuging the sample to obtain a membrane pellet that was subjected to free flow electrophoresis to separate plasma membranes of basal lateral (serosal) surfaces of proximal tubule cells from brush border (luminal) surfaces. AC activity of basal lateral plasma membranes was stimulated by PTH and Gpp(NH)p. 50% of the maximal stimulations were elicited by .01uM PTH and by 0.9uM Gpp(NH)p. Ca^{++} inhibited basal, Gpp(NH)p-stimulated or PTH-stimulated AC activity. Adding Ca^{++} depressed the PTH dose-response curve so the maximal AC activity observed was less than that seen with a control PTH dose-response curve; the PTH concentration giving 50% of the maximal activities, respectively, was the same for both curves. The maximal AC activity elicited by 1uM PTH or 10uM Gpp(NH)p was progressively inhibited by increasing Ca^{++} concentrations; 50% inhibition occurred at 50uM Ca^{++}. The Hill coefficients were $.95\pm.18$ in the former case and $1.01\pm.06$ in the latter case. The Ca^{++} inhibition is 1)independent of the nature of the agonist, 2)mediated by a single Ca^{++} ion at each AC enzyme complex (indicated by the Hill coefficients close to unity), and 3)does not alter the apparent affinity of PTH for its receptor.

362 STUDIES ON PANCREATIC ISLET CELL MEMBRANES: CATION-ACTIVATED PHOSPHATASE ACTIVITIES IN RELATION TO INSULIN SECRETION, Ake Lernmark, Department of Biochemistry, The University of Chicago, Chicago, Illinois 60637
Studies on the pancreatic β-cells have suggested that secretion of insulin may be regulated by transmembrane fluxes of cations. Enzymes catalyzing cation-dependent cleavage of ATP or certain organophosphate indicators have been implicated in cation transport in many cells. However, such enzyme activities are poorly understood in the pancreatic islets. A plasma membrane enriched fraction was prepared from isolated rat or mouse islets by sucrose-gradient centrifugations. Using ^{125}I-wheat germ agglutinin as a plasma membrane probe, a fraction was obtained at a sucrose density of about 1.10 that was enriched in 5'-nucleotidase, Mg^{2+}-ATPase and alkaline phosphatase. The fraction contained little, if any, monoamine oxidase activity, insulin or DNA. Hydrolysis of fluorescein phosphate or p-nitrophenyl phosphate was stimulated by K^+ or Rb^+ at pH 8 in the presence of Mg^{2+}. The K^+-induced activity was inhibited by ouabain at concentrations that stimulate insulin secretion and inhibit Rb^+ uptake. The K^+-stimulated p-nitrophenyl phosphatase was markedly inhibited by ATP, Na^+, Ca^{2+} or p-chloromercuribenzene sulfonic acid. Hydrolysis of ATP was optimum at pH 7.0 and 8.2. At the latter pH, a small fraction of the total Mg^{2+} ATPase activity was inhibited by ouabain in the presence of Na^+ and K^+. Similarities of the properties of these enzyme activities to the characteristics of Rb^+ uptake in pancreatic β-cells suggest that univalent cation transport may be coupled to an ATP-dependent phosphoryl-transfer reaction.
[Supported by NIH grants AM 17046, AM 13914 and the Swedish Medical Research Council.]

Inhibitors of Adenylate Cyclase

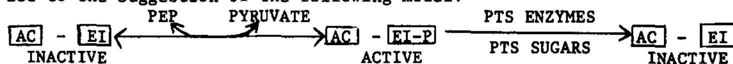
363 CONTROL OF ^{35}S PROTEOGLYCAN SYNTHESIS OF CHONDROCYTES. R.P. Miller and S.Lohin. St. Mary's Hosp. and U. of Rochester Sch. Med. Dent., Rochester N.Y. 14611
We probed the relationship between replication and the 24h synthesis of sulfated proteoglycans of chondrocytes grown in monolayer culture with vasopressin (LVP) and N^6O^2 dibutyryl adenosine 3'5' monophosphate (dbcAMP). LVP (0.5-10 ng/ml in 2% calf serum (C.S.) stimulated replication and enhanced both medium soluble and cell layer ^{35}S proteoglaycans. However, the label incorporation was directly related to cell number. When 5×10^{-4}M dbcAMP in 2% C.S. was utilized, no replication occurred and synthesis of medium and cell layer ^{35}S proteoglycans was enhanced. If LVP and dbcAMP were added together, hormone stimulated replication was suppressed and no enhancement of ^{35}S proteoglycans was noted above that caused by dbcAMP. Therefore, LVP is mitogenic but fails to enhance ^{35}S proteoglycan synthesis except for that which appears obligatory to cell division. Stimulation with 16% C.S. and dbcAMP inhibited replication, slightly suppressed medium ^{35}S proteoglycans and dramatically enhanced cell layer ^{35}S proteoglycans. When dbcAMP and graded amounts of C.S. were added, the lower concentrations of serum failed to increase ^{35}S incorporation to that of 16% C.S., suggesting dbcAMP was enhancing C.S. components, presumably serum hormones. The dose response noted with graded amounts of C.S. when cultures were sacrificed at 24h was not present when cultures were sacrificed at 6h, indication a delayed cellular event. Pulse addition of label for 30m localized the onset of dbcAMP effect to 8-12h after C.S. stimulation, a time prior to thymidine incorporation, confirming a localized event late in the G_1 compartment of the cell cycle. We conclude that chondrocytes synthesize 2 groups of proteoglycans, one which is obligatory to cell division and a second type, which is late in the G_1 compartment and under control of adenytate cyclase.

364 EFFECTS OF CHOLESTEROL INCORPORATED INTO PLATELETS ON AGGREGATION, α-ADRENORECEPTORS, AND ADENYLYL CYCLASE. P.A.Insel,P.Nirenberg,S.J.Shattil, U.of Pa. & U.of Cal., S.F.

Human platelets offer a useful model system to examine effects of changing membrane cholesterol content on adrenergic response and adenylyl cyclase(AC). Platelets incubated in vitro with cholesterol-rich(CR)phospholipid dispersions acquire membrane cholesterol, their membrane lipids become less fluid(as measured by fluorescent probes), and they aggregate in response to lower concentrations of epinephrine(epi)than do normal platelets. In addition, these platelets have increased basal AC activity but decreased AC responsiveness to several stimulants. Since epi-stimulated aggregation is an α-adrenergic event, we examined α-adrenoreceptors on intact and homogenized platelets to determine whether the increased sensitivity of CR platelets to epi is attributable to a change in affinity or number of membrane binding sites for epi. To characterize α-adrenoreceptors we used ^{3}H-dihydroergocryptine(DHE), a radiolabeled antagonist. DHE binding to normal platelets at 37° reaches equilibrium by 50 min, is reversible and saturable (K_d=1-4 nM by both kinetic and equilibrium studies)to one class of binding sites(330±53 sites/platelet). CR platelets(C↑ 44%)aggregate in response to a 35x lower concentration of epi than do normal platelets. However, the time course of DHE binding, the K_d and the number of binding sites/platelet are similar to those of normal platelets. Since CR platelets have increased sensitivity to epi at a time when effector-stimulated AC is inhibited, we conclude: 1)the increase in epi-stimulated platelet aggregation is not due to an increased number or affinity of α-adrenoreceptors; 2)cholesterol or its effects on membrane fluidity may modify transmission of signals for aggregation(or for AC stimulation)distal to sites of receptor binding on the membrane.

365 MECHANISM OF REGULATION OF E. COLI ADENYLATE CYCLASE (AC) BY PHOSPHOENOLPYRUVATE (PEP). Alan Peterkofsky and Celia Gazdar, NIH, Bethesda, Md. 20014.

E. coli AC is controlled by a transmembrane signalling mechanism involving a sugar transport system (the PEP:sugar phosphotransferase system or PTS). When external sugar is transported, AC activity is inhibited. We previously reported (PNAS 72, 2920(1975)) that E. coli carrying a leaky mutation in Enzyme I (EI) of the PTS had abnormally low cAMP levels. Toluene-treated cells (TTC) of this strain had low AC activity which was markedly stimulated by added PEP. Those data led to the suggestion of the following model:



The model was supported by the following data: 1) PEP does not activate AC in TTC of the leaky EI mutant by regenerating ATP, since the rates of ATP utilization in mutant and wild-type TTC are comparable. 2) Exogenous PEP increases intracellular cAMP levels in both wild-type and leaky EI mutant. 3) AC in TTC is inhibited by glucose or pyruvate presumably by decreasing the concentration of EI-P; the inhibition is partially relieved by PEP, which should increase the concentration of EI-P. 4) AC in TTC is inhibited by the addition of ATP and pyruvate kinase, suggesting that TTC contain a pool of PEP essential for AC activity. 5) The leaky EI mutant has a higher than wild-type Km for PEP in the PTS pathway, suggesting that the requirement for PEP for AC activation in this strain reflects the low affinity for PEP for phosphorylation of EI. Taken together, these studies further fortify the scheme for regulation of AC activity based on the above described phosphorylation-dephosphorylation mechanism.

366 MULTIPLE, CYCLIC AMP-MEDIATED RESPONSES TO A SINGLE HORMONE, Richard M. Hays, Albert Einstein College of Medicine, New York, NY 10461

Vasopressin (antidiuretic hormone) activates multiple, cyclic nucleotide-mediated transport systems in receptor cells such as those of the toad urinary bladder. The permeability of the luminal cell membrane to water, amides (notably urea), lipophilic molecules and sodium is greatly increased when vasopressin binds to receptors in the opposite (basolateral) membrane. These permeability changes can all be duplicated by exogenous cyclic AMP. They appear to take place in the same cell, but do not occur simultaneously, and do not appear to be activated from a single adenylate cyclase-cyclic AMP system. Instead, they occur in sequence as hormone binding increases, and exhibit significantly different control and effector mechanisms. The water permeability system involves the action of microtubules or microfilaments, and induces the aggregation of membrane-associated particles at the luminal membrane. It is highly sensitive to prostaglandins, and is in a state of relative suppression within the cell. The amide transport system, on the other hand, is far more sensitive to vasopressin and relatively insensitive to prostaglandins; it does not appear to involve microtubules or microfilaments, or induce membrane particle aggregation. A variety of inhibitory agents, including phloretin, rotenone, dinitrophenol and methylene blue, selectively inhibit the transport system for water, urea, or other classes of solutes, at early or late points in the hormone-adenylate cyclase-membrane sequence, again indicating that these transport systems are functionally distinct.

Thus, in this system, a single hormone activates multiple cyclic AMP-mediated pathways whose relationship to cell metabolism and microtubules differ significantly.

367 MOLECULAR RESOLUTION AND RECONSTITUTION OF THE GPP(NH)P AND NAF SENSITIVE ADENYLATE CYCLASE SYSTEM, Harry Le Vine III, Claus J. Schmitges, Naji Sahyoun , and Pedro Cuatrecasas, Department of Molecular Biology, Wellcome Research Laboratories, Research Triangle Park, N.C. 27709
A particulate adenylate cyclase virtually non-responsive to Gpp(NH)p (guanylyl-5'-iminodiphosphate) or NaF has been prepared from rat brain by a detergent extraction regimen. Addition of exogenously derived detergent solubilized membranes or water soluble supernatants of homogenates from various tissues restores stimulation of the enzyme by both Gpp(NH)p and NaF while contributing only a few percent of the total cyclase activity. The reconstituted membranes may be washed without loss of activation. These findings indicate that the stimulation caused by these agent(s) is mediated by one or more regulatory component(s) physically distinct from the enzyme itself. The regulatory component(s) appear to be sensitive to trypsin and to SH-reactive reagents. The properties of the reconstituted system resemble those of the original particulate brain adenylate cyclase. This system may serve as a convenient tool to probe the molecular properties of adenylate cyclase and the basis of its regulatory control.

368 POSSIBLE ROLE OF CALCIUM AS A MEDIATOR OF GLUCOCORTICOID ACTIONS IN LYMPHOID CELLS Donald A. Young and Mary L. Nicholson, Univ. of Rochester School of Medicine, Rochester, New York 14642
Calcium influx has been shown to be essential for the evolution (over the course of 12 hrs) of the lethal actions of glucocorticoid hormones on thymus cells [N. Kaiser and I.S. Edelman: Proc. Nat'l. Acad. Sci; 74, 638, 1977]. We have therefore examined the possibility that hormone-induced increases in calcium influx might _initiate_ cellular deterioration in P1798 lymphoid tumor cells. Increases in "nuclear fragility" (the inability of nuclei to survive lysis of the cells by hypotonic shock) were used as the first sign of cell deterioration, since in thymus cells [cf, S.J. Giddings and D.A. Young: J. Steroid Biochem. 5, 587, 1974] and in lymphosarcoma cells changes in this parameter generally preceed other indices of cell deterioration (spontaneous release of DNA, the inability to exclude trypan blue dye, or decreases in cell numbers) by several hours. Glucocorticoid-sensitive tumor lines are less able than resistant cells to withstand incubation _in vitro_. The differences in cell survival were predicted by earlier changes in nuclear fragility, and also by early differences (at 1 hr) in calcium uptake. However, so far we have been unable to detect early glucocorticoid effects on calcium uptake that preceed or coincide with the hormone-induced increases in nuclear fragility present at 2 hrs. It thus seems unlikely that hormone-induced changes in calcium uptake _initiate_ the lethal effects of glucocorticoids in these cells. [Supported by grants from the United Cancer Council (of Rochester) and N.I.H. - AM 16177.]

369 REGULATION OF THE RECEPTOR-MEDIATED CYCLIC AMP RESPONSE OF KIDNEY TO PARATHYROID HORMONE, Leonard R. Forte and Constantine S. Anast, Depts. of Pharmacology and Child Health, University of Missouri and Harry S. Truman Memorial Veterans Hospital, Columbia, Mo.
The renal cyclic AMP response to parathyroid hormone (PTH) is markedly reduced in rats fed diets deficient in either vitamin D (-D) or calcium (-Ca). Both the -D and -Ca animal models exhibit hypocalcemia with a secondary increase in circulating immunoreactive (i)PTH. This refractory response of the kidney to PTH is associated with a substantial decrease in the PTH-dependent adenylate cyclase activity of cortical plasma membranes isolated from -D or -Ca animals. The impaired renal cyclic AMP response was specific for PTH since the basal, F-, calcitonin- and vasopressin-stimulated enzyme activities were unchanged in membranes from -D or -Ca rats. The mechanism of this refractory response to PTH in animals with elevated circulating PTH could conceivably be mediated by a "down-regulation" of PTH receptors or alternatively through a modification in translation of the hormone-receptor binding signal to the catalytic component of the adenylate cyclase. Either mechanism is a viable possibility since we have shown that both PTH infusion into rats and exposure of renal slices to PTH _in vitro_ results in significant depression of the PTH-dependent adenylate cyclase. Therefore, elevated PTH _per se_ does induce a state of renal refractoriness (tachyphylaxis) to subsequent PTH activation of the cyclic AMP system. On the other hand, other studies in our laboratory suggest that both Ca and vitamin D are involved in the regulation of the kidney's response to PTH. At this point, we are not able to resolve the relative importance of the elevated circulating PTH, Ca deficiency and reduced vitamin D metabolites as they contribute to the decrease in receptor-mediated cyclic AMP responsiveness to PTH in the renal tissue of -D or -Ca rats.

370 EXCITATION-SECRETION COUPLING IN ISOLATED ADRENAL CHROMAFFIN CELLS: DIFFERENCES BETWEEN ACETYLCHOLINE AND EXCESS POTASSIUM STIMULATION, Allan S. Schneider and Hollis Cline, Sloan-Kettering Institute for Cancer Research & Cornell University, Graduate School of Medical Sciences

Acetylcholine (ACh) and excess K^+ both increase adrenal chromaffin cell membrane permeability to extracellular calcium which in turn induces secretion of catecholamines by exocytosis. We have compared the secretory response of isolated bovine adrenal chromaffin cells to ACh vs. K^+ stimulation in terms of: a) kinetics of release, b) calcium dependence, c) local anesthetic blocking, d) dose-response curves, and e) cytoskeletal elements. Comparison of our kinetic results with published data on the electrophysiological response of chromaffin cells (Brandt, B.L. et.al., J.Physiol., London 263, 417 (1976)) suggests that differences between ACh and K^+-stimulated secretion may be related to their different effects on membrane action potential spike frequency. This could also explain their different calcium dependence and have implications for calcium entry occurring in a pulsed manner with each action potential. Vinblastine was found to block ACh- but not K^+-stimulated catecholamine secretion from the isolated chromaffin cells,thus indicating a lack of involvement of microtubules in a common mechanism of exocytosis. Cytochalasin B had no significant effect on secretion.

371 THE USE OF BIOPHYSICAL AND BIOCHEMICAL MEASUREMENTS TO DETERMINE SYNAPTIC VESICLE STRUCTURE. John A. Wagner, Steven S. Carlson & Regis B. Kelly. Department of Biochemistry & Biophysics, University of California, San Francisco 94143

We have purified synaptic vesicles from the electric organ of Narcine brasiliensis. These vesicles have been shown to be homogeneous by three independent criteria: mobility in an electric field, density in a glycerol density gradient and sedimentation velocity. The resulting measurements were used to define the structure of the synaptic vesicle and the synaptic vesicle membrane. From the $S_{20,w}$ and the density of synaptic vesicles, we can calculate a particle weight of 176 million daltons and a radius of 410 Å. From the vesicle density in glycerol density gradients (1.119 g cm^{-3}) and in sucrose density gradients (1.050 g cm^{-3}), we estimate that 74% of the vesicle is water. Synaptic vesicles have a high lipid to protein ratio (6:1 by weight) which is consistent with their density in both glycerol and sucrose gradients. Further, the density of the isolated synaptic vesicle membrane (1.09 g cm^{-3}) suggests that most of the protein is membrane bound. They contain twenty proteins of which three are selectively associated with vesicles. Synaptic vesicles contain high concentrations of acetylcholine (500mM) and ATP (160mM) and GTP (20mM).

372 MUSCARINIC CHOLINERGIC AGONISTS INCREASE β-ADRENERGIC RECEPTOR AFFINITY FOR CATECHOLAMINES AND INHIBIT CATECHOLAMINE STIMULATION OF ADENYLATE CYCLASE. August M. Watanabe, Mona M. McConnaughey, Robert A. Strawbridge, John W. Fleming, Larry R. Jones and H.R. Besch, Jr. Indiana University School of Medicine, Indianapolis, Indiana 46202.
Guanine nucleotides (Gpp(NH)p > GTP) stimulated adenylate cyclase activity and potentiated isoproterenol activation of adenylate cyclase activity in membrane vesicle preparations from dog hearts. Methacholine had no effect on basal, NaF stimulated, Gpp(NH)p stimulated or Gpp(NH)p plus isoproterenol stimulated activity. In contrast, methacholine (10^{-6} M) essentially abolished GTP (0.3 to 100 μM) stimulated activity [increment in activity due to GTP (pmol cAMP/mg/min): GTP (30 μM) 240 ± 20; GTP + methacholine 22 ± 8]. Methacholine also markedly attenuated isoproterenol stimulation of adenylate cyclase activity in the presence of GTP [adenylate cyclase activity (pmol cAMP/mg/min) with GTP plus isoproterenol, 545 ± 26; GTP plus isoproterenol plus methacholine 405 ± 10]. These effects of methacholine were reversed by atropine. In parallel experiments, the effects of methacholine on β-adrenergic receptor binding was examined, using ^{3}H-dihydroalprenolol to label β-adrenergic receptors. As observed in other tissues, Gpp(NH)p and GTP (10^{-4} M) both decreased the affinity of β-adrenergic receptors for receptor agonists, without altering the affinity of the receptors for receptor antagonists. Methacholine (10^{-6} M) abolished the agonist-specific effects of both Gpp(NH)p and GTP on β-adrenergic receptor binding. These results suggest that muscarinic cholinergic receptor agonists can modulate both β-adrenergic receptor affinity for agonists and adenylate cyclase activity, by an effect mediated through guanine nucleotides.

373 RELEASE OF AZUROPHILIC AND SPECIFIC GRANULE CONSTITUENTS FROM HUMAN GRANULOCYTES BY THE CALCIUM IONOPHORE A23187. James D. Simon, William E. Houck and Maurice M. Albala. Rhode Island Hospital and Brown University, Providence, Rhode Island 02902.

Although it is well known that the secretion of a number of granule-associated substances from human granulocytes (PMN's) is Ca^{++}-dependent, there is no definitive evidence for the differential effect of Ca^{++} on azurophilic (Az) and specific (Sp) granules. Utilizing A23187, we investigated the role of Ca^{++} in the release of β-glucuronidase, a marker for Az granules, unsaturated vitamin B12 binding capacity (UBBC), a Sp granule marker, and lysozyme, a constituent of both granules. PMN's were isolated from buffy coats of ACD blood by density gradient centrifugation. Ten million cells were incubated at 37 C in 3.0 ml of phosphate buffered saline with varying concentrations of ionophore. The cell-free supernatants were assayed for UBBC, lysozyme, and β-glucuronidase. Maximal release of UBBC (92% of total) was seen at 1.0 μM A23187. The % of release of lysozyme and β-glucuronidase was 32% and 5%, respectively. At 10.0 μM ionophore, 59% of UBBC, 73% of lysozyme and 31% of β-glucuronidase were secreted. Further increases in the concentration of A23187 elicited minor increases in the release of lysozyme and β-glucuronidase. Ca^{++} (up to 50 mM) without ionophore did not evoke the release of any granule constituent. Cell viability was not altered, as indicated by the lack of release of cytoplasmic lactate dehydrogenase and trypan blue exclusion. These results suggest that Sp granules are more sensitive than Az granules to Ca^{++}ionophore-induced release of their constituents.

374 SPIN-LABEL STUDIES ON RAT LIVER AND HEART PLASMA MEMBRANES: EFFECTS OF TEMPERATURE, CALCIUM AND LANTHANUM ON MEMBRANE FLUIDITY, Richard D. Sauerheber, Larry M. Gordon and Judy A. Esgate, Scripps Clinic and Research Foundation, La Jolla, CA 92037

The structures of rat liver and heart plasma membranes were studied with the 5-nitroxide stearic acid spin probe, I(12,3). The polarity-corrected order parameters (S) of liver and heart plasma membranes were independent of probe concentration only if experimentally-determined low I(12,3)/lipid ratios were employed. At higher probe/lipid ratios, the order parameters of both membrane systems decreased with increasing probe concentration and these effects were attributed to enhanced nitroxide radical interactions. Examination of the temperature dependence of both S plots and probe-probe interactions indicated that lipid phase separations occur in liver (between 19 and 28°C) and heart (between 21 and 32°C) plasma membranes. The possibility that a wide variety of membrane-associated functions may be influenced by these thermotropic phase separations is considered.

Addition of 2.0 mM $CaCl_2$ to I(12,3)-labelled liver plasma membrane decreased the fluidity as indicated by a 6% increase in S at 37°C. Similarly, titrating I(12,3)-labelled heart plasma membranes with either $CaCl_2$ or $LaCl_3$ decreased the lipid fluidity at 37°C, although the magnitude of the La^{3+} effect was larger and occurred at lower concentrations that that induced by Ca^{2+}; addition of 0.2 mM La^{3+} or 3.2 mM Ca^{2+} increased S by 6 or 4%. The above cation effects reflected only alterations in the membrane fluidity and were not due to changes in probe probe interactions. Ca^{2+} or La^{3+} at these concentrations decrease the activities of such plasma membrane enzymes as Na^+,K^+-ATPase and adenylyl cyclase, and it is suggested that the inhibition of these enzymes is the result of cation-mediated decreases in the lipid fluidity.

375 CONCANAVALIN A INDUCES RELEASE OF MITOCHONDRIAL BOUND CA^{2+}IN THYMOCYTES. Ross B. Mikkelsen, Dept. of Therapeutic Radiology, Tufts-New England Med. Ctr., Boston, MA. 02111

The role of extracellular Ca^{2+} in Con A stimulation of lymphocytes has been extensively studied. Another major source of cellular Ca^{2+} is the mitochondrion. Chlorotetracycline (CTC)has been successfully used to monitor Ca^{2+}-mitochondrial membrane interactions since CTC fluorescence in nonpolar environments, e.g. biomembranes, is enhanced by low $[Ca^{2+}]$ or $[Mg^{2+}]$. I have used CTC fluorescence to study Con A-induced changes in mitochondrial Ca^{2+}binding in rabbit thymocytes. If 2 x 10^8 cells/ml are equilibrated for 90 min at 37° in Dulbecco's phosphate buffered saline plus 5mM glucose and 0.2mM CTC and then diluted 10-fold into buffer minus CTC, the enhanced CTC fluorescence decreases ~30% to a new equilibrium level in 10 min. If Con A (5-20 μg/ml) is then added, a fluorescence decrement (~20%) occurs within 10 secs that is independent of extracellular Ca^{2+} and inhibited by .01M α-methylglucoside. A new equilibrium value is achieved within ~3 min. The excitation and emission wavelengths are characteristic of CTC-Ca^{2+} complexes (396nm and 529nm) with minimal response at wavelengths diagnostic for Mg^{2+} (379nm and 524nm). Antimycin A (5μM), an inhibitor of mitochondrial Ca^{2+} binding and transport, blocks the Con.A effect. Ruthenium Red (10-20μM), an inhibitor of mitochondrial Ca^{2+} transport but not binding to mitochondrial "low affinity" Ca^{2+} sites, does not inhibit the Con A induced CTC fluorescence decrement. No effect of Con A on CTC emission is observed with isolated microsomes or plasma membranes. These results suggest that one immediate consequence of Con A-plasma membrane interactions is a transmembrane signal for release of mitochondrial bound Ca^{2+} into the cytosol. This Ca^{2+} may trigger later events in the lectin stimulation of lymphocytes. Research supported by NIH Grant 1-R-01 CA22269-01.

376 PROTEINS DESTINED FOR FAST AXONAL TRANSPORT ARE TRANSFERRED FROM GOLGI APPARATUS TO THE TRANSPORT SYSTEM BY A CALCIUM-DEPENDENT PROCESS. Richard Hammerschlag and Pierre-André Lavoie, Div. of Neurosciences, City of Hope Nat. Med. Ctr., Duarte, CA 91010.
The neuronal axon, which has virtually no capacity for protein synthesis, continually receives proteins from its soma by an active intracellular transport process. The initiation of this process in the soma shows a marked calcium dependence in that the amount of axonal transport of [^{3}H]protein is depressed when peripheral nerve ganglia, but not when nerve trunks, are exposed in vitro to either a calcium-free medium or to a cobalt-supplemented normal medium. These conditions have no effect on the rate of transport, or on protein synthesis or ATP levels within the ganglia (Hammerschlag, Dravid & Chiu, Science 188:273,1975; Hammerschlag et al, J. Neurobiol. 8:439,1977). These results imply that the calcium-dependent, cobalt-sensitive step occurs after protein synthesis and prior to the onset of anterograde transport. Since most newly-synthesized proteins appear to migrate through the Golgi apparatus where glycosylation of selected proteins is completed, the effects of cobalt ions on glycosylation and on fast axonal transport of glycoproteins were examined to circumscribe better the intracellular sequence of events during which the calcium-dependent step must occur. Transport of [^{3}H]glycoproteins was depressed by 87 $\pm$ 2% (mean $\pm$ SEM; n=6) when 0.18 mM $CoCl_2$ was added to the incubation medium after exposure of ganglia to [^{3}H]fucose and [^{3}H]glucosamine. As judged by total TCA-insoluble radioactivity in ganglia and nerve trunks, protein glycosylation was unaffected by this level of Co^{++}. These findings suggest that calcium ions are required at a step during the transfer of proteins from the Golgi apparatus to the fast-transport system. (Supported by NSF Grant BNS75-17640, and by MRC of Canada Fellowship to P.-A.L.)

377 IONOPHORE A23187 AS A PROBE IN STUDYING THE ROLE OF Ca^{2+} IONS IN PANCREATIC ACINAR CELL METABOLISM, Manjit Singh, V.A. Hospital (FHD), Augusta, Georgia 30904
There are no reports available on the effect of Ca^{2+} on metabolic functions in pancreas except secretion and the "phospholipid effect". To study the role of Ca^{2+} in acinar cell metabolism, pancreatic fragments were preincubated for 1 hour with $5x10^{-6}$M A23187 and 2 mM EGTA in Ca^{2+}-free Krebs-Henseleit buffer. The fragments were then equilibrated for 30 minutes with 10 µM, 100 µM, 1 mM and 2.5 mM Ca^{2+} buffers containing $5x10^{-6}$M A23187. Final incubation was with the same Ca^{2+} and ionophore concentration with or without CCK-PZ and with the addition of tracer required. In the absence of CCK-PZ, fragments incorporated 2880 $\pm$ 95, 3183 $\pm$ 71, 3103 $\pm$ 74 and 3449 $\pm$ 67 cpm/mg protein of ^{14}C-phenylalanine; oxidized 3.40 $\pm$ 0.3, 3.89 $\pm$ 0.3, 3.86 $\pm$ 0.2 and 4.93 $\pm$ 0.3 cpm/µg DNA of ^{14}C-glucose; secreted 19.4 $\pm$ 0.6, 21.8 $\pm$ 0.9, 30.9 $\pm$ 1.1 and 36.6 $\pm$ 0.9% of total counts incorporated following a 5-minute pulse of ^{14}C-phenylalanine at 10 µM, 100 µM, 1 mM and 2.5 mM concentrations of Ca^{2+} respectively. Incorporation of ^{32}P into phospholipids and ^{14}C-thymidine into DNA was not influenced. In the presence of CCK-PZ, incorporation of ^{14}C-phenylalanine was depressed and that of ^{32}P was increased. The data suggest that metabolic events like protein synthesis, transport, secretion and glucose oxidation are dependent on Ca^{2+} and are mimicked by the ionophore. Ionophore A23187 is thus a useful probe in studying the role of Ca^{2+} in pancreatic acinar cell metabolism.

378 PURIFICATION OF SYNAPTIC VESICLES. Steven C. Carlson, John A. Wagner & Regis B. Kelly. Biochemistry & Biophysics, University of California, San Francisco 94143

We have purified an important nerve terminal organelle, the synaptic vesicle, by gentle procedures which allow the integrity of the membrane to be maintained. The retention of the internal contents (acetylcholine and ATP) is used as an assay for the vesicles and a measure of the intactness of the membrane. Several fractionation methods are used to isolate the synaptic vesicles and to evaluate the purity of the preparation. These methods should be of general use in organelle purification.

The purification scheme involves differential centrifugation, sucrose density flotation centrifugation, and exclusion chromatography on glass beads containing 3000 Å pores. The synaptic vesicles elute from the glass bead column as a single peak in the included volume with a constant specific activity of 8000±800 nmoles acetylcholine/mg protein which is 300 fold higher than the original starting material. These vesicles are stable for several weeks.

Additional separation techniques are used to assess the purity of the vesicle preparation. These methods (electrophoresis, sedimentation velocity centrifugation and glycerol density gradient centrifugation) rely on different properties to separate particles than the original purification procedures, and thus are independent criteria of purity. Vesicular acetylcholine, phospholipid phosphorus, and protein all behave as a single component when subjected to these procedures. We conclude that our organelle preparation is homogeneous; and, based on the sensitivity of our assay methods are >90% pure.

379 PROTEOLYTIC PROCESSING AS A BIOSYNTHETIC MECHANISM. C. Patzelt, S.J. Chan, J.R. Duguid, B. Schaffer and D.F. Steiner, Dept. of Biochemistry, Univ. of Chicago, Chicago Il 60637

In the last decade since the discovery of proinsulin, many studies have shown that limited intracellular proteolysis plays an important role in the biosynthesis of many secreted proteins. It is now evident that proteolytic processing occurs not only at the stage of packaging of the products during their transit through or near the Golgi region of the cell, but also occurs during the translational stage in association with the vectorial discharge and segregation of the protein product within the rough endoplasmic reticulum. Studies on insulin precursors which illustrate many aspects of these systems will be presented. Proteolysis of precursors is also increasingly being recognized as an important mechanism in the generation of supramolecular structures such as collagen, viruses, organelles and enzyme complexes. The varieties and roles of intracellular protealytic processing mechanisms will be briefly summarized. (Work in this laboratory has been supported by grants from the NIH (AM 13914, AM 17046, CA 19265), the Lolly Coustan Memorial Fund and The Kroc Foundation.)