INTERNATIONAL SOCIETY FOR
FREE RADICAL RESEARCH

VI Biennial Meeting

Free Radicals: from Basic Science to Medicine

Torino (Italy) June 16-20, 1992

Volume of Abstracts

University of Torino
C.N.R. - Center of immunogenetics and experimental oncology
Regione Piemonte

On behalf of the International Society for Free Radical Research, the Organizing Committee extends to you its cordial invitation to attend the 6th Biennial Meeting in Torino, June 16th-20th, 1992.

It will represent a further step in the constitution of a single big community with American, Asian, Australasian and European Regional branches.

Fifteen plenary lectures and twenty afternoon sessions with two invited and four selected oral presentations per session, as well as an unique poster session extended for the whole duration of the Meeting, should cover the majority of the areas of research, even if a special emphasis will be given to problems of biomedical interest.

Beside the scientific program, we will try to do our best in involving you in a warm and friendly social atmosphere, offering the history, the culture, the arts and of course the foods and the wines of Piemonte.

Welcome in Torino !

Mario Umberto Dianzani
President Local Organizing Committee

ORGANIZING SCIENTIFIC COMMITTEE

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SCIENTIFIC PROGRAM

PLENARY SESSIONS

- Radical Reactions in Ageing
- Free Radicals and Cancer
- Reactive Species in Metabolic Disorders
- Pharmaceutical Intervention in Free Radical-Based Pathology

AFTERNOON PARALLEL SESSIONS

1 Free Radicals in Radiation-Exposed Systems
2 Oxidized Lipoproteins
3 Free Radicals inArachidonic Acid Cascade
4 Free Radicals in Drug Activation and Metabolism
5 Free Radicals in Medicine I (Lung,Kidney)

6 Redox Chemistry and Antioxidants
7 Gene Expression and Oxidative Damage
8 Oxidant and Antioxidant Reactions in Plants
9 Ischemia-Reperfusion
10 Free Radicals in Medicine II (Nervous System, Muscle)

11 Antioxidants in Food
12 Lipid Peroxidation
13 NO and Related Radicals
14 Carcinogenesis
15 Free Radicals in Medicine III (Liver, Eye,Skin)

16 Antioxidant Defences in Eukariotic Cells
17 Inflammation
18 Reactive Species in Metabolic Disorders
19 DNA Damage and Repair
20 Free Radicals in Medicine IV (Red Cells,Heart)

FORMAT OF THE MEETING

	9,00-12.30	12,30-13,30	13,30-14,30	14,30-16,30	16,30-19,00
Tue June 16th				Premeeting Conference	Get-together Party
				14,30-18,00	**18,00-19,00**
Wed June 17th	Opening Lecture Plenary Session on Radical Reactions in Ageing	Lunch	Poster viewing	Parallel oral sessions 1 - 5	Poster viewing
Thu June 18th	Plenary Session on Free Radicals and Cancer	Lunch	Poster viewing	Parallel oral sessions 6 - 10	Poster viewing
Fri June 19th	Plenary Session on Reactive Species in Metabolic Disorders	Lunch	Poster viewing	Parallel oral sessions 11 - 15	SFRR International General Assembly
Sat June 20th	Plenary Session on Pharmaceutical Intervention in Free Radical-Based Pathology	Lunch	Poster viewing	Parallel oral sessions 16 - 20	

PRELIMINARY SCIENTIFIC PROGRAM

TUESDAY JUNE 16

PRE-MEETING CONFERENCE

Free Radical Mechanisms of Cell Injury

14:30-14:45	M.U.Dianzani, University of Torino (Italy) V.Vannini, University of Pavia (Italy) Opening remarks.
14:45-15:30	S. Orrenius, Karolinska Institutet (Sweden) Mechanisms of oxidative cell damage. (abstract PM.1)
15:30-16:15	H.Esterbauer, University of Graz (Austria) Cytotoxic and genotoxic effects of lipid peroxidation. (abstract PM.2)
16:15-16:30	Conclusions.
16:30	Get-together Party.

OPENING ADDRESSES

9:00-9:20	M.U. Dianzani (Magnificus Rector, University of Torino).
9:20-9:40	E. Niki (President of SFRR International).

PLENARY SESSION ON RADICAL REACTIONS IN AGING

9:40-9:50	D. Harman, University of Nebraska (U.S.A.). Chairperson's remarks.
9:50-10:30	R.G. Cutler, National Institute on Aging (U.S.A.). Free radicals in aging and longevity mechanisms. (abstract A.1)
10.30-11:00	**Coffee break**
11.00-11:10	B. Chance, University of Pennsylvania (U.S.A.). Chairperson's remarks.
11:10-11:50	R.T. Dean, Heart Research Institute (Australia). Protein oxidation as marker and mechanism in aging and atherosclerosis. (abstract A.2)
11:50-12:30	V. Monnier, Case Western Reserve University (U.S.A.). Maillard reaction and oxidative stress are interrelated stochastic mechanisms of aging. (abstract A.3)
12:30-13-30	**Lunch Break**
13:30-14-30	**Poster Viewing**
13:30-14:30	**Lunch Time Seminar** New Therapeutical Implication of NAC in Lung Pathologies *(Sponsored by Zambon Group)*
13:30-13-40	J.Viña, University of València (Spain). Introductory remarks.
13:40-13:55	B.S. van Asbeck, University Hospital Utrecht, (The Netherlands). Hydrogen peroxide dependent inflammatory mechanisms in acute lung injury. (abstract A.4)

13:55-14:10 S. De Flora, M.Bagnasco, R.M. Balansky, C. Bennicelli, A. Camoirano, F. D'Agostini and A. Izzotti, University of Genova (Italy). Antigenotoxic and anticarcinogenic effects of N-acetylcysteine in lung cells. (abstract A.5)

14:10-14:25 F. Bistoni, Dottorini, Cociani, Pistrella, Todisco, Vecchiarelli, University of Perugia (Italy). Enhancement of candidacidal activity of peripheral blood mononuclear cells after "in vitro" treatment with N-acetylcysteine. (abstract A.6)

14:25-14:30 Conclusions

AFTERNOON PARALLEL SESSIONS

Session 1. ***Free Radicals in Radiation-Exposed Systems***

Chairperson: R. Bisby, University of Salford (U.K.).

14:30-15:00 C. Von Sontag, Max Planck Institut, (Germany). (abstract 1.1)

15:00-15:30 K. Neriishi, Radiation Effect Research Foundation (Japan). Oxidative stress in atomic bomb survivors. (abstract 1.2)

15:30-16:00 D.E. Cabelli and R.A. Hallewell, Brookhaven National Laboratory, New York and Scripps Research Institutes, La Jolla (U.S.A.). Studies of modified superoxide dismutases. (abstract 1.3)

16:00-16:30 **Coffee break**

16:30-17:00 J.A. Simpson, S.P. Gieseg and R.T. Dean The Heart Res. Inst. (Australia). Long lived reductive moieties on free radical damaged proteins. (abstract 1.4)

17:00-17:30 H. Utsumi, H. Kawabe, S. Masuda, K. Takeshita, Y. Miura, T. Ozawa, T. Hashimoto, H. Ikehira, K. Ando, O. Yukawa and H. Hamada Showa University of Tokyo and Nat. Inst. Rad. Sci, (Japan). In vivo ESR studies on radical reactions in whole mice - Effect of radiation exposure. (abstract 1.5)

17:30-18:00 M.T. Leccia, M.J. Richard, J.C. Béani, H. Faure, J. Cadet, P. Amblard and H. Favier Centre d'Etude Nucléaires de Grenoble (France). Protective effect of selenium and zinc on UV-A damage on human skin fibroblasts. (abstract 1.6)

Session 2.	***Oxidized Lipoproteins.***
Chairperson:	C. Rice-Evans, University of London (U.K.).
14:30-15:00	H. Puhl, G. Waeg, F. Tatzber, A. Krebs, H. Esterbauer, University of Graz (Austria). Effect of vitamin E and other antioxidants on the oxidation resistance of low density lipoprotein. (abstract 2.1)
15:00-15:30	B. Kalyanaraman, The Medical College of Wisconsin (U.S.A.). Effect of ascorbate on the production of free radicals during oxidative modification of low density lipoprotein - An electron spin resonance study. (abstract 2.2)
15:30-16:00	R. Stocker, V.W. Bowry and D. Mohr, The Hearth Research Institute (Australia). The role of ubiquinol-10 in the inhibition of the early stages of lipoprotein lipid oxidation. (abstract 2.3)
16:00-16:30	**Coffee break**
16:30-17:00	N. Noguchi, N. Gotoh, E. Niki and H. Shimasaki, University of Tokyo (Japan). Oxidative modification of low density lipoprotein induced by free radicals generated in its inside or outside. (abstract 2.4)
17:00-17:30	G. Paganga and C. Rice-Evans, University of London (U.K.). Haem proteins as mediators of oxidative modification of low density lipoproteins. (abstract 2.5)
17:30-18:00	N. Hogg, M. Wilson, V. Darley-Usmar and V. O'Leary, University of Essex and Wellcome Research Laboratories (U.K.). Kinetic simulation of copper induced oxidation of low density lipoprotein. (abstract 2.6)
Session 3.	***Free Radicals in Arachidonic Acid Cascade.***
Chairperson:	S. Nigam, Free University of Berlin (Germany).
14:30-15:00	C.R. Pace-Asciak, O. Lancuville, A. Margalit and A.A. Livne, University of Toronto (Canada) and University of Negev (Israel). New biological properties of the hepoxilins derived through the transformation of 12-HPETE. (abstract 3.1)

15:00-15:30 R. Pella, A. Kretz-Rommel, H. Schawmberger and V. Ullrich, University of Konstanz (Germany). Vitamin C and E oxidation by cyclooxygenase and lipoxygenase reactions. (abstract 3.2)

15:30-16:00 K.V. Honn, B. Liu, D. Tang, Y.O. Chen Wayne State University of Detroit (U.S.A.). Eicosanoid modulation of signal transduction: importations for cancer metastasis. (abstract 3.3)

16:00-16:30 **Coffee break**

16:30-17:00 C.S. Boyer, G.L. Bannenberg, A. Ryrfeldt, P. Moldeus, Karolinska Institute (Sweden). Hydrogen peroxide-mediated liberation of arachidonic acid in endothelium: lack of evidence for free radical mechanisms. (abstract 3.4)

17:00-17:30 E.M. Link, London University College and Middlesex School of Medicine (U.K.). Mechanism of H_2O_2 cytotoxicity and inflammation. (abstract 3.5)

17:30-18:00 G.N. Semenkova, U.V. Zakrevskaja, S.N. Cherenkevich, Byelorussian State University (Belarus). Recombinant interleukin-1β enhances the active oxygen forms production in neutrophils. (abstract 3.6)

Session 4. ***Free Radicals in Drug Activation and Metabolism.***

Chairperson: F. De Matteis, Medical Research Council, Carshalton (U.K.).

14:30-15:00 R.P. Mason, R.V. Lloyd, H. Monteiro and V. Fischer, Research Triangle Park (U.S.A) and Drug Safety Dept. Sandoz Pharma Ltd. (Switzerland). Possible role of free radical formation in clozapine(clozaril)-induced agranulocytosis. (abstract 4.1)

15:00-15:30 A. Tomasi, D. Constantin, P. Moldeus, V. Vannini, University of Modena (Italy) and Karolinska Institute (Sweden). (BI) sulfite metabolism in human granulocytes and macrophages. (abstract 4.2)

15:30-16:00 A.J. Kettle and C.C. Winterbourn, Christchurch School of Medicine (New Zealand).
Oxidation of hydroquinone by mycloperoxidase. (abstract 4.3)

16:00-16:30 **Coffee break**

16:30-17:00 E. Cavalieri and E. Rogan,
University of Nebraska (U.S.A.).
Metabolic activation of the potent carcinogens benzo-[a]pyrene (BP) and 7,12-dimethylbenz[a]anthracene (DMBA) by one-electron oxidation. (abstract 4.4)

17:00-17:30 J. Butler, J.A. Hartley and B.M. Hocy,
CRC Paterson Institute for Cancer Research and London University College and Middlesex School of Medicine (U.K.).
Redox studies on diaziridinyl benzoquinones; DNA site specific damage as a consequence of reductive activation. (abstract 4.5)

17:30-18:00 M.M. Kostic, B. Ognjanovic, K.V. Zikic, A. Stajn,
Faculty of Medicine and Faculty of Sciences, Kragujevac (Yugoslavia).
Activity of antioxidant enzymes of interscapular brown adipose tissue (IBAT) in cadmium-treated rats. (abstract 4.6)

Session 5. ***Free Radicals in Medicine I (Lung, Kidney).***

Chairperson: A. Ciaccia, University of Ferrara (Italy).

14:30-15:00 C.E. Cross, M.L. Hu, P. Motchnik, S. Louie, A. Reznik, L. Packer, B. Halliwell, UC Davis
Medical Center of Sacramento and Univ. of California, Berkeley (U.S.A.).
Molecular targets of biological damage by inhaled environmental oxidants. (abstract 5.1)

15:00-15:30 F.J. Kelly and S.C. Langley,
St. Thomas' Hospital, London (U.K.).
Role of glutathione in oxygen-induced lung injury of the newborn. (abstract 5.2)

15:30-16:00 M. Lopez-Torres, R. Perez-Campo, C. Rojas, S. Cadenas, G. Barja de Quiroga, R. Madrid and R.M. Arahuetes,
University of Madrid (Spain).
Pulmonary antioxidants in the fetus at term. Influence of maternal adrenal steroids. (abstract 5.3)

16:00-16:30	**Coffee break**
16:30-17:00	L. Baud, B. Fouqueray, C. Philippe and R. Ardaillou, INSERM - Hopital Tenon (France). The glomerulus as a source of reactive oxygen metabolites (ROM). (abstract 5.4)
17:00-17:30	J. Pincemail, J.O. Defraigne, C. Franssen, C. Philippart, D. Serteyn, C. Deby, M. Lamy and M. Meurisse, University of Liege (Belgium). Free radicals production during in vivo ischemia/reperfusion of rabbit kidney. (abstract 5.5)
17:30-18:00	J.K. Orak, G.S. Dhaunsi, S. Gulati, A.K. Singh, P.R. Rajagopalan and I. Singh Medical University of South Carolina (U.S.A.). Comparative activity of redox enzymes in cold renal ischemia utilyzing Euro-Collins(EC) vs. University of Wisconsin(UW) preservation solution in a whole organ model. (abstract 5.6)
18:00-19-00	**Poster Viewing**

THURSDAY JUNE 18

PLENARY SESSION ON FREE RADICALS AND CANCER

9:00-9:10	I. Emerit, Institute Biomedical des Cordeliers (France). Chairperson's remarks.
9:10-9:50	P. Cerutti, Swiss Institute for Experimental Cancer Research (Switzerland). Cellular response to oxidative stress. (abstract B.1)
9:50-10:30	B. Goldstein, Rudgers University and UMDNJ Medical School (U.S.A.) Free radicals and cancer. (abstract B.2)
10:30-11:00	**Coffee break**
11:00-11:10	T. Galeotti, Catholic University of Roma (Italy). Chairperson's remarks.
11:10-11:50	C. Hennekens, Harward Medical School (U.S.A.). Current and future epidemologic perspectives on vitamin E and beta-carotene in prevention of cardiovascular disease
11.50-12:30	K.H. Cheeseman and T.F. Slater, Brunel University (U.K.). Lipid peroxidation and cell division. (abstract B.4)
12:30-13:30	**Lunch Break**
13:30-14.30	**Poster Viewing**

AFTERNOON PARALLEL SESSIONS

Session 6.	***Redox Chemistry and Antioxidants.***
Chairperson:	L. Ernster, University of Stockholm (Sweden).
14:30-15:00	E. Cadenas, University of Southern California (U.S.A.). Reactivity of thiols towards the high oxidation state of myoglobin, ferrylmyoglobin. Importance of electron transfer and alkylation reactions. (abstract 6.1)

15:00-15:30	K.D. Asmus, A. Aced, S.A. Everett and Ch. Schoneich, Hahn Meitner Institute of Berlin (Germany). Recent aspects in peroxyl and perthiyl radical chemistry. (abstract 6.2)
15:30-16:00	R.H. Bisby and A.W. Parker, University of Salford and Rutherford Appleton Laboratory of Chilton (U.K.) Properties of the α-tocopheroxyl radical in phospholipid bilayer membranes. (abstract 6.3)
16:00-16:30	**Coffee break**
16:30-17:00	L. Landi, D. Fiorentini, L. Cabrini and A.M. Sechi, University of Bologna (Italy). Lipid and water soluble azoinitiators as a tool for the study of the antioxidant properties of ubiquinols with different chain length. (abstract 6.4)
17:00-17:30	D.R. Allen and P.B. McCay, University of Oklahoma, College of Medicine and Oklahoma Medical Research Foundation (U.S.A.). Formation by epinephrine of an iron-dependent reactive species that is not HO· but is capable of efficient hydrogen atom abstraction. (abstract 6.5)
17:30-18:00	F. Ursini, M. Maiorino, A. Roveri, P. Morazzoni, G. Pifferi, University of Udine, University of Padova and Inverni della Beffa, Milano (Italy). A novel antioxidant flavonoid (IdB 1031) affecting molecular mechanisms of cellular activation. (abstract 6.6)

Session 7.	***Gene Expression and Oxidative Damage.***
Chairperson:	K.J.A. Davies, The Albany Medical College (U.S.A.).
14:30-15:00	K.J.A. Davies, The Albany Medical College (U.S.A). Regulation of gene expression in oxidative stress. (abstract 7.1)
15:00-15:30	R. Tyrell, A. Nascimento and S. Basu-Modak, Swiss Institute for Experimental Cancer Research (Switzerland). Oxidant-inducible expression of the human heme oxygenase gene. (abstract 7.2)

15:30-16:00 S. Farr, X. Gidrol, D. Johnstone, M. Chernova
Harvard School Public Health of Boston (U.S.A.).
Characterization of the soi28 gene product in E. Coli.
(abstract 7.3)

16:00-16:30 **Coffee break**

16:30-17:00 S. Borrello, M.E. De Leo, T. Galeotti,
Catholic University, Roma (Italy).
Regulation of manganese superoxide dismutase expression in normal and tumor cells. (abstract 7.4)

17:00-17:30 A. Azzi, D. Boscoboinik and E. Chatelain,
University of Bern (Switzerland).
Regulation of cell growth by α-tocopherol. (abstract 7.5)

17:30-18:00 M. Parola, G. Leonarduzzi, E. Albano, G. Barrera, F. Biasi, M.E. Biocca, I. Dianzani, R. Muraca, G. Poli and M.U. Dianzani,
University of Torino (Italy).
Protection against CCl_4-induced liver fibrosis by dietary vitamin E supplementation is accompanied by the decrease of oxidative damage and by the inhibition of TGFB1 and procollagen I expression.
(abstract 7.6)

Session 8. ***Oxidant and Antioxidant Reactions in Plants.***

Chairperson: W. Bors, GSF Research Center, Neuherberg (Germany).

14:30-15:00 K. Asada, Kyoto University, Japan.
Microlocalization of scavenging enzymes for active oxygen in chloroplasts.
(abstract 8.1)

15:00-15:30 M. W. Sutherland, University of Southern Queensland (Australia).
A review of oxygen radical generation during the response of plants to pathogenic challenge. (abstract 8.2)

15:30-16:00 A. Puppo and M. Davies,
Université de Nice-Sophia Antipolis (France) and University of York (U.K.)
Detection of a globin-derived radical from leghemoglobin: possible protective effect of glutathione.
(abstract 8.3)

16:00-16:30 **Coffee break**

16:30-17:00 R.F. Haseloff, H. Hartel, B. Ebert, I.E. Blasig,
Research Institute of Molecular Pharmacology, Humboldt University,
Physikalisch-Technische Bundesanstalt, Berlin (Germany).
Methyl viologen-induced formation of free radicals in chloroplasts.
(abstract 8.4)

17:00-17:30 M.J. Alcaraz, M.L. Ferrandiz, M.J. Sanz, C. Montesinos, M. Paya,
Universidad de Valencia (Spain).
Effects of 7,8-dihydroxyflavone on glutathione depletion and lipid
peroxidation in bromobenzene intoxication. (abstract 8.5)

17:30-18:00 R. Carini, A. Comoglio, E. Albano, H. Basaga and G. Poli,
University of Torino (Italy) and M.E.T.U., Ankara (Turkey).
Free radical scavenging, antioxidant and antihepatotoxic activity of
Silipide. (abstract 8.6)

Session 9. ***Ischemia-Reperfusion.***

Chairperson: F. Ursini, University of Padova (Italy).

14:30-15:00 J.J. Lemasters, University of North Carolina (U.S.A.).
Cellular changes leading to cell death from hypoxia and oxidative
stress. (abstract 9.1)

15:00-15:30 G. Gerber, F.-R. Ungemach, W. Siems, T. Grune, S. Klee,
University of Berlin (Germany).
Oxidative stress during ischaemia/anoxia and reoxygenation: similarities
and divergencies depending on the experimental model. (abstract 9.2)

15:30-16:00 S. Bradamante, E. Monti, L. Paracchini, F. Piccinini,
CNR Center and University of Milano (Italy).
Involvement of free radicals in the reperfusion injury: prevention by
infusion of spin traps. (abstract 9.3)

16:00-16:30 **Coffee break**

16:30-17:00 S. Masuda, H. Utsumi and A. Hamada, Showa University of Tokyo (Japan).
In vivo ESR studies on radical reaction during femoral ischemia-reperfusion
injury of mice. (abstract 9.4)

17:00-17:30 L.C. Benov, V.I. Markova, Med. Inst. of Stara, Zagora,Pleven (Bulgaria).
Kinetics of activated oxygen production and lipid peroxidation in ischemia
and reperfusion. (abstract 9.5)

17:30-18:00 C. Guarnieri, R.M. Borzi, B. Grigolo, A. Facchini, T. Portsmann, I. Caldarera, A. Branzi, University of Bologna (Italy). Plasma Cu-Zn superoxide dismutase concentrations after acute myocardial infarction. (abstract 9.6)

Session 10. ***Free Radicals in Medicine II (Nervous System, Muscle).***

Chairperson: L. Packer, University of California, Berkeley (U.S.A.).

14:30-15:00 A.Z. Reznick, E.H. Witt, P. Starke-Reed and L. Packer, Technion-Israel Institute of Technology (Israel), University of California, Berkeley and George Washington University School of Medicine, Washington (U.S.A.). Oxidative stress and damage indicators in animals and human exercise. (abstract 10.1)

15:00-15:30 G.G. Duthie, Rowett Research Institute (U.K.) Free radicals, antioxidants and calcium homeostasis with reference to malignant hyperthermia. (abstract 10.2)

15.30-16.00 F.F. Oldfield, University of Missouri (U.S.A.). Free radicals, dopamine, levodopa and Parkinson's disease. (abstract 10.3)

16:00-16:30 **Coffee break**

16:30-17:00 G. Bruchelt, M. Schenk, G. Boos, J. Treuner and D. Niethammer, University of Tubingen (Germany). Induction of poly(ADP-ribose)polymerase in neuroblastoma cells by antibody-glucose oxidase conjugates and its enhancement by ascorbic acid. (abstract 10.4)

17:00-17:30 T. Hasegawa and M. Ichiba, Saga Medical School, Nabeshima (Japan). Active oxygen radical species as a first signal stress. (abstract 10.5)

17:30-18:00 G.A.C. Murrell, R.D. Goldner, A.V. Seaber, T.M. Best, Duke University, Durham (U.S.A.). The effects of the free radical modulator, tumor necrosis factor-α, on Achilles tendon healing. (abstract 10.6)

18:00-19:00 **Poster Viewing**

FRIDAY JUNE 19

PLENARY SESSION ON REACTIVE SPECIES IN METABOLIC DISORDERS

9:00-9:10	J. Gebicki, Macquarie University (Australia). Chairperson's remarks.
9:10-9:50	M. Navab, UCLA (U.S.A.). Atherosclerosis as an inflammatory reaction: artery wall cell and lipoprotein interactions. (abstract C.1)
9:50-10:30	G.M. Chisolm, Case Western Reserve University (U.S.A.). Free radicals and diabetes. (abstract C.2)
10:30-11:00	**Coffee break**
11.00-11:10	U.M. Marinari, University of Genova (Italy). Chairperson's remarks.
11:10-11:50	M. Ingelman-Sundberg, Karolinska Institute (Sweden). Ethanol-inducible cytochrome P450 2E1. Mechanisms of regulation, radical formation and toxicological importance. (abstract C.3)
11:50-12:30	B. Bacon, St. Louis University (U.S.A.). Hepatotoxicity of experimental hemochromatosis. (abstract C.4)
12:30-13:30	**Lunch Break**
13:30-14:30	**Poster Viewing**
13:30-14:30	**Lunch Time Seminar** Glutathione in Free Radical Pathology *(Sponsored by Boehringer Mannheim Italia)*
13:30-13-40	P.F. Mannaioni, University of Firenze (Italy). Introductory remarks.
13:40-13:55	G.Annoni, B. Arosio, D. Santambrogio, A. De Vincentiis, C. Vergani, University of Milano (Italy). Glutathione (GSH) supplementation prevents rat's liver damage from the acute CCl_4 intoxication. (abstract C.5)

13:55-14:10	D.P. Jones, P.S. Saniec, L.J. Dahm, W.J. Eley, E.W. Flagg and R.J. Coates, Emory University (USA). Utilization of oral glutathione (GSH). (abstract C.6)
14:10-14:25	A. Altomare, G. Vendemiale, P. Angelini, F. Cirelli, University of Bari (Italy). Role of glutathione as a therapeutic agent. (abstract C.7)
14:25-14:30	Conclusions

AFTERNOON PARALLEL SESSIONS

Session 11. ***Food Antioxidants.***

Chairperson:	A.T. Diplock, London University (U.K.).
14:30:15:00	M.G. Simic and S.V. Jovanovic, University of Maryland (U.S.A.) and the Boris Kidric Institute, Beograd (Yugoslavia). Mechanisms of food antioxidants. (abstract 11.1)
15:00-15:30	S. Banni, M.A. Dessì, M.P. Melis and F.P. Corongiu, University of Cagliari (Italy). Changes of vitamin E content in liver, plasma and adipose tissue of rats following carbon tetrachloride intoxication. (abstract 11.2)
15:30-16:00	F.R. Hewgill, University of Western Australia (Australia). BHA-precursor of a chemical menu. (abstract 11.3)
16:00-16:30	**Coffee break**
16:30-17:00	P. Lambelet, F. Saucy and J. Löliger, Nestlé Research Centre (Switzerland). Radical exchange reactions between vitamin E, vitamin C and phospholipids in autooxidizing polyunsaturated lipids. (abstract 11.4)
17:00-17:30	J. Terao and F. Ojima, National Food Research Institute, Tsukuba and Kagome Research Institute, Tochigi (Japan). Antioxidant effect of carotenoids on photosensitized oxidation of human blood plasma and low density lipoprotein. (abstract 11.5)
17:30-18:00	H. Korpela, R. Salonen, K. Nyyssönen, M. Parviainen, M. Kantola, J.T. Salonen, University of Kuopio (Finland). Low plasma beta-carotene, alpha-tocopherol and selenium levels associate with accelerated carotid atherogenesis in hypercholesterolemic men. (abstract 11.6)

Session 12. ***Lipid Peroxidation.***

Chairperson: M. Comporti, University of Siena (Italy).

14:30-15:00 I.B. Afanas'ev, Vitamin Research Institute, Moscow (Russia).
Effects of pro- and antioxidants on superoxide production and lipid peroxidation. (abstract 12.1)

15:00-15:30 W.G. Siems, T. Grune, H. Zollner and H. Esterbauer,
University of Berlin (Germany) and University of Graz (Austria).
Metabolism of the lipid peroxidation product 4-hydroxynonenal in liver and small intestine. (abstract 12.2)

15:30-16:00 L. Masotti, E. Casali, N. Gesmundo, G. Cavalli, A. Spisni,
University of Parma and University of Bologna (Italy).
Hydroxystearic acid: an unusual lipid peroxidation product affecting in vitro cell proliferation. (abstract 12.3)

16:00-16:30 **Coffee break**

16:30-17:00 S. Takeda, D. Horrobin, G. Sim and M. Manku,
Efanol Research Institute and National Research Council (Canada).
Polyunsatured fatty acid peroxidation mechanism specific to human breast cancer cells (ZR-75-1) producing selective cancer cell-killing in response to gamma-linolenic acid + Fe(II). (abstract 12.4)

17:00-17:30 D. Blache, INSERM, Bron (France).
Effects of oxysterols on arachidonate metabolism and calcium flux in platelet. (abstract 12.5)

17:30-18:00 E. Lissi, M. Slim-Hanna, L.A. Videla
USACH, Universidad de Chile, Santiago, Chile
Spontaneous visible urinary chemilluminescence and oxidative stress (abstract 12.6)

Session 13. ***NO and Related Radicals.***

Chairperson: H. Nohl, University of Wien (Austria).

14:30-15:00 V.M. Darley-Usmar, V.J. O' Leary, M.T. Wilson, N. Hogg and S. Moncada,
Wellcome Research Laboratories and University of Essex (U.K.).
Hydroxyl radical formation from the simultaneous generation of superoxide and nitric oxide: mechanisms and implications for the pathogenesis of atherosclerosis. (abstract 13.1)

15:00-15:30 M.J. Murphy, University of Vermont (U.S.A.)
Interactions between nitric oxide (NO) and oxygen radicals.
(abstract 13.2)

15:30-16:00 M. Saran, C. Dalke, C. Michel, W. Bors and U. Andrae,
GSF-Institute für Strahlenbiologie and GSF-Institute für Toxikologie
(Germany).
The formation of NO_2 radicals can explain the genotoxicity of 2-nitro-
propane. A pulse radiolysis study. (abstract 13.3)

16:00-16:30 **Coffee break**

16:30-17:00 J.S. Beckman, H. Ischiropoulos, L. Zhu, J. Chen, M. Tsai, J.C. Martin and
C.D. Smith, University of Alabama (U.S.A.)
Production of peroxinitrite by activated rat alveolar macrophages.
(abstract 13.4)

17:00-17.30 Y. Naito, T. Yoshikawa, T. Kaneko, S. Iinuma, S. Mishimura, S. Kokura,
K. Mastuyama and M. Kondo, Hikone Central Hospital and Kyoto
Prefectural University of Medicine (Japan).
Synergism between superoxide dismutase (SOD) and nitric oxide in gastric
mucosal protection following ischemia-reperfusion in rats.
(abstract 13.5)

17:30-18:00 V.K. Koltover, Russian Academy of Sciences (Russia).
Stimulation of transcription in mouse liver cells by nitric-oxide radicals.
(abstract 13.6)

Session 14. ***Carcinogenesis.***

Chairperson: R. Burdon, University of Strathclyde (U.K.).

14:30-15:00 P.A. Riley, University College and Middlesex School of Medicine (U.K.).
Derangements of cellular metabolism in the pre-malignant syndrome.
(abstract 14.1)

15:00-15:30 R. Lindhal, University of South Dakota (U.S.A.).
Roles of aldehyde dehydrogenases in carcinogenesis.
(abstract 14.2)

15:30-16:00 O.I. Aruoma and B. Halliwell, University of London, King's College (U.K.)
Iron and copper induced DNA damage. (abstract 14.3)

16:00-16:30 **Coffee break**

16:30-17:00 B. Fubini, A. Astolfi, S.Boasso, E. Giamello, M. Volante and E. Belluso, University of Torino (Italy).
Role of iron in asbestos carcinogenicity. (abstract 14.4)

17:00-17:30 M. Gerber, C. Ségala, J. Simony-Lafontaine, C. Astre, A.B. Guizard, H. Mathieu-Daudé and H. Pujol, CRLC Epidaure, Montpellier Cedex (France).
The relationship between plasma oxidant-antioxidant status and aggressiveness characteristics of breast cancer in young and aged patients. (abstract 14.5)

17:30-18:00 R.H. Burdon, University of Strathclyde (U.K.).
Released active oxygen species as intercellular signals - their role in regulation of normal and tumour cell proliferation. (abstract 14.6)

Session 15. ***Free Radicals in Medicine III (Liver, Eye, Skin).***

Chairperson: G. Bellomo, University of Pavia (Italy).

14:30-15:00 J. Fehér, Semmelweis University (Hungary).
Free radical scavengers in toxic liver lesions. (abstract 15.1)

15:00-15.30 V. Kagan and L. Packer, University of Pittsburgh and University of California, Berkeley (USA).
Protective or photosensitizing role of vitamin E? Light-induced and dark reactions of vitamin E radical in liver, eye and skin. (abstract 15.2)

15:30-16:00 P. Odetti, G. Noberasco, M.A. Pronzato, L. Cosso, A. Bellocchio, D. Cottalasso, U.M. Marinari, University of Genova (Italy).
Correlation between lipoperoxidation adducts and non enzymatic glycation in collagen during aging. (abstract 15.3)

16:00-16.30 **Coffee break**

16:30-17:00 M.A. Goss-Sampson, A. Kriss, K.J. Lindley and D.P.R. Muller, Institute of Child Health, London (U.K.).
Vitamin E and retinal function. (abstract 15.4)

17:00-17:30 M.R. Liles, M.V. Miceli, P.D. Oliver and D.A. Newsome, Touro Infirmary and Tulane University, New Orleans (U.S.A.).
An in vitro model of lipofuscinogenesis in human retinal pigment epithelium cells. (abstract 15.5)

17:30-18:00 A.L. Nieminen, B. Herman and J.J. Lemasters,
University of North Carolina (U.S.A).
Laser scanning confocal microscopy (LSCM) and digitized video microscopy (DVM) of cultured hepatocytes after oxidative stress with t-BuOOH: protection by 1,10,-phenanthroline. (abstract 15.6)

18:00-19:00 **SFRR International General Assembly**

SATURDAY JUNE 20

PLENARY SESSION ON PHARMACEUTICAL INTERVENTION IN FREE RADICAL-BASED PATHOLOGY

9:00-9:10	H. Sies, University of Düsseldorf (Germany). Chairperson's remarks.
9:10-9:50	A. Meister, Cornell University Medical College (U.S.A.). Approaches to the therapy of glutathione deficiencies. (abstract D.1)
9:50-10.30	M. Mino, Osaka Medical College (Japan). Metal-catalyzed free radical injuries in childhood disorders and pharmaceutical intervention. (abstract D.2)
10:30-11:00	**Coffee break**
11:00-11:10	G. Burton, National Research Council (Canada). Chairperson's remarks.
11:10-11:50	G.B. Bulkley, The Johns Hopkins Hospital, Baltimore (U.S.A). Pharmaceutical intervention for the prevention of post ischemic reperfusion injury. (abstract D.3)
11:50-12:20	***Discussion on Antioxidant Interactions***
Chairperson:	L. Packer, University of California, Berkeley (U.S.A).
12:20-12:30	**Closing Remarks** G. Poli, University of Torino (Italy).
12:30-13.30	**Lunch Break**
13:30-14:30	**Poster Viewing**
13:30-14:30	**Lunch Time Seminar** Lipid peroxidation: role of silymarin *(Sponsored by Istituto Biochimico Italiano, Giovanni Lorenzini, IBI)*
13:30-13:40	K.H. Cheeseman, Brunel University (U.K.). R. Fantozzi, University of Novara (Italy). Introductory remarks.

13:40-14:00 F. Minisci, A. Citterio, M. Dattilo, Politecnico di Milano and IBI, (Italy). Antioxidant activity of silymarin (abstract D.4).

14:00-14:20 J. Feher, Semmelweis University, Hungary. Antioxidant protection by silymarin in toxic liver lesions. (abstract D.5)

14:20-14:30 Conclusions.

AFTERNOON PARALLEL SESSIONS

Session 16. ***Antioxidant Defences in Eukaryotic Cells.***

Chairperson: E. Niki, University of Tokyo (Japan).

14:30-15:00 G. Burton, National Research Council of Canada (Canada). Antioxidant mechanisms of vitamin E and ß-carotene. (abstract 16.1)

15:00-15:30 J. Viña, M. Asensi, V.Anton, G. Estrela, F.V. Pallardó, J. Sastre, J.A. Ferrero, University of València (Spain). Oral antioxidant administration partially prevents exercise-induced glutathione oxidation in rats and humans. (abstract 16.2)

15:30-16:00 M. Maiorino, A. Roveri and F. Ursini, University of Padova and University of Udine (Italy). Phospholipid hydroperoxyde glutathione peroxidase is the major selenoperoxidase in nuclei and mitochondria of rat testis. (abstract 16.3)

16:00-16:30 **Coffee break**

16:30-17:00 M. Miki and M. Mino, Osaka Medical College (Japan). Involvement of free radicals catalyzed by metals in Wilson's disease and antioxidant defence. (abstract 16.4)

17:00-17:30 J. Emerit, F. Congy, D. Bonnefont, M.C. Jaudon, J. Delattre, Hopital de la Salpetrière, Paris (France). Oxidative stress status (OSS) in the elderly. (abstract 16.5)

17:30-18:00 A.F. Casini, E. Maellaro, B. Del Bello, L. Sugherini and M. Comporti, University of Siena (Italy). Redox cycle of ascorbate in the liver: a study of dehydroascorbate-reductase activities. (abstract 16.6)

Session 17. *Inflammation.*

Chairperson: B. Halliwell, UC Davis Medical Centre (U.S.A.).

14:30-15:00 T. Yoshikawa, Kyoto Prefectural University of Medicine (Japan).
Role of neutrophil-dependent oxygen radical in inflammatory reactions and cancer treatments. (abstract 17.1)

15:00-15:30 N.H. Hunt, T.M. Jeitner, J.-C. Fragonas, C.L. Kneale, D.M. van Reyk, University of Sidney (Australia).
Redox mechanisms in T cell activation. (abstract 17.2)

15:30-16:00 J. Sandström, L. Carlsson, H. Ohlsson, S.M. Marklund, T. Edlund, University of Umeå (Sweden).
Expression of human EC-SOD in pancreatic ß-cells of the non-obese diabetic (NOD) mouse. (abstract 17.3)

16:00-16:30 **Coffee break**

16:30-17:00 J.J.M. van den Berg, F.A. Kuypers, E. Roitman and C.C. Winterbourn, Children's Hospital Oakland Research Institute (U.S.A.) and Christchurch School of Medicine (New Zealand).
Identification of the reaction products of unsaturated fatty acids with the neutrophil oxidant, hypochlorous acid. (abstract 17.4)

17:00-17:30 M.L. Hu, S. Louie, P. Motchnik, C.E. Cross, B. Halliwell, UC Davis Medical Center, Sacramento and University of California, Berkeley (U.S.A.).
Oxidation of plasma constituents by hypochlorite. (abstract 17.5)

17:30-18:00 J.M.S. Davies, D.A. Horwitz and K.J.A. Davies, Albany Medical College and University of Southern California, Los Angeles (U.S.A.).
Collagen breakdown by hypochlorous acid and N-chloroamines: possible role in synovitis. (abstract 17.6)

Session 18. *Reactive Species in Metabolic Disorders.*

Chairperson: G. Nanni, University of Genova (Italy).

14:30.15:00 Y.A. Vladimirov, A.N. Osipov and A.V. Kozlov
Medical University, Moscow (Russia).
Free radical producing reactions of Fe^{2+} ions in biomembranes and lipoproteins. (abstract 18.1)

15:00-15:30 A. Boveris, University of Buenos Aires (Argentina).
Oxidative stress in ischemia-reperfusion
(abstract 18.2)

15:30-16:00 I.S. Young, S. Tate and E.R. Trimble, Queen's
University of Belfast (U.K.).
The effect of ascorbate and desferrioxamine treatment on oxidative stress in diabetes. (abstract 18.3)

16:00-16:30 **Coffee break**

16:30-17:00 G. Minotti and M. Ikeda-Saito, Catholic
University of Roma (Italy) and Case Western
Reserve University, Cleveland (U.S.A.).
Oxyradical generation and iron sequestration by a novel microsomal protein: a double edged sword? (abstract 18.4)

17:00-17:30 M. Williams, B.B.H. Krootjes, J. Van der Zee and J. Van Steveninck,
State University Leiden (The Netherlands).
Effects of protoporphyrin on the susceptibility of human erythrocytes to oxidative stress. (abstract 18.5)

17:30-18:00 M. Ferrali, C. Signorini, L. Ciccoli and M. Comporti,
University of Siena (Italy).
Iron release as a mechanism of membrane damage in erythrocytes exposed to oxidant agents. (abstract 18.6)

Session 19. ***DNA Damage and Repair.***

Chairperson: R. Meneghini, University of San Paolo (Brasil).

14:30-15:00 J. Cadet, M. Berger, G. Buchko, J.L. Ravonat and H. Kasai,
Centre d'Etudes Nucléaires, Grenoble Cedex (France) and National Cancer Center Research Institute of Tokyo (Japan).
Oxidation reactions of the purine and pyrimidine moieties of DNA and model compounds. (abstract 19.1)

15:00-15:30 M. Dizdaroglu, R. Olinski and Z. Nackerdien,
National Institute of Standards and Technology, Gaithersburg (U.S.A.).
DNA base damage and DNA-protein cross-links in chromatin of gamma-irradiated or H_2O_2-treated cultured human cells. (abstract 19.2)

15:30-16:00 C.G. Fraga, P.A. Motchnik and B.N. Ames,
University of Buenos Aires (Argentina) and University of California, Berkeley (U.S.A.).
Oxidative stress in human sperm: DNA damage and antioxidants. (abstract 19.3)

16:00-16:30 **Coffee break**

16:30-17:00 E.L. Nalvarte and D.M. Yourtee,
University of Missouri, Kansas City (U.S.A.).
Mutagenecity of doxorubicin in a free radical generating system. (abstract 19.4)

17:00-17:30 D. Touati, B. Tardat and I. Compan,
Institute Jacques Monod, Paris Cedex (France).
DNA oxidative damage and mutagenesis in E. Coli. (abstract 19.5)

17:30-18:00 D.I. Feig and L.A. Loeb,
University of Washington, Seattle (U.S.A.).
Mutagenesis by oxygen free radicals. (abstract 19.6)

Session 20. ***Free Radicals in Medicine IV (Red Cells, Heart).***

Chairperson: P. Hochstein, University of Southern California (U.S.A.).

14:30-15:00 W.D. Flitter, J.G. Coghlan, S.M. Clutton, A. Rees, C.D. Ilsley and T.F. Slater,
Brunel University, Uxbridge and Harefield Hospital (U.K.).
The role of free radicals and vitamin E in human reperfusion injury. (abstract 20.1)

15:00-15:30 G. Rotilio, M. Paci, M. Setta, A. Bozzi and M.R. Ciriolo,
"Tor Vergata" University of Roma and University of L'Aquila (Italy).
Glutathione and redox reactions of the erythrocyte membrane. (abstract 20.2)

15:30-16:00 H. Nohl, K. Stolze and L. Weiner,
Veterinary University of Wien (Austria) and Weizmann Institute of Science, Rehovat (Israel).
Detection of GSH-levels in perfused rat hearts by means of a novel ESR-method. (abstract 20.3)

16:00-16:30	**Coffee break**
16:30-17:00	O. Augusto, M. da Silva Morais and J. Vásquez-Vivar, University of San Paolo (Brasil). Peroxidation of the antimalarial primaquine: characterization of a benzidine-like metabolite with methemoglobin-forming activity. (abstract 20.4)
17:00-17:30	A. Arduini, V. Tyurin, Y. Tyurina, G. Dottori, F. Molayoni and R.R. Ramsay, "G. D'Annunzio" University of Chieti (Italy) and University of California, San Francisco (U.S.A.). Carnitine and carnitine palmitoyltransferase, key modulators of the acyl-trafficking in the course of the secondary antioxidant response to oxidative stress. (abstract 20.5)
17:30-18:00	I.B. Zavodnik, Academy of Sciences of Belarus (Belarus). The process of human hemoglobin autooxidation as source of free radicals in red cells. (abstract 20.6)

Pre-Meeting Conference

Free Radical Mechanism of Cell Injury

PM1 MECHANISMS OF OXIDATIVE CELL DAMAGE

Sten Orrenius, Department of Toxicology, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden

Exposure of mammalian cells to oxidative stress induced by redox-active compounds results in thiol and NAD(P)H oxidation followed by ATP depletion and the loss of cell viability. Protein thiol modification is associated with effects on various cell functions, including alteration of enzyme activities, impairment of signal transduction, and perturbation of cytoskeletal organization. The latter effect appears to be responsible for the formation of the numerous plasma membrane blebs, typically seen in cells exposed to cytotoxic concentrations of prooxidants. Following the disruption of thiol homeostasis in prooxidant-treated cells, there is a perturbation of intracellular Ca^{2+} homeostasis and subsequent Ca^{2+} overload. This Ca^{2+} increase can cause activation of various Ca^{2+}-dependent degradative enzymes (phospholipases, proteases, endonucleases) and may also be responsible for the mitochondrial damage seen in oxidative stress. Severe oxidative stress is also associated with extensive DNA damage which may result from direct radical attack and/or endonuclease activation. Under certain experimental conditions the DNA damage may lead to excessive stimulation of poly(ADP-ribose)polymerase activity and subsequent NAD+ and ATP depletion which may contribute to cell killing. Oxidative cell death is normally of the necrotic type, but in some experimental systems, moderate oxidative stress can induce apoptotic cell death.

CYTOTOXIC AND GENOTOXIC EFFECTS OF LIPID PEROXIDATION PM2

H. Esterbauer, F. Tatzber, H. Puhl, H. Zollner, J. Schaur, G. Waeg

Institute of Biochemistry, University of Graz
Schubertstr. 1, A-8010 Graz, Austria

A number of pathological effects associated with free radicals and oxidative stress appear to be mediated by lipid peroxidation and reactive aldehydic lipid peroxidation products, such as 4-hydroxynonenal, malonaldehyde and others (1). The findings, which support this hypothesis, will be discussed with particular emphasis to the cytotoxic antiproliferative and genotoxic effects of 4-hydroxynonenal (2). Research on lipid peroxidation and lipid peroxidation products received a remarkable stimulus in the recent years through the discovery, that oxidatively modified low density lipoprotein is highly cytotoxic and causes foam cell formation and is probably involved in the pathogenesis of atherosclerosis (3). These studies also led to the development of antibodies, which allowed the demonstration that 4-hydroxynonenal and malonaldehyde modified proteins are indeed produced in vivo.

1. H. Esterbauer et. al., Free Rad. Biol. Med. 11,81-128, 1991
2. H. Esterbauer et. al., in: Oxidative stress. Oxidants and antioxidants (H. Sies ed.), Academic Press 1991, pp. 337-369.
3. D. Steinberg et. al., N. Engl. J. Med. 320, 915-924, 1989.

Plenary Session on Radical Reactions in Aging

A.1 Free Radicals in Aging and Longevity Mechanisms.

R.G. Cutler, Gerontology Research Center, NIA, NIH, Baltimore, MD 21224

Most evidence indicates that aging is a result of normal metabolic processes that are essential for life. Thus an important approach in biogerontology is to identify specific metabolic reactions necessary for life but which could also lead to aging. A unique characteristic of this approach is an explanation of what governs aging rate or longevity of a species or even individuals within a species. These would be mechanisms that would act to reduce the long-term toxic or aging effects of the normal metabolic and developmental reactions. The reactions involving oxygen metabolism clearly fit into this model for they are essential for life yet can potentially cause many of the dysfunctions associated with aging. Such a model can also account for differences in aging rate or longevity of different animal species by differences that may exist in their innate ability to reduce oxidative stress state. Our laboratory has been testing this oxidative stress state (OSS) hypothesis of aging and longevity by determining if a positive correlation exists between OSS of an animal and its aging rate. Much of our data has found such a positive correlation, yet there are some indications that separate causive mechanisms may exist in determining aging rate as opposed to those related to age dependent specific diseases such as cancer or cardiovascular disease.

A.2 PROTEIN OXIDATION AS MARKER AND MECHANISM IN AGING AND ATHEROSCLEROSIS.

Roger T.Dean

Heart Research Institute, 145 Missenden Road, Camperdown, Sydney, NSW 2050, Australia.

There has been much emphasis in the literature on the accumulation during aging of inactive proteins, containing stable modified or lipid-derivatised amino acids. Many of these modifications may derive directly (protein oxidation) or indirectly (protein derivatisation by lipid oxidation products) from radical processes. While discussing the information on mechanisms of generation of such entities, I would also like to present new information on two novel protein modifications which give rise to reactive species: protein hydroperoxides and protein-bound reducing moieties including DOPA.

These species can, respectively, consume cellular reductants, and reduce transition metals such as iron and copper, whether present in low molecular weight or protein-bound forms. Their possible importance in influencing the progression of radical events, especially on adjacent protein surfaces, will be discussed. Data on their occurrence in certain aged materials is being gathered, and will be discussed. Kinetic considerations reveal that the steady-state pool of even a reactive molecule can be substantial, depending on both rates of generation and removal. Thus I will discuss how damage these species inflict may relate to the progression of aging.

A.3 MAILLARD REACTION AND OXIDATIVE STRESS ARE INTERRELATED STOCHASTIC MECHANISMS OF AGING

V.M.Monnier, D.R.Sell, R.H.Nagaraj, and P.Odetti.

Institute of Pathology, Case Western Reserve University, Cleveland, Ohio 44106, USA.

The incubation of proteins with reducing sugars leads to nonenzymatic browning Fluorescent and UV active products are formed which link covalently the proteins together. The formation of these crosslinks can be dramatically slowed down by removing O_2 from the reaction mixture.

Our laboratory has isolated a fluorescent molecule (335/385 nm) from aging human collagen and elucidated its structure which revealed presence of an imidazo-4,5b-pyridinium molecule involving a lysine and an arginine residue crosslinked by a pentose. The molecule, baptized "pentosidine", can be synthesized most rapidly from ribose, but even hexoses, threoses, trioses as well as ascorbate can act as pentosidine precursors. In every case, removal of O_2 dramatically reduces pentosidine formation rate.

Quantitation of pentosidine in various biological tissues revealed 1) pentosidine increases with age ubiquitously in the extracellular matrix 2) that diabetes and especially uremia promote its formation 3) that its formation in cataractous lenses is dependent on the integrity of the ascorbate/glutathione system and 4) that dietary restriction decreases its formation rate.

These data, together with work on carboxymethyl-lysine by Baynes and colleagues, those from Wolff et al. on autooxidative glycosylation and those from Namiki & Hayashi on sugar-derived pyrazine radical formation during browning reactions suggest that much of the damage resulting from free radicals in aging may in fact be mediated by oxidative stress on reducing sugars which engage in the Maillard reaction.

A.4 HYDROGEN PEROXIDE DEPENDENT INFLAMMATORY MECHANISMS IN ACUTE LUNG INJURY

BS van Asbeck, Department of Medicine, University Hospital Utrecht, PO Box 85500, 3508 GA Utrecht, The Netherlands

The damage to lung tissue that occurs during ARDS is a consequence of an inflammatory process that is set in motion by various stimuli, such as endotoxin and high concentrations of oxygen. Under the influence of vasoactive, chemotactic and adhesion-promoting substances produced by damaged endothelial cells and activated alveolar macrophages, activated platelets and granulocytes are sequestered in the pulmonary microcirculation, which leads to occlusion and pulmonary hypertension. This results in an increased permeability of the alveolar-capillary membrane, leading to hemorrhage and protein- and cell-rich interstitial and intra-alveolar edema. There is strong evidence that toxic oxygen species play a major role in this process. In particular hydrogen peroxide. This molecule is not only the precursor of hydroxyl radical and myeloperoxidase derived oxidants, but also appears to serve as a messenger in mediating the release of inflammatory factors. Therapeutic interventions aimed at controlling the effects of H_2O_2 by increasing intracellular concentrations of glutathione are thus indicated.

A.5 ANTIGENOTOXIC AND ANTICARCINOGENIC EFFECTS OF N-ACETYLCYSTEINE IN LUNG CELLS

S. de Flora, M. Bagnasco, R.M. Balansky, C. Bennicelli, A. Camoirano, F. D'Agostini and A. Izzotti

Institute of Hygiene and Preventive Medicine, University of Genoa, I-16132 Genoa, Italy

The thiol N-acetylcysteine (NAC) displayed antigenotoxic and anticarcinogenic properties in a variety of in vitro and in vivo test systems, and is now assayed as a cancer chemopreventive agent in clinical trials performed both in Europe and USA. NAC has been shown to work through multiple mechanisms, among which the ability to act as a nucleophile and antioxidant, inhibiting the genotoxicity of O_2^-, H_2O_2, and products of the reaction between $^1O_2$ and cell components. NAC exhibited several in vivo protective effects in lung cells. In urethan-treated mice, oral NAC prevented to a large extent the formation of lung tumors. In pulmonary alveolar macrophages (PAM) and lung parenchymal cells of rats, i.p. NAC stimulated detoxifying biochemical pathways. In rats receiving i.t. instillations of benzo[a]pyrene (BP), NAC by gavage prevented the induction of micronuclei in PAM and inhibited the formation of BPDE-DNA adducts in liver and lungs. In rats exposed whole-body to mainstream cigarette smoke for 40 consecutive days, NAC by gavage protected the respiratory tract form smoke-induced histopathological changes prevented the induction of micronuclei in PAM, exerted antitoxic effects in bone-marrow erythrocytes, inhibited the formation of BPDE-DNA adducts in heart and lungs, and modulated biochemical parameters in liver and lung cells.

Supported by CNR FATMA and CNR-ENEL Projects

A.6 ENHANCEMENT OF CANDIDACIDAL ACTIVITY OF PERIPHERAL BLOOD MONONUCLEAR CELLS AFTER IN VITRO TREATMENT WITH N-ACETYL CYSTEINE

Bistoni, Dottorini*, Cociani, Pietrella, Todisco*, Vecchiarelli. Dept. Exper. Med. & Biochem. Sci., Microbiology Sec., University and *Pulmonary Unit, R. Silvestrini, Perugia, Italy

N-acetyl cysteine (NAC) is a thiol containing compound used as a mucolytic drug. It is able to reduce mucus viscosity, acts as a gluthatione precursor and increases cysteine levels in cells. It is employed as a direct free radical scavenger: in particular, it has an excellent action on hydroxyl radicals (OH). The beneficial effect has been studied in chronic bronchitis. Recently NAC has been involved in the inhibition of stimulatory effects of TNF-α on replication of HIV in T cells and peripheral blood mononuclear cells (PBMC). In the present study, we selected 10 patients with chronic obstructive pulmonary disease and 10 with lung cancer. Ten assumed NAC (600 mg/die) for 15 days and ten received placebo consecutive for 15 days. We analyzed candidacidal activity in alveolar macrophages (AM), PBMC and polymorphonuclear cells (PMN) from these patients before and after treatment. The results show that candidacidal activity of the cells before and after NAC treatment were similar to placebo-treated patients. On the contrary in an other group of lung cancer patients (n. 5) the addition of NAC in vitro was able to enhance candidacidal activity. In our opinion, the phenomenon observed in vitro could be reproduced in vivo, through appropriate dosage and monitoring, especially in immunodepressed patients, when opportunistic infection become frequent and life-threatening.

Session 1

Free Radicals in Radiation-Exposed System

1.1 FREE RADICALS IN RADIATION - EXPOSED SYSTEMS

Clemens von Sonntag

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D 4330 Mullheim /R Germany

Radiation - chemical techniques have made major contributions to our presen knowledge of free-radical chemistry, especially to that in a aqueous environment Here it is recalled that the living cell consists of 70% water.
When ionizing radiation is absorbed by matter,radical cations, electrons anc excited molecules are generated. In water this leads to the formation of OH-radicals, solvated electrons and H-atoms as reactive intermediates. The solvated electron can be converted with nitrous oxide into further OH-radicals. The system then consists of 90% OH-radicals and only 10% H-atoms. It will be shown that in a similar manner the water radical can be converted quite specifically into a whole series of radicals, e.g. R, $R^{\bullet}$, $CO_2^{\bullet-}$, RS, $PhO^{\bullet}$, $O_2^{\bullet-}$ or $ROO^{\bullet}$.
The reactions of such radicals and their kinetics can be followed by pulse radiolysis using a variety of detection systems, e.g. optical spectroscopy, conductometry, e.s.r., and (with polymers) light-scattering . With the help of these techniques a whole body of reaction rate constants and redox-potentials of free radicals has been obtained.
In the radiotherapy of cancer use is made of the ability of ionizing radiation to kill cells. It has been shown that this effect is mainly caused by DNA damage. Some of the DNA damage can be repaired by the cell's thiol pool, largely glutathione. Examples will be given, high radiation-chemical techniques can be used to elucidate the underlying free radical reactions.
Ionizing radiation provides a unique tool to generate free radicals in crystalline material. Under favourable conditions chain reactions may be induced whose propagation are determined by the structure of the crystal.

1.2 OXIDATIVE STRESS IN ATOMIC BOMB SURVIVORS

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Based on accumulated epidemiological data for the atomic bomb (A-bomb) survivors in Hiroshima and Nagasaki, two possibilities with regard to radiosensitivity are discussed, i.e., cell turnover and an oxidative stress to the host. In previous studies, significant relationships between an acute symptom of irradiation (epilation) and leukemia mortality and between epilation and chromosome aberrations were observed, suggesting radiation estimation errors and/or individual differences in radiosensitivity. There is evidence that epilation is observed more in younger survivors than in older survivors. Taking into consideration that rapid cell turnover is related to radiosensitivity, and that there is a similarity between hair cells and bone marrow stem cells in terms of rapid cell turnover, the evidence is more likely to be due to radiosensitivity. In addition, since the oxidative stress of inflammation damages DNA and/or cell membranes, it is possible that the oxidative stress of inflammation that has been observed in A-bomb survivors might play an important role in the late effects of A-bomb irradiation. Neutrophilia, observed in A-bomb survivors, could be typical evidence of both the rapid cell turnover and the oxidative stress to the host.

1.3 STUDIES OF MODIFIED SUPEROXIDE DISMUTASES*

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Superoxide dismutase (SOD) is an enzyme that catalyzes the dismutation of O_2^- to oxygen and peroxide. Site directed mutagenesis has been successful in affording the preparation of SOD in which selected amino acid residues have been altered. The activity of these enzymes, as measured by pulse radiolysis, when compared to that of the unmodified enzymes, allows us to assess the importance of certain residues to the electrostatically facilitated direction of O_2^- to the active site and to the overall activity of the enzymes. Modifications were carried out to the residues: Arg 143, Thr 137, Lys 136, Glu 133 and Glu 132. The effects of these modifications upon catalytic activity will be discussed. In addition, some results demonstrating the effects structural changes at the active site and in the active site channel have on the activity will be presented. Finally, an anomalous result concerning the effect of phosphate on enzymes modified at Glu 132 and Glu 133 will be described.

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1.4 LONG LIVED REDUCTIVE MOIETIES ON FREE RADICAL DAMAGED PROTEINS

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Interactions between free radical species and protein, with resulting modification of the latter are well known. As part of our strategy to define protein oxidation more fully and to permit adequate assessment of its physiological relevance, we report some studies on novel long lived reactive products in oxidised protein.

In contrast to the native form, BSA exposed to free radicals generated by gamma irradiation, UV irradiation, and Fe or Cu/H_2O_2 systems, significantly reduced cytochrome c as assessed by its increase in absorbance at 550 nm. The reductive capacity, termed protein bound reducing moiety(ies, PBRedM), was stable for many days, and the amount of PBRedM generation was related to the dose of UV or gamma irradiation of the protein.The reductive capacity was consumed after approximately two hours of incubation with cytochrome c at room temperature and was stoichiometric with the concentration of the irradiated protein. The radical modified BSA also reduced free ferric and cupric ions which were detectable by coupling to neocuproine and ferrozine dyes respectively. PBRedM formation was not specific to BSA but was found on soybean trypsin inhibitor, insulin, lysozyme and human serum albumin after gamma irradiation.

Irradiations under different gassing regimes and the inclusion of radical scavenger studies have indicated that PBRedM is formed by hydroxyl radicals nteracting with aromatic amino acids. Tyrosine was found to produce higher levels of reductive capacity than the other common amino acids. Recent studies have indicated that PBRedM is probably protein bound DOPA. PBRedM may play a role in the replenishment of reduced transition metal ions involved in potentially physiological and pathological free radical reactions. The stability of PBRedM should permit them to diffuse through biological systems, in contrast to the transient radicals themselves.

Simpson, J. A., Narita, S., Gieseg, S., Gebicki, S., Gebicki, J.M. and Dean, R.T. (1992) Biochem. J. In press.

1.5 *IN VIVO* ESR STUDIES ON RADICAL REACTION IN WHOLE MICE -EFFECT OF RADIATION EXPOSURE-

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Recent development of L-band ESR spectrometer made possible to measure radical species in whole animal noninvasively. Nitroxide radicals are known to react with various redox systems including active oxygens, and *in vivo* ESR measurement using nitroxide radicals as probes were investigated by several groups to estimate the radical reaction in whole animals. We previously reported nitroxide radical reactions in head, lung, muscle, and blood in whole mice by *in vivo* ESR spectroscopy. Here, we demonstrate the relation of spin-clearance of nitroxide to biological phenomena, and the effect of radiation exposure on nitroxide radical reaction in whole body.

Nitoroxide radicals, TEMPO and PROXYL derivatives, were dissolved in isotonic buffer. The solution was administered to female ddY or A/J mice anesthetized with pentobarbital, and then ESR spectrum was successively observed with *in vivo* ESR spectroscopy.

Nitroxide radicals lost their paramagnetism in whole mice obeying first order kinetics. The reduction rate of the radicals i.v. administered depended on oxygen concentration of the inspired gas, and the influence of oxygen differed between in head and in abdomen, indicating that inspired oxygen should affect radical reaction in different manner.

Nitroxide radical injected into lung also reduced its paramagnetism by one-electron reduction. Treatment of lung with SH modifying reagents inhibited the reduction and lipid-soluble nitroxide radicals were reduced much faster than lipid-insoluble one, suggesting that membranous components should take part in the radical reduction in lung.

The radiation exposure to whole mice also influenced on spin-clearance of nitroxide radicals i.v. administered. High dose (15 Gy) reduced spin-clearance to the half, but low dose (7.5 Gy) reduced a little, indicating that radiation exposure caused damage in some redox systems of mice.

The present studies suggest that *in vivo* ESR measurements with nitroxide radicals as probes should reflect biological activities such as radical generation and reduction.

1.6 PROTECTIVE EFFECT OF SELENIUM AND ZINC ON UV-A DAMAGE ON HUMAN SKIN FIBROBLASTS

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Ultraviolet A radiation participates in cytotoxicity and carcinogenesis of the skin by a mechanism involving the generation of reactive oxygen species. Endogenous antiradical defense systems utilize metalloenzymes including selenium-dependent glutathione peroxidase (GPX) and copper and zinc superoxide dismutase (SOD). We determined the protective effect of selenium (Se) and zinc (Zn) on human fibroblasts exposed to UV-A radiation (λ=375nm). Se in the culture medium (0.1 mg/L) in the form of sodium selenite increased the synthesis and activity of GPX by 60.5% and 35% in the absence of irradiation and after irradiation with 5 J/cm2 (P=0.043). The presence of this element significantly increased the survival of UV-A-irradiated fibroblasts (P<0.0001). In addition, malondialdehyde (TBARs) decreased in the presence of exogenous selenium: -19% and -22% without irradiation and after irradiation with 5 J/cm2 (P=0.056). When Zn was added at the dose of 6.5 mg/L as $ZnCl_2$, fibroblasts subjected to oxidizing stress induced by UV-A were protected (P<0.0001). TBARs production decreased significantly: -33% and -34% without irradiation and after irradiation with 5 J/cm2 (P=0.0085). SOD activity, however, decreased after supplementing with zinc: -26% and -20% without irradiation and after UV-A (P=0.017). These results confirm the essential role of Se in the detoxifying activity of the GPX whereas the antioxidant properties of Zn are thus apparently independent of SOD activity.

Ref.: Dudek E.J. and al., 1990, J. Free Rad. Biol. Med. 9, 76. Richard M.J. and al., 1990, Nouv. Dermatol. 9, 1-7. Thomas J.P. and al., 1990, J. Biol. Chem., 265, 454-461. Tyrrell R.M. and al., 1990, Photochem.photobiol. 4, 349-361.

1.7 FREE RADICALS IN IRRADIATED SPICES

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Twenty five different spices were irradiated to a dose of 20 k Gray by a Cs^{137} source. The ESR free radical spectra of all samples were measured 2, 8, 22, 42, 72 and 403 days post irradiation, using a carefully standardised technique, with a Varian E12 spectrometer. Wide low field scans were also used to check transition metal ion spectra. ESR intensity decay curves can be explained either on the basis of a second order process, or on the basis of different radicals decaying at different rates. For some spices, permanent (against subsequent re-irradiation) changes in transition metal ion signals occur; some retain quite large stable free radical signals. Difficulties in detecting "irradiated" form "non-irradiated" species will be discussed.

1.8 INDIRECT EVALUATION OF IRRADIATION GENERATED FREE - RADICALS BY MEASUREMENT OF TBA - REACTIVE SUBSTANCES AND LIPOFUSCINOGENESIS

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The ionizing radiations damage biological structures in different ways but one of the most important is the production of free radicals, particularly oxyradicals, that can subtract electrons to biomolecules triggering a process taking to the compromision of whole cellular areas. Well known indexes of oxidative stress are the evaluation of total TBA (thiobarbituric acid) reactive substances and fluorimetric levels associated to the so called pigment "lipofuscin".

In this work we have tried to quantify, from a biochemical point of view, the consequence of a single dose of 4Gy on a marine organism, Torpedo marmorata, characterised by the lack of antioxidant defenses in central nervous system (CNS).

1.9 THE MECHANISM OF NORDIHYDROGUAIARETIC ACID ACTION IN MICE AFTER IRRADIATION

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Our earlier experiments showed improved hemopoietic recovery in mice irradiated for one hour after application of nordihydroguaiaretic acid (NDGA). It is known that NDGA acts as a cyclooxygenase and lipoxygenase (enzymes regulating hemopoiesis) blocker and has also been reported as a strong antioxidant (AO). This study was designed to decide which of these two mechanisms determines the radioprotective effect of NDGA.
AO effects of NDGA in a concentration range of 0.03 - 30 ,ug/ml were studied on bone marrow phagocytes of CBAxC57Bl mice. Luminol-amplified chemiluminescence (CL) was employed to detect reactive oxygen species generated by phagocytes activated with opsonized zymosan.
While NDGA applied *in vitro* strongly reduced the CL response, no changes were detected in sera samples of mice investigated one hour after i.p. application when compared with controls.
These differences suggest that NDGA is probably metabolized *in vivo*. Therefore, the improved hemopoietic recovery may be a consequence of NDGA interference with regulatory events rather than due to direct AO effects.

1.10 THE REACTIVITY OF NITRO BLUE TETRAZOLIUM WITH CO_2^-/O_2^- RADICALS*

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The generation and disappearance of the one-electron reduced intermediate of NBT^{2+}, $NBT^{\bullet+}$, was investigated. The kinetic results indicate that, under anaerobic conditions, the latter process involves a NBT^{2+}-dependent second-order reaction at high pH (pH>8), resulting in the production of parent NBT^{2+} and the protonated two-electron reduced species, MF^+. At lower pH, a similar kinetic process was observed, followed by a NBT^{2+}-dependent first-order reaction, resulting in much smaller production of MF^+. A plausible mechanism to fit these results is suggested. In the presence of high concentrations of O_2, the reaction between $NBT^{\bullet+}$ and O_2 was measured ($k = 1.1 \times 10^5\ M^{-1}\ s^{-1}$), yielding an equilibrium constant for $NBT^{\bullet+} + O_2 \rightleftharpoons NBT^{2+} + O_2^-$ of K = 0.31. A reaction between O_2^- and $NBT^{\bullet+}$ was not observed, leading to a limiting rate of $k<10^8\ M^{-1}\ s^{-1}$. Finally, the reaction between O_2^- and MF^+ was found to be much faster than that of O_2^- with NBT^{2+}.

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1.11 Oxidative damage to lysozyme by the hydroxyl radical; comparative effects of scavengers

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The hydroxyl radical (OH)° is considered the most toxic reactive oxygen species, given its high reactivity with molecules present at its site of formation and the generation of secondary, potentially toxic, free radical formation. This study was aimed at determining if (OH)° scavenging by uric acid, pentoxifylline (Ptx, a methylxanthine) or thymine could fully or partially protect a protein (lysozyme).
When lysozyme was incubated with (OH)°, it was inactivated with a yield of 7 moles (OH)° per mole of lysozyme; acrylamide gel electrophoresis showed the presence of dimer and trimer aggregates. Inactivation was associated with a loss of the major protein band (14.4 kDa) corresponding to native lysozyme; tryptophan fluorescence was lost before aggregation became detectable in terms of bityrosine formation. Densitometric analysis of electrophoretic gels of irradiated lysozyme also revealed specific fragmentation. Increasing concentrations of (OH)° scavengers gave increasing protection of lysozyme activity, showing that none of the scavengers used were toxic. Although all three compounds scavenge (OH)° with high rate constants, their effects were different: uric acid and Ptx prevented aggregation and preserved enzyme activity, whereas thymine preserved activity but did not prevent aggregation. The protective effect appeared to be associated with the quantity of reducing secondary radicals formed.

1.12 Gamma and pulse radiolysis study of pentoxifylline, a methylxanthine

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Pentoxifylline (Ptx) is a trisubstituted purine with antiinflammatory properties thought to be due, in part, to oxygen radical scavenging. We reports an investigation of the reaction of Ptx with hydroxyl radical [(OH)°], superoxide anion, azide radical and hydrogen peroxide generated by pulse and γ-radiolysis, which are carried out to determine the scavenging properties of Ptx towards oxygen radicals.

In γ-radiolysis, the action of (OH)° on Ptx at pH 7.4 gave rise to an end-product separated by high performance liquid chromatography and identified by nuclear magnetic resonance and mass spectrometry as C-8-OH-Ptx (yield of $0{,}12 \times 10^{-6}$ mol. J^{-1}).
The reaction of Ptx with (OH)° after pulse radiolysis at pH 7-7.4 occurred with a rate constant of $(7.7 \pm 1.0) \times 10^9\ dm^3\ mol^{-1}\ s^{-1}$, forming time-dependent transient radicals. The initial spectrum (2 μs after the pulse) showed three maxima. A decrease in the absorbance around 500 nm and an increase around 310 nm reflected a first-order reaction suggesting a unimolecular rearrangement. It was shown by redox titration that at least two OH-adducts were formed; one with reducing and the other with oxidizing properties.These results suggest that the reducing radical may be (C-8-OH-Ptx)°.

1.13 ON THE SPIN TRAPPING OF PEROXYL RADICALS BY DMPO. A STUDY COMBINING PULSE RADIOLYSIS AND EPR SPECTROSCOPY.

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Conflicting data exist on the spin trapping of peroxyl radicals by the cyclic nitrone spin trap 2,2-dimethyl-pyrrolidine-N-oxyl (DMPO). On the one hand, the peroxyl radicals $CH_3OO^{\cdot}$ and $(CH_3)_3COO^{\cdot}$ can be clearly identified as moderately stable adducts with hyperfine splitting constants distinct from those of other adducts of oxygen-centered radicals (e.g. alkoxyl, hydroxyl or superoxide). On the other hand, several studies reported on the instability of peroxyl adducts of DMPO which should eventually lead to an adduct which is identical to or closely resembles the DMPO-OH adduct.

To resolve this discrepancy we determined the rate constants of peroxyl radicals derived from alcohols with DMPO. While we obtained quite high trapping rate constants, no EPR-detectable adducts were observed both after pulse-radiolytic and photolytic generation of the peroxyl radicals.

We propose therefore that alkyl peroxyl radicals with alpha-hydroxygroups form highly unstable adducts, whereas simple alkyl peroxyl radicals can be clearly identified by their DMPO-adducts.

Session 2

Oxidized Lipoproteins

2.1 EFFECT OF VITAMIN E AND OTHER ANTIOXIDANTS ON THE OXIDATION RESISTANCE OF LOW DENSITY LIPOPROTEIN

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The Cu^{2+}-stimulated oxidative modification of LDL can be measured by continuously monitoring the 234nm absorbance, which shows three consecutive phases: a lag, propagation and decomposition phase. We use the lag phase measurement to define the oxidation resistance (OR) of different LDL samples. During the lag phase the antioxidants in LDL (vitamin E, carotenoids, ubiquinol-10) are consumed with α-tocopherol (α-T) as the first and ß-carotene as the last one. α-T is the most prominent antioxidant of LDL (0.4±1.8 mol/mol LDL), whereas the concentration of the others is only 1/10 to 1/300 of α-T. In a screening study with 78 subjects the lag phase varied from 34 to 114 min. Interestingly, only a weak correlation was found between the α-T content and the lag phase ($r=0.2, p<0.01, n=78$). Increasing the α-T content of individual LDL samples in vitro or by oral supplementation led always to a proportional increase of OR, according to the equation $y=kx+a$. The slope **k** is the efficacy of vitamin E and the intercept **a** represents a vitamin E indipendent parameter. Strong individual variations were observed for **k** and **a** (0.7 to 17 for **k** and 68 to 108min for **a**), which probably explains that the vitamins E content alone is not predictive for the OR of an individual LDL.
Other antioxidants not contained in LDL (probucol, α- and gamma -tocotrienol, trolox C, ascorbate, glutathione) were also tested in vitro. All of them prolonged the lag phase, with probucol as the most efficient one. In contrast to ascorbate, trolox C and glutathione, probucol did not spare vitamin E in LDL.

EFFECT OF ASCORBATE ON THE PRODUCTION OF FREE RADICALS DURING OXIDATE MODIFICATION OF LOW DENSITY LIPOPROTEIN- AN ELECTRON SPIN RESONANCE STUDY 2.2

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There is overwhelming evidence that oxidatively modified low density lipoprotein (OX-LDL) is atherogenic. Ascorbate supplementation has been shown to inhibit significantly the formation of OX-LDL. Electron spin resonance (ESR) studies have shown that the _- tocopheroxyl radical is a primary free radical formed during oxidation of LDL. In some oxidation systems, a secondary free radical, presumably derived from the other endogenous anti-oxidants in LDL, also appeared with time. ESR studies have also demonstrated that both _-tocopheroxyl and probucol phenoxyl radicals are formed during oxidation of LDL-probucol. These lipophilic antioxidant-derived radicals undergo recycling in the presence of ascorbic acid, with the concomitant formation of ascorbate radicals. The ESR results are consistent with the finding that ascorbate supplementation inhibits degradation of OX-LDL by macrophages. In this talk, ESR evidence for the prooxidant and antioxidant mechanism of action of ascorbic acid in LDL-oxidation will be discussed.

2.3 THE ROLE OF UBIQUINOL-10 IN THE INHIBITION OF THE EARLY STAGES OF LIPOPROTEIN LIPID OXIDATION

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Oxidation of low-density lipoprotein (LDL) lipid is important as oxidatively modified LDL is thought to contribute to the formation of lipid-laden cells (foam cells) in atherosclerotic lesions.

Using ultra-sensitive HPLC assays for lipid hydroperoxides (LOOH), we have shown recently that (i) ubiquinol-10 ($CoQ_{10}H_2$) is associated with LDL, and (ii) under conditions of constant rate of initiation the oxidation chain length in LDL was low (0.2-0.4) as long as $CoQ_{10}H_2$ was present, but increased ≈25-fold upon its consumption even though 80-95% of α-tocopherol (α-Toc) and carotenoids were still present (Stocker, R. et al. *Proc. Natl. Acad. Sci. USA* **88**, 1646). We now show that dietary supplementation of human volunteers with ubiquinone-10 (CoQ_{10}) resulted in increased [$CoQ_{10}H_2$] within circulating LDL and such supplemented LDL was more resistant towards the initiation of lipid oxidation, to an extent that was proportional to the LDL's initial [$CoQ_{10}H_2$]. In sharp contrast to the situation with the LDL particle, the peroxyl radical-mediated oxidation chain length of *extracted* LDL lipids in homogeneous system was very low (≈0.03) and not significantly influenced by the addition of physiological [$CoQ_{10}H_2$]. Also in contrast to LDL, oxidation of isolated high-density lipoprotein (HDL) resulted in LOOH formation without delay and at a linear rate throughout the incubation. Competition experiments carried out in fresh plasma showed that HDL lipids were oxidized before those in LDL. This was likely due to the absence of $CoQ_{10}H_2$ and α-Toc from most freshly isolated HDL particles. Indeed, in plasma these antioxidants were preferentially located in LDL and this was reflected in an uneven distribution of plasma LOOH: 85% of the detectable plasma cholesterylester-OOH (CEOOH) were carried in HDL particles and only 15% in LDL. Linked with high [CEOOH] was (i) the presence of phospholipid hydroperoxides in HDL, and (ii) a low $CoQ_{10}H_2$:CoQ_{10} ratio. Neither $CoQ_{10}H_2$ nor α-Toc alone correlated with plasma LOOH. The results will be discussed in the light of the role of $CoQ_{10}H_2$ in preventing the early stages of lipoprotein lipid oxidation.

OXIDATIVE MODIFICATION OF LOW DENSITY LIPOPROTEIN INDUCED BY FREE RADICALS GENERATED IN ITS INSIDE OR OUTSIDE 2.4

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The oxidative modification of LDL induced by copper, water-soluble radical initiator, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) or lipid-soluble radical initiator, 2,2'-azobis(2,4-dimethylvaleronitrile (AMVN), has been studied by following oxygen consumption and the changes in vitamin E, lipid hydroperoxides, thiobarbituric acid reactive substances (TBARS), relative electrophoretic mobility (REM), and aggregation and fragmentation of apolipoprotein B. These initiators all induced the free radical-mediated, chain oxidation of LDL and gave phosphatidylcholine hydroperoxide (PCOOH) and cholesteryl ester hydroperoxide (CEOOH) as major products. When compared at the same extent of oxidation, AMVN gave CEOOH in the highest yield, suggesting that AMVN initiates lipid peroxidation in the core part of LDL. Furthermore, the rates of accumulation of hydroperoxides were enhanced after depletion of vitamin E in the oxidations induced by copper or AAPH but those induced by AMVN were less dependent on the presence or absence of vitamin E, implying that vitamin E does not suppress the oxidation taking place in the core efficiently. TBARS also increased in all oxidation systems and copper gave higher TBARS than both azo compounds. Good correlation was observed between TBARS and REM or fragmentation of apolipoprotein B in all oxidation systems, although the manner observed in the oxidations induced by AMVN was different from those induced by copper or AAPH. These results suggest that the oxidative modification of LDL is dependent on the type of chain initiation, above all the presence or absence of metal ions and site of radical formation.

2.5 HAEM PROTEINS AS MEDIATORS OF THE OXIDATIVE MODIFICATION OF LOW DENSITY LIPOPROTEINS

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We are interested in the potential for haem proteins to mediate the oxidative modification of low density lipoproteins. The interaction of ruptured erythrocytes and myocytes with LDL induces oxidative damage to the LDL as detected by alterations in electrophoretic mobility and the peroxidation of the polyunsaturated fatty acyl chains. Difference spectroscopy reveals that the induction of the oxidative process by the haem proteins is apparently dependent on the transition of the oxidative state of the haemoglobin in the erythrocyte lysate from the oxy [X-Fe^{II}-O_2] to the ferryl [X-Fe^{IV}=C] state. The timescale of this haem conversion is related to the antioxidant status of the LDL and the erythrocyte lysate. The incorporation of lipid-soluble antioxidants at specific time points during the LDL-erythrocyte interaction prolongs the lag phase to oxidation, eliminates the oxy to ferryl conversion of the haemoglobin, and prevents the oxidative modification of the LDL.

KINETIC SIMULATION OF COPPER INDUCED OXIDATION OF LOW DENSITY LIPOPROTEIN 2.6

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It has long been known that incubation of low density lipoprotein (LDL) with copper ions results in oxidative modifications of the LDL particle. Modification can be continuously monitored by observing both the production of conjugated dienes and the uptake of oxygen. We have shown that oxygen uptake and conjugated diene formation follow synchronous time courses consisting of a slow 'lag' period followed by a fast phase. We have used kinetic simulation software to model a proposed mechanism of copper dependent peroxidation. From these simulation studies we have made the following conclusions: 1) The length of the lag period is dependent upon the concentration of endogenous peroxide, the presence of antioxidant and the concentration of copper. 2) In the absence of antioxidants the lag period is proportional to the log of the concentration of peroxide when copper concentration is constant and in excess over peroxide. 3) The observed inhibition of LDL oxidation by antioxidants is strongly dependent on the content of endogenous peroxide and on the point during the oxidation at which the addition of antioxidant is made.

2.7 SYNERGISTIC INHIBITION OF THE OXIDATION OF LIPID MICROSPHERE BY UBIQUINOL-10 AND α-TOCOPHEROL

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The oxidation of low density lipoprotein (LDL) has received much attention since it may play a very important role in atherosclerosis. α-Tocopherol (VE) has been suggested to be the best antioxidant in LDL, however, we reported at the previous SFRR meeting that the decrease of ubiquinol-10 (UQ-10) preceded that of VE and the formation of lipid hydroperoxides was significant only after the depletion of UQ-10 in the cupric ion-induced oxidation of LDL. Stocker et al. also reported similar results when the oxidation of LDL was initiated with other sources of free radicals [PNAS (1991) **88**, 1646]. In order to clarify the role of UQ-10, we have compared the antioxidant activities of UQ-10 and VE in lipid microsphere (LM), since the preparation of VE-free LDL is difficult. LM was prepared by dispersing a mixture of soybean phosphatidylcholine (PC), methyl linoleate (18:2 Me), and stearylamine (SA) into water. In the oxidation of LM initiated with oil-soluble azo-initiator at 37°C in air, constant rates of the formation of hydroperoxides of PC and 18:2 Me were observed. The addition of VE or UQ-10 gave a distinct induction period and the stoichiometric number for peroxyl radical trap by UQ-10 was calculated as 1 by the comparison of the lengths. The rates of oxidation (Rinh) of 18:2 Me during the induction period were similar for two antioxidants, suggesting the rates of peroxyl radical trap by VE and UQ-10 are similar. On the other hand, VE inhibited the oxidation of PC better than UQ-10. When both antioxidants were present, UQ-10 spared VE and Rinh of two substrates decreased with increasing VE concentration. However, in the absence of UQ-10, Rinh did not decrease with increasing VE concentration, indicating that VE needs reductants for its maximum antioxidant activity. These results and the fact that UQ-10 content is much less than VE in LDL suggest that UQ-10 mainly acts as a reductant for VE radical rather than a direct trap of oxygen radicals.

EFFECTS OF VITAMIN E AND ITS ANALOGUES ON THE OXIDATION OF LOW DENSITY LIPOPROTEIN MEDIATED BY FREE RADICALS 2.8

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It is now accepted that the oxidatively modified low density lipoprotein (LDL) plays a significant role in the progression of atherogenesis. α-Tocopherol, the major antioxidant in LDL, does not prevent the formation of phosphatidylcholine hydroperoxide (PC-OOH) and cholesterol ester hydroperoxide (CE-OOH) as efficiently as it does in the homogeneous solution. In this study, we oxidized LDL treated with several kinds of α-tocopherol analogues, having different length of isoprenoid side chain in order to elucidate the weak antioxidant activity of α-tocopherol in LDL. The oxidations were induced by either AAPH or Cu(II) in pH7.4 phosphate buffer. When the LDL treated with 2,2,5,7,8-pentamethyl-6-chromanol(PMC) was oxidized by AAPH or Cu(II), the formation of PC-OOH and CE-OOH was not observed while PMC existed in LDL. On the other hand, LDL treated with other kinds of analogues gave the hydroperoxides even when they were present in LDL and the rates of formation of hydroperoxides increased with increasing length of side chain. These results indicate that the antioxidant activity of chroman ring is high even in LDL but that the side chain affects its antioxidant activity. We conclude that the weak antioxidant activity of endogenous α-tocopherol in LDL is ascribed to a reduced mobility due to its long phytyl side chain.

2.9 FATE OF LIPID HYDROPEROXIDES IN HUMAN PLASMA

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We could detect about 3 nM cholesteryl ester hydroperoxide (CE-OOH) in healthy human plasma [BBRC (1989) **165**, 988] as a direct evidence of lipid peroxidation *in vivo*. However, phosphatidylcholine hydroperoxide (PC-OOH) was not found in methanol extracts of human plasma [Free Rad Biol Med (1987) **3**, 359], and neither in methanol/chloroform extracts. This difference is likely due to the instability of PC-OOH in plasma [Frei et al., PNAS (1987) **85**, 988]. Lecithin:cholesterol acyltransferase (LCAT) in HDL or phospholipase A2 (PLA2) activity in LDL has been suggested to convert PC-OOH to CE-OOH and lysoPC, and free fatty acid hydroperoxide (FFA-OOH) and lysoPC, respectively. In order to examine the above hypothesis, we have studied the fate of PC-OOH, CE-OOH, and FFA-OOH in plasma. Soybean PC-OOH and hydroperoxides of cholesteryl linoleate and linoleic acid were used as substrates. Hydroperoxides were added as small volume of alcohol solution to human heparinized plasma and analyzed by an HPLC/CL assay. Alcohols (PC-OH and CE-OH) were estimated by their absorption at 234 nm by subtracting PC-OOH and CE-OOH, respectively. FFA-OH was determined by the precolumn fluorescence derivatization method. First of all, CE-OOH was stable in plasma but FFA-OOH was rapidly reduced to FFA-OH. PC-OOH also decayed rapidly in plasma and was converted to PC-OH as the major product. At the same time, small amount of CE-OOH and CE-OH were also produced, but little FFA-OH. No significant decrease of antioxidants in plasma was observed by the addition of hydroperoxides, indicating that hydroperoxides were reduced enzymatically. Furthermore, PC-OH was slowly converted to CE-OH in plasma. These results indicate that the peroxidase is likely to be the major defense against PC-OOH in the plasma. PLA2 activity is not important in decomposing PC-OOH in plasma. However, LCAT activity is not negligible if PC-OOH is formed in HDL.

2.10 LIPID PEROXIDATION OF LOW DENSITY LIPOPROTEINS AND INHIBITION BY MICROCOMPONENTS OF DIET: QUENCHING OF PARINARIC ACID FLUORESCENCE

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Several lines of evidence suggest that lipid peroxidation of low density lipoproteins (LDL) occurs *in vivo* and that this event plays a central role in the mechanism of atherosclerotic damage to blood vessels. Therefore, nevertheless the presence of antioxidant compounds in LDL, these lipoproteins are an important target for free radicals *in vivo*, possibly because of their high content in polyunsaturated fatty acids. Since plasma composition is diet dependent, a rapid and simple methodology to assess chain-breaking activities of some compounds is of obvious interest regarding LDL protection against peroxidation. The fluorescent free fatty acid parinaric acid (PnA) recently introduced as a membrane peroxidation probe, has been used in our laboratory in LDL peroxidation studies to get precise and reliable quantitative indices of antioxidant activity. In this assay, LDL with PnA incorporated are subject to a constant flux of peroxyl radicals by thermal decomposition of 2,2'-azobis(2-amidinopropane hydrochloride) (AAPH) in the aqueous phase. The peroxidation reaction is followed by PnA fluorescence decrease. The antioxidant activity (chain-breaking) of added compounds is evaluated as the inhibition period corresponding to the supression of fluorescence decay. The antioxidant is consumed at a constant rate and, when depleted, the inhibition period ends and the fluorescence decay rate is resumed. Previously we have shown that for common plasma antioxidant compounds, namely ascorbate, urate and cysteine, a close relationship between inhibition periods of PnA acid fluorescence decay and O_2 consumption is observed (1). In this study, the chain-breaking efficiencies of some microcomponents of diet, namely ellagic, caffeic and chlorogenic acids were measured and compared with the efficiency of ascorbate by the PnA assay. Data also correlate well with inhibition periods of LDL oxidation as measured by O_2 consumption. Even at nM concentrations, the compounds exhibit a strong chain-breaking antioxidant activity according to the sequence in terms of inhibition period: ellagic > chlorogenic > caffeic.

(1) João A.N. Laranjinha, Leonor M. Almeida and Vítor M.C. Madeira. *Arch. Biochem. Biophys.* (submitted)

2.11 DETERMINANTS OF LIPID PEROXIDATION [LPO] IN CYSTIC FIBROSIS [CF]

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Polyunsaturated fatty acids [PUFAs] of LDL are protected against LPO by vitamin E and carotenoids. In vitro exposure of LDL to a prooxidant leads to consumption of these antioxidants [AO] (lag-phase [lag]), resulting in formation of conjugated dienes [CD]. In 5 patients CD generation in LDL was monitored up to complete LDL oxidation and lag was measured. α-, γ-tocopherol [T], ß-carotene [C] and other carotenoids were determined in plasma and LDL. 3 patients showed a short lag (30,57,60 min) compared to others (135,152 min) and to healthy controls, indicating a suboptimal supply of AO. They also had decreased plasma α-T (median) (11.0 µmol/L) and LDL α-T (1.4 nmol/mg). ß-C was very low in all patients (0.08 µmol/L), as were other carotenoids. The length of the lag was more closely (r^2=0.84) related to LDL α-T than in controls (r^2=0.51), indicating the lack of other AO. CD absorbance in fully oxidized LDL was considerably lower in patients (0.4-0.6) compared to controls (0.8-1.0), suggesting a limited amount of oxidizable PUFAs in LDL. In conclusion, LDL-LPO in CF is almost exclusively prevented by α-T. Low oxidizable PUFAs in LDL due to essential FA deficiency lead to limited CD formation even in severe AO deficiency.

2.12 REACTION OF ACTIVATED HAEMPROTEINS WITH LIPOPROTEINS

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Reaction of the iron(III) form of certain haemproteins with H_2O_2 is known to generate a ferryl [iron(IV)-oxo] species, which is one oxidising equivalent above the initial level, and a protein (globin) radical. Previous studies have shown that these species can induce oxidative damage to biological membranes, though the exact mechanisms by which this occurs has not been fully elucidated.

The oxidation of LDL molecules by systems containing Metmyoglobin or methaemoglobin and H_2O_2 have been investigated by use of both direct ESR spectroscopy and ESR spin trapping and a number of radical species which might be involved in the initiation of LDL oxidation and the subsequent propogation of damage detected. The structure and identity of these radical species will be discussed together with their possible roles in the generation of damage. Correlation of the observed radicals and their concentrations with other markers of LDL damage suggest that these species play a crucial role in LDL oxidation.

2.13 FORMATION OF PROTEIN PEROXIDES BY FREE RADICAL GENERATING SYSTEMS

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The demonstration that some proteins exposed to gamma radiation in presence of oxygen acquire peroxide moieties (1) prompted us to examine other radical-generating systems for their potential effectiveness. We found that BSA, lysozyme and a range of susceptible amino acids exposed to a variety of oxidizing conditions gave positive tri-iodide peroxide tests. The effective systems included those which generate hydroxyl or similarly reactive species directly, as in the Fenton reaction, or indirectly, from less potent reactants such as superoxide. Of the latter, we found that the systems xanthine/xanthine oxidase/iron, NADH/NADH oxidase/iron, ascorbate/iron and neutrophils activated with phorbol myristoyl acetate induced formation of peroxide groups on BSA with varying efficiencies. A more surprising finding was that AAPH (2,2'-Azobis(2-Amidinopropane)Hydrochloride), which generates peroxyl radicals, was also able to induce protein peroxidation. In cases where the superoxide radical was the initial reactant, SOD was not able to offer complete protection from peroxidation, but catalase was an effective inhibitor of the process.

(1) Simpson J., Narita S., Gieseg S., Gebicki S., Gebicki J.M. and Dean R.T., Biochem. J., in press.

2.14 FORMATION OF PROTEIN PEROXIDES ON LOW DENSITY LIPOPROTEIN EXPOSED TO FREE RADICALS OR COPPER

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Oxidation of LDL results in modifications of its lipid and protein components, some of which may be responsible for its atherogenic properties. A possible protein modification, not studied so far, is acquisition of peroxide groups. To test this, we oxidized LDL by free radicals generated with gamma rays or by exposure to Cu(II). The LDL protein (apo B) was isolated by the removal of lipids, using four techniques: extraction with methanol/chloroform or dissolution in Nonindet P-40 (J. Lipid Res. 1979,20:631), and extraction with ether/ethanol with or without Gu.HCl (Biochem. 1982,21:4503; PNAS 1977,74:5150). The protein recovered in the Nonindet method was tested for presence of hydroperoxide groups with the tri-iodide test (Atherosclerosis 1990,81:175). Both free radicals and Cu(II) induced apo B peroxidation which was linear with time of irradiation and incubation with Cu(II). The kinetics of the process suggest that endogenous antioxidants are unable to protect the apo B from this form of modification.

2.15 TOTAL PEROXYL RADICAL-TRAPPING CABAPILTIY OF HUMAN LDL.

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In order to study antioxidant contents of human low-density lipoprotein a new luminescent method was developed. The precipitated LDL fraction was extracted with choloform:methanol and devided for determinations of organic phosphorus and for a chemiluminescent assay of chain-breaking antioxidants. The sample was exposed to peroxyl radicals produced by the thermal decomposition of 2,2´-azobis(2,4-dimethylvaleronitrile). The rate of peroxyl radical production in the thermal decomposition of AMVN was followed by the luminol enhanced chemiluminescence. The addition of an antioxidant to the reaction mixture extinguishes the chemiluminescence and its duration has a linear correlation to the radical trapping cabapility of the sample. D-α-tocopherol was used as a standard. The duration of the extinction caused by 1 nmol of D-α-tocopherol is about 1100 sec. Normal human LDL has total radical-trapping cabapility of 19±4 (SD) mmol per mole of organic phosphorus. If human LDL contains 700 molecules of phospholipids per LDL particle this antioxidant capacity can be achieved with the tocopherol composition of 7 molecules per LDL particle.

2.16 CAROTENOID EFFECTS ON OXIDANT INDUCED HUMAN LOW-DENSITY LIPOPROTEINS SURFACE CHARGE SUBPOPULATIONS DETECTED BY LASER DOPPLER ELECTROPHORESIS

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Oxidative modifications of human low density lipoproteins (LDL) is believed to be the first of many steps in atherosclerotic plaque formation. Oxidants deplete LDL antioxidants, cause lipid and protein oxidation and increase Rf on agarose gel electrophoresis (AGE). Characterization of oxidants-induced LDL surface charge subpopulations and antioxidant prevention require sensitive methods. In this study, Laser Doppler Electrophoresis (LDE) was used to detect modifications in LDL surface charges occurring before and after oxidant stress. In the presence of O_2 and the water-soluble azo initiator of peroxyl radicals, 2,2'-azobis (2-amidinopropane)HCl (AAPH), or cobalt γ-radiolysis ($^{\cdot}OH$, $O_2^{\cdot}$) increased negative surface charges of LDL. Mean mobilities measured varied from -1,7 to -5μms^{-1}Vcm^{-1} according to treatment. AAPH effects were partially prevented by pretreatment with betatene (Henkel) and ß-carotene (Roche), the latter observed by Jialal et al., BBA, 1086: 134-138,1991 by AGE. In an LDL sample from a vitamin E deficient patient, ß-carotene was extremely effective in preventing formation of oxidant induced surface charge subpopulations. Thus, LDE revealed unambiguously that oxidative modifications increased negative surface charge density and verification by light-scattering showed that LDL size was not affected. Beyond the results, an important technical point is the comparison between LDE and AGE. LDE reveals immediate parameters of physico-chemical properties and surface charge subpopulations (at least 4 may be distinguished). Moreover, LDE allows for recovery of LDL subpopulations.

2.17 REVERSAL OF CIRCULATING HIGH PLASMA OXIDIZED LDL (LDL⁻) WITH ANTIOXIDANTS

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Abdominally obese cynomolgus monkeys on ad lib atherogenic diet (AD) for 12 months were compared to lean control diet (CD) monkeys. Magnetic resonance imaging revealed visceral fat accumulation in AD primates associated with elevations of insulin, insulin resistance, glucose, blood pressure, heart rate, plasma triglycerides, LDL, AND LDL⁻. LDL⁻ correlated with body weight and was shown to correlate stronger with atherosclerosis than unmodified LDL. Four AD monkeys were treated with oral antioxidants for 8 weeks: 2 with the powerful vitamin E analog (VEA) U-81556; 2 with the lazeroid (L) U-74,389T. This resulted in the following mean lipoprotein changes (mg/dl). In VEA: LDL⁻ 11.1-1.5, LDL 459-62, HDL 97-377; in L: LDL⁻ 11.0-0.8, LDL 385-45, HDL 83-321. Conclusion: Exogenous antioxidants reversed atherogenic LDL⁻ to normal and improved remarkedly two other risk factors: LDL and HDL. Thus, antioxidant therapy may be a powerful tool in prevention and reversal of cardiovascular disease in a high risk group.

2.18 HUMAN SERA CONTAINING AUTOANTIBODIES BINDING TO OXIDIZED LOW DENSITY LIPOPROTEIN PARTIALLY ALSO BIND TO NATIVE LDL

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Screening of 500 atherosclerosis patients and control individuals by a self developed ELISA method gave positive results for IgG molecules binding to oxidized low density lipoprotein (oLDL) in 30% of all samples. oLDL autoantibody positive sera were then investigated for their binding properties to native LDL (nLDL). Most of these sera gave binding signals to nLDL in range of 10 to 30% of the signals obtained in oLDL ELISA. Storage and stressing of these sera resulted in reduction of IgG binding to oLDL, but not nLDL. Similar results were obtained in case of polyclonal animal antisera raised against oLDL in rabbits. The highest signals in nLDL ELISA were always lower than in oLDL ELISA, which indicates a smaller number of IgG antibodies bound per molecule nLDL compared to oxidized LDL. From these data we suggest that oLDL is probably the trigger for development of autoantibodies. The fact that we also observe binding to nLDL strongly suggests that autoantibodies are also generated against unmodified parts of oLDL. The recent finding that positive oLDL autoantibody signals are strongly associated with sonographically measured increase of blood vessel intimal thickness further supports the data presented.

2.19 OXIDATION OF LDL BY BOVINE MICROVASCULAR CELLS.

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Oxidized low density lipoprotein (oxLDL) is thought to promote the formation of fatty streaks during atherogenesis in large and medium arteries. The in vivo oxidation of LDL is believed to occur in fatty streaks, since all cell types present in these lesions can oxidize LDL in vitro. However, it is possible that LDL oxidation occurs in the microvasculature. We report that cloned bovine adrenal microvascular endothelial cells (BMEC, Furie et al., JCB 98:1033, 1984) can oxidize LDL in HAM's F10 medium. OxLDL was more mobile than unoxidized LDL on agarose gel electrophoresis. After 4 hr incubation of LDL with BMEC, increased TBARS were seen; TBARS rose through 24 hr. of incubation. The BMEC-induced oxidation was inhibited by alpha-tocopherol (20 μM) and probucol (10 μM). These results open the possibility that some oxidation of LDL may occur in the microcirculation when lipoproteins interact with endothelial cells under slow flow conditions existing in these vascular beds.

Session 3

Free Radicals in Arachidonic Acid Cascade

3.1 NEW BIOLOGICAL PROPERTIES OF THE HEPOXILINS DERIVED THROUGH THE TRANSFORMATION OF 12-HPETE

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We have previously shown that arachidonic acid (AA) is transformed into 12-HPETE via 12-lipoxygenase(12-LOX) and that this product is further transformed into hydroxy-epoxide metabolites which we termed 'hepoxilins' (HxA_3). 12-LOX is present in a variety of tissues, while the transformation of 12-HPETE into the hepoxilins is catalysed by ferric iron, making a variety of ferric-containing substances candidates to carry out this transformation in vivo. Hepoxilins are indeed found in the circulation. The hepoxilins are metabolised via two independent pathways, one involving their inactivation via an epoxide hydrolase into trihydroxy metabolites termed 'trioxilins' ($TrXA_3$), the second involving inactivation, retention or further activation of the biological activity through conjugation with glutathione to form hepoxilins A_3-C via glutathione S-transferase. We previously showed that the hepoxilins are capable of inducing the release of insulin from pancreatic islets, the transport and mobilisation of calcium in human neutrophils, and the potentiation of vasoconstriction in isolated aortic strips. Novel actions of the hepoxilins on vascular permeability and regulation of cell volume will be presented.

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3.2 VITAMIN C AND E OXIDATION BY CYCLOOXYGENASE AND LIPOXYGENASE REACTIONS

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Incubations of human blood platelets ex vivo with arachidonic acid or thrombin resulted in an oxidation of endogenous ascorbic acid (vitamin C) and α-tocopherol (vitamin E), whereas glutathione was not considerably reduced. Experiments with inhibitors of the oxygen activating enzymes cyclooxygenase/prostaglandin H synthase and the 12-lipoxygenase (acetylsalicylic acid, esculetin, 5,8,11,14-eicosatetraenoic acid) and their corresponding products (prostaglandin G_2, 12-HPETE) showed that the major part of the vitamins is oxidized by prostaglandin H synthase. 12-lipoxygenase contributes less to the consumption of the vitamins. We consider the observed effects as co-oxidations induced by the arachidonic acid dioxygenase reactions. Since the two vitamins are not inhibitors of cyclooxygenase or 12-lipoxygenase their oxidation should occur apart from the catalytic cycle. Preliminary experiments support the hypothesis of an auxiliary peroxide cycle for the activation of the enzymes as potential source of the oxidation equivalents.

3.3 EICOSANOID MODULATION OF SIGNAL TRANSDUCTION: IMPORTATIONS FOR CANCER METASTASIS

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Metastasis the process of tumor cell dissemination involves multiple cell-cell interactions, e.g. tumor cells (TC)-platelets (PLT)-endothelial cells (EC). Eicosanoids produced during these cell-cell interactions have a profound effect on the development of a successful metastatic lesion. The effect of these various eicosanoids on cell-cell interactions are mediated by modulation of cell signaling, via enzymes involved in signal transduction [i.e. protein kinase C (PKC) and protein kinase A (PKA)]. A lipoxygenase (LOX) metabolite of arachidonic acid [i.e. 12(S)-HETE] positively effects the metastatic process whereas a LOX metabolite of linoleic acid [i.e. 13(S)-HODE] has a negative impact on metastasis. 12(S)-HETE enhances the surface expression of the integrin receptor αIIbß3 on tumor cells and $\alpha v\beta_3$ on ECs, induces EC retraction and enhances tumor cell spreading on subendothelial matrix. These effects are mediated by activation of PKC. 12(S)-HETE increases membrane-bound PKC activity. Quantitative Western blotting using PKC iso form specific antibodies reveals that 12(S)-HETE induces protein translocation. This PKC activation in C results in phosphorylation of cytoskeletal proteins (i.e. vimentin) and cytoskeletal associated proteins (myosin light chain, myosin heavy chain and vinculin). Similar results were observed in EC with the exception of an additional phosphorylation of actin. All of the above effects are antagonized by 13(S)-HODE which blocks 12(S)-HETE induced PKC translocation to membrane. In addition prostacyclin by activation by PKA also inhibits the effects of 12(S)-HETE and demonstrates synergy with 13(S)-HODE for inhibition of these effects. Therefore, various oxygenization products of arachidonic and linoleic acid can bidirectionally modulate tumor cell metastasis through activation of A and C kinases.

3.4 HYDROGEN PEROXIDE-MEDIATED LIBERATION OF ARACHIDONIC ACID IN ENDOTHELIUM: LACK OF EVIDENCE FOR FREE RADICAL MECHANISMS

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Administration of hydrogen peroxide to the isolated perfused lung system *via* the systemic circulation results in marked enhancement in both the circulatory and airway resistance reflecting a general increase in the pulmonary smooth muscle tonus. Previous experiments have indicated that this effect is mediated through the actions of arachidonic acid metabolites, particularly thromboxane A_2. The present study assesses the liberation of arachidonic acid in pulmonary endothelial cells in response to hydrogen peroxide. In particular, the possibility that intracellular arachidonate is released by free radical-mediated events is addressed. Significant release of arachidonic acid can be seen from [^{3}H]-arachidonate-loaded bovine pulmonary artery endothelium 5 minutes after administration of 250μM hydrogen peroxide and reaches a maximum between 10 and 15 minutes. Pretreatment of the cells with mannitol or the iron chelator desferal had no effect on the extent of arachidonate liberation. Similarly, pretreatment of the cells with the antioxidant DPPD had no effect. The possibility that hydrogen peroxide could act *via* the stimulation of intracellular thiol oxidation was also investigated using the thiol oxidant diamide. At diamide concentrations ranging from 1-500μM, no enhancement in arachidonic acid release was found. Thus while it is clear that in bovine pulmonary endothelium hydrogen peroxide can stimulate significant arachidonic acid liberation, these findings do not support the possibility for the contribution of free radical-mediated processes in this event.

3.5 MECHANISM OF H_2O_2 CYTOTOXICITY AND INFLAMMATION

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Erythema and inflammation are first symptoms occurring after intracutaneous injection of H_2O_2 or radiotherapy suggesting that the same cytotoxic pathway(s) is triggered by both or H_2O_2 contribution to damage caused by ionizing radiation is significant. H_2O_2 is known to be involved in inflammatory processes. All forms of inflammatory reaction are also associated with increased prostanoid synthesis. The appearance of both H_2O_2 and prostanoids in inflammation is suggestive of a causative link between them. A mechanism of H_2O_2 cytotoxicity might, therefore, help to elucidate such an association. Cyclooxygenase is crucial in prostanoids formation. Exogenous hydroperoxides including H_2O_2 activate the enzyme. Cyclooxygenase, therefore, by using H_2O_2, not only increases synthesis of prostanoids but should protect cells against H_2O_2 cytotoxicity. Indeed, cyclooxygenase alone was crucial in the protection of cells exposed to H_2O_2 at the concentrations not exceeding $3.5x10^{-10}$M per cell. An interaction of cyclooxygenase with glutathione peroxidase when both enzymes were fully activated resulted in a further protection against even higher concentrations of H_2O_2 (up to $9x10^{-10}$ M per cell); surviving fraction of almost 100% was obtained as compared with 20% in the presence of a very limited activity of both cyclooxygenase and glutathione peroxidase. The findings help to explain why both H_2O_2 itself and ionizing radiation induce erythema and inflammation.

3.6 RECOMBINANT INTERLEUKIN-1β ENHANCES THE ACTIVE OXYGEN FORMS PRODUCTION IN NEUTROPHILS

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The influence of recombinant interleukin-1β (r IL-1β) on active oxygen forms generation by human blood neutrophils and aggregation of these cells were studied.

After the addition of r IL-1β to neutrophil suspensions the enhancment of luminol-dependent chemiluminescence (LDCL) at adhesion to glass and the action of Con A was observed. The sharp increase of the rate of the spontaneous and Con A-induced cell aggregation was also established. Specific scavengers (1,4-diazo-2,2,2-bicycleoctan and catalase) inhibited essentially and superoxidedismutase (SOD) abolished totally the r IL-1β effects on neutrophils. It was found that interaction of r IL-1β with neutrophils led to the activation superoxide-generated systems localized on cell surfase and plaied a key role in the regulation of intracellular myeloperoxidase system. It was shown that increase of LDCL yield in neutrophils under the r IL-1β influenceis receptor-mediated process.

The effects observed were independent of the presence of glucose in cell incubation medium and supressed essentially by ingibitors of arachidonate metabolism. It was suggested that the generation of active oxygen forms induced by r IL-1β connected mainly with the lipoxygenase pathway of arachidonate metabolism.

3.7 A PHYSIOLOGIC ROLE FOR THE HYDROXYL RADICAL? TRIGGERING OF "PRIMED" PLATELET ACTIVATION BY A FENTON-LIKE REACTION.

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We have previously shown that human platelets "primed" with arachidonic acid or collagen can be activated by nanomolar concentrations of H_2O_2, either generated from platelet derived O_2- by SOD or added directly. We now report that H_2O_2 activation of "primed" platelets is actually mediated by OH° formed in a Fenton-like reaction. H_2O_2 effects on platelets are, in fact, prevented by OH° scavengers mannitol, deoxyribose and salicylate, and by Desferal. Also OH° generated by the redox cycling of iron activated human platelets. ESR spin trapping experiments confirmed the formation of OH° during SOD-, H_2O_2- and iron (II) driven platelet activation. The DMPO-OH° spin adduct was detected only in conditions that led to platelet aggregation and was prevented by catalase, mannitol and deoxyribose. OH° induced arachidonic acid mobilization from platelet membranes blocked by mepacrine, suggesting a radical reaction mechanism linked to phospholipase A_2. These results indicate a possible messanger-like function in transmembrane signalling for the hydroxyl radical.

Session 4

Free Radicals in Drug Activation and Metabolism

4.1 POSSIBLE ROLE OF FREE RADICAL FORMATION IN CLOZAPINE(CLOZARIL)-INDUCED AGRANULOCYTOSIS.

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The use of clozapine, a unique antipsychotic drug, has been restricted due to a 1-2% incidence of drug-induced agranulocytosis. Metabolic activation of clozapine to a free radical in neutrophils or stem cells could be the molecular mechanism underlying this side effect. Evidence for the intermediate formation of a clozapine radical during the peroxidase-mediated metabolism of clozapine stems from the observation of thiyl and ascorbyl radicals in the presence of glutathione and ascorbate, respectively. The ascorbyl radical was detected by direct ESR spectroscopy in a myeloperoxidase system. Its steady-state concentration was significantly increased in the presence of clozapine. Glutathionyl radical formation was also demonstrated by radical trapping with DMPO. Again, the radical adduct concentration was significantly increased in the presence of clozapine. It can be concluded from these data that radical scavengers such as ascorbic acid, when coadministered with clozapine to patients, may reduce the clozapine-derived free radical to regenerate the parent drug and

4.2 (BI) SULFITE METABOLISM IN HUMAN GRANULOCYTES AND MACROPHAGES

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Sulfur oxides are important atmospheric pollutants; their presence in the air is mainly related to car exhaust and household heating. Inhalation of sulfur oxides causes hyperreactivity and inflammation of the respiratory airways, along with the inhibition of macrophage and granulocyte phagocytosis.

In aqueous solution at pH 7, sulfur oxides exist primarily as sulfite (SO_3^{2-}) and bisulfite (HSO_3^-), to a minor extent as hydrated SO_2. A free radical intermediate, sulfur trioxide radical anion ($SO_3^{\cdot-}$); is produced during (bi)sulfite autoxidation and is involved in a number of reactions of biological significance, including oxidation of methionine, addition to double bonds of alkenes and peroxidation of fatty acids.

In this work, sulfite metabolism has been studied in human granulocytes isolated from fresh blood. Sulfite disappearance rate and sulphate accumulation were measured using HPLC and spectrophotometry. Electron spin resonance spectroscopy coupled to the spin trapping technique demonstrated the formation of the radical anion $^{\cdot}SO_3^-$. Oxygen consumption was recorded in the presence of sulfite and TPA stimulation. GSH cosumption was determined also in the same model system.

Similar experiments have been carried out on human lung macrophages obtained through lavage.

4.3 OXIDATION OF HYDROQUINONE BY MYELOPEROXIDASE

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Myeloperoxidase is a prime candidate for mediating the inflammatory tissue damage of neutrophils because it converts Cl^- to the potent oxidant hypochlorous acid. It also oxidizes xenobiotics to reactive free radicals. Peroxidation of hydroquinone displayed a distinct lag phase, which was practically abolished by excluding O_2 and was eliminated by adding benzoquinone at the start of the reaction. Superoxide dismutase increased the rate of peroxidation by 40% but did not eliminate the lag phase. Spectral investigations revealed that during the initial phase of the reaction, MPO was converted to oxy-MPO, or compound III, by a mechanism that was not reliant on superoxide. Benzosemiquinone, however, was able to convert ferric-MPO to compound III. Both compound III and ferro-MPO reacted with benzoquinone to regenerate ferric-MPO. We propose that the lag phase occurs because benzosemiquinone reduces ferric-MPO to ferro-MPO, which rapidly binds O_2 to form compound III. Since compound III is outside the peroxidation cycle, conversion of hydroquinone to benzoquinone is retarded. However, as benzoquinone accumulates, it oxidizes ferro-MPO and compound III to ferric-MPO, thereby increasing the rate of peroxidation. There is a minimal lag phase under an atmosphere of N_2 because ferro-MPO would be rapidly oxidized by benzoquinone, without formation of compound III. We conclude that when substrates produce radicals capable of reducing ferric-MPO, they will be peroxidized efficiently only if oxy-MPO is readily recycled. Furthermore, these radicals will prevent MP^{3+} from reacting with H_2O_2, and thereby prevent the enzyme from oxidizing Cl^- to hypochlorous acid. Thus, this mechansim could be exploited to prevent hypochlorous acid-mediated inflammatory tissue damage.

4.4 METABOLIC ACTIVATION OF THE POTENT CARCINOGENS BENZO-[A]PYRENE (BP) AND 7,12-DIMETHYLBENZ[A]ANTHRACENE (DMBA) BY ONE-ELECTRON OXIDATION

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The DNA adducts of BP and DMBA formed after activation by 3-methylcholanthrene-induced rat liver microsomes in vitro were analysed. Adducts were formed from either radical cation or diol epoxide (DE) intermediates, and the adducts were stable in DNA or lost from DNA by depurination. Stable adducts were quantitated and partially identified by the ^{32}P-postlabeling method, whereas depurination adducts were separated by HPLC, identified by fluorescence line narrowing spectroscopy and quantitated by a radiation flow monitor. For BP, 81% were depurination adducts: BP bound at C-6 to the N-7 of Ade (BP-6-N7Ade, 58%), BP-6-N7Gua (10%), BP-6-C8Gua (12%) and BPDE-10-N7Ade (0.5%). Stable adducts (19%) included BPDE-10-N^{2}dG (15%). For DMBA, 99% were depurination adducts: 7-MBA-12-CH_2-N7Ade (82%) and 7-MBA-12-CH_2-N7Gua (17%). The stable adducts (1.2%) included 0.2% that may arise from the DMBA DE. The 12-CH_3 group of DMBA is critical in metabolic activation of DMBA, a finding consistent with the results of carcinogenicity studies in rodents. These results show that with activation by cytochrome P-450, the BP-DNA and DMBA-DNA adducts are predominantly formed by one-electron oxidation and are lost from DNA by depurination. Similar profiles of adducts are observed in the target tissue mouse skin treated with BP or DMBA, suggesting that one-electron oxidation plays the central role in the carcinogenicity of these compounds. (NIH grants RO1-CA25176, RO1-CA44686 and PO1-CA49210)

4.5 Redox studies on diaziridinyl benzoquinones; DNA site specific damage as a consequence of reductive activation.

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Several diaziridinyl benzoquinones have undergone clinical trials as antitumour drugs. These compounds can be activated after reduction by several enzymes including cytochrome P450 reductase and DT-diaphorase. We have previously shown (Dzielendziak *et al*, 1990, Cancer Res., 50, 2003) that the cytotoxicity of a series of novel diaziridinyl benzoquinones can be related to the ease of one-electron reduction, the intensity of the ESR signals produced when the drugs are incubated with cells, and the ability of the reduced forms of the drugs to cross-link DNA.
Recent results have shown that certain novel diaziridinyl benzoquinones can react selectively at certain specific sequences in linearised pBR322 DNA segments. Essentially, all of the compounds react at the N7-positions of guanine and the alkylkations are random in the absence of reduction. However, some diaziridinyl benzoquinones display a preference for TGC sites, but only when reduced to the hydroquinone/semiquinone forms. Computer modelling of the drug/DNA interactions for different diaziridinyl benzoquinones has been used to explain the selectivity.

4.6 ACTIVITY OF ANTIOXIDANT ENZYMES OF INTERSCAPULAR BROWN ADIPOSE TISSUE (IBAT) IN CADMIUM-TREATED RATS

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A little is known about antioxidant status of brown adipose tissue, a unique termogenic system. Therefore, the aim of this work was the elucidation of this system in nontreated (control) and rats expossed to dietary Cd.
Male Wistar albino rats, 2 months old, were given Cd-supplemented drinking water for 1 month. Average Cd intake was about 15 mg/d/kg b.m. After 30 days of treatment animals were sacrified, IBAT was isolated, its mass measured and activity of following antioxidant enzymes of IBAT were determined: SOD (total, CuZnSOD and MnSOD), catalase, GSH-POX and GST.
The average mass of IBAT amounted in contol group of rats to 317±42 mg was significatly reduced in Cd-treated rats (228±15 mg, $p<0.025$) Somatic index of IBAT was not, whereas its protein content was significantly reduced (3.92 vs 2.68 mg) in Cd-treated animals. In comparison to the control all antioxidant enzymes determined were increased: total SOD for 47% ($p<0.005$), CuZnSOD for 78% ($p<0.005$), catalase for 26% ($p<0.005$), GSH-PX for 45% ($p<0.005$) and GST for 15% ($p<0.005$), respectively. Dietary selenium supplementation (7 μg Se/d/kg b.m.) during 30 days altered nonsignificantly activites of all mentioned enzymes in IBAT of either Cd-treated or control rats.

4.7 NAD(P)H: QUINONE-ACCEPTOR OXIDOREDUCTASE (QAO, DT-DIAPHORASE)ENHANCES THE ALKYLATING ACTIVITY OF THE ANTITUMOR AGENT DIAZIQUONE (AZQ)

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There is evidence to suggest that the mechanism of AZQ cytotoxicity is due to: a) redox cycling leading to reactive oxygen species, and b) aziridine alkylation enhanced by reduction of the quinone. We reduced AZQ by $1e^-$ and $2e^-$ and assessed alkylating activity by the nitrobenzylpyridine (NBP) assay. Free radical production was assessed by ESR. Two electron reduction was accomplished chemically with borohydride and enzymatically with the diaphorase-rich S9 fraction from MCF-7 cells, a human breast cancer cell line. One electron reduction was carried out with NADPH cytochrome C reductase and the mild $1e^-$ reducing agent NADPH. Results showed that upon reducing AZQ with $2e^-$, the alkylating activity increased 3 fold over parent compound. This activity was totally abrogated by dicumarol. In contrast, $1e^-$ reduction did not increase the alkylating activity over the oxidized drug, and dicumarol had no effect. ESR measurements indicated that free radicals are produced in both, the $1e^-$ and $2e^-$ reduction. The latter reduction results in the hydroquinone which can be oxidized $1e^-$ at a time with the production of reactive oxygen species. Dicumarol was shown to protect MCF-7 cells from the cytotoxic effect of AZQ when incubated simultaneously for 1 hr. These results show that in MCF-7 cells, part of the cytotoxicity of AZQ is due to the $2e^-$ reduction by QAO because this toxicity can be modulated by dicumarol. These data begin to explain the variability of toxic effects of AZQ in different cell lines. For example, in lines with high QAO content, the damage to DNA was almost exclusively interstrand cross-linking with no detectable strand breaks (D. Ross, et al.). QAO is a particularly important enzyme in the bioactivation of AZQ.

4.8 "IN VIVO" ELECTRON PARAMAGNETIC RESONANCE IMAGING OF A NITROXIDE FREE RADICAL IN WHOLE RAT.

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Low frequency (280 MHz) electron paramagnetic resonance spectroscopy has been used to follow uptake, spatial distribution and reduction of a pyrrolidine nitroxyl spin label, PCA, in the rat. No difference of half life was found in seven normotensive rats submitted to three administrations of 0.44 mmol/Kg PCA (11.3±0.4; 11.0±0.6 and 11.5±0.7 min). Transversal two-dimensional images of PCA distribution in the living rat body were obtained over 6 min by means of field gradients and imaging reconstruction techniques. The observed regions are attributable to the superimposition of all the sites where the PCA signal was present. Three not completely separate regions of PCA were observed by projections along the longitudinal axis of the rat. PCA accumulation was found in the lower abdomen 12 min after the start of the PCA injection.
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4.9 MECHANISM OF $CBrCl_3$ TOXICITY: INFLUENCE ON INTRACELLULAR THIOL GROUPS, CYTOSOLIC FREE Ca^{++} AND CELLULAR MEMBRANE INTEGRITY IN ISOLATED RAT HEPATOCYTES.

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Radical species, as induced by $CBrCl_3$ metabolism, are able to attack cellular membranes and thiol groups and probably to increase cytosolic free Ca^{++}-concentration. An interrelationship between these events is still obscure.
In isolated hepatocytes, the influence of 0.5, 1.0, 1.5 mmol/l $CBrCl_3$ on MDA-production, thiol homeostasis (GSH, protein thiols), damage of lysosomal and plasma membranes (release of glucoronidase and LDH, trypan blue uptake and plasma membrane blebbing) and cytosolic free Ca^{++}-concentrations were comparatively investigated.
After an initial 50% decrease of the GSH-concentration, the cells retained an amount of about 4 mmol/l cellular water at each $CBrCl_3$-concentration used. Total protein thiols showed no apparent changes. The parameters reflecting membrane damage reacted dose-dependently and their time course correlated well with glutathione depletion. In contrast, a distinct rise in cytosolic free Ca^{++}-concentration was not observed until 45 minutes after $CBrCl_3$ addition.
The observed GSH depletion does not explain the dose-dependent membrane damage and therefore membrane damage is a coincidence rather than the result of GSH consumption. On the other hand membrane damage might result in loss of GSH, followed by an increase of cytosolic Ca^{++}. Thus, the late rise of cytosolic free Ca^{++} seems to be a subsequent and not a causative event in $CBrCl_3$ mediated toxicity.

This study was supported by the Deutsche Forschungsgemeinschaft.

4.10 ON THE ROLE OF CYTOCHROME P-4502E1 PHOSPHORYLATION FOR LIPID PEROXIDATION AND MODULATION OF ENZYMATIC ACTIVITIES

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Posttranslational modification of some hepatic cytochromes P-450 due to the cAMP-dependent phosphorylation has been shown to regulate their level and relative enzymatic activities. The goal of current study was to follow the phosphorylation-induced changes in enzymatic activities, including lipid peroxidation (LP), dependent on the ethanol-inducible cytochrome P-450 (CYP2E1). CYP2E1 was induced by treatment of rats with intragastric acetone combined with starvation. Phosphorylation of CYP2E1 was followed by its immunoprecipitation from incubation media containing microsomes, [^{32}P]ATP, catalytic subunit of cAMP-dependent protein kinase and 0.05% Na cholate with subsequent SDS-PAGE and autoradiography. The time-course of ^{32}P incorporation into CYP2E1 was found to be linear up to 30 min. Detegents increased the extent of phosphorylation in a dose- dependent manner. Although in the control rat microsomes NADPH-dependent LP (assayed by TBA test) was increased by 35% after incubation with kinase, the effect was opposite (20% inhibition) in microsomes with high level of CYP2E1. Studies of CYP2E1-dependent enzymatic activities showed similar effect of inhibition in the two cases. The most expressed inhibition was observed in case of CCl_4 dependent lipid peroxidation (about 40%). Para-nitrophenol hydroxylation as well as NADPH utilization rate followed by its disappearance at 340 nm were also depressed, but slightly less. This data are consistent with the previously shown phosphorylation-induced rapid degradation of CYP2E1 both in hepatocytes and microsomes.

4.11 EFFECT OF PROSTAGLANDIN E_1 ON RADICAL GENERATION IN RAT LIVER MICROSOMES

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One of the mechanisms of hepatoprotective action of some prostaglandins (PG) in alcoholic liver injury can be realized via their antioxidative properties. NADPH-dependent production of radicals by the cytochrome P-450 systm is an important source of peroxide species. Chronic alcohol intoxication (5 g/kg, i.g., 30 days) leads to increased NADPH oxidation rate, cytochrom P-450 content, superoxide dismutase (SOD) and microsomal ethanol oxidizing system (MEOS) activities as well as NADPH-stimulated radical production in liver microsomes. PGE_1 (4 g/kg, i.p., 7 days) decreases the cytochrome P-450 content and MEOS activity lower the control value and normalizes the NADPH oxidation rate, NADPH-induced chemoluminiscence and SOD activity in the liver of alcohol-treated rats. PGE_1 develops similar effects in the liver of rats after phenobarbital induction (80 mg/kg, i.p., 3 days) of the microsomal systems. We can conclude that the antioxidative properties of PGE_1 are related to a decrease of NADPH-dependent free radical generation in liver microsomes.

4.12 ENHANCEMENT OF ETHANOL-INDUCED LIVER INJURY DURING ISOLATED HEPATOCYTE POISONING WITH CCL4 OR 1,2-DICHLOROETHANE.

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Chronic ethanol consumption results in stricking structural and functional alterations in hepatocyte membranes, including endoplasmic reticulum, Golgi apparatus and plasma membranes. These effects may be ascribed to oxidative damage. Since free radical mechanisms are involved in CCl4 and 1,2-dichloroethane(DCE)-mediated liver damage, the aim of the present study was to determine the effects produced by the acute concomitant administration of these xenobiotics to hepatocytes isolated from chronically-ethanol treated rats. Rats were fed with chronic ethanol diet for 8 weeks, the isolated hepatocytes were prelabelled both with lipid (3H Na Palmitate) and glycoprotein (14Cd Glucosamine) precursors, and subsequentely poisoned with CCl4 or DCE for different times. The radioactivity significantly increased in total Golgi apparatus of hepatocytes isolated from chronic ethanol intoxicated rats and exposed to the tested xenobiotics in respect to the untreated hepatocytes. These data show that the chronic ethanol consumption potentiates the CCl4- or DCE-induced hepatotoxicity, suggesting that the synergic action of these compounds may be dependent on common prooxidant mechanisms as already hypothesized.

4.13 USE OF TWO METABOLICALLY DIFFERENT HEPATOMA CELL LINES IN THE ASSESSMENT OF ALDEHYDE CYTOTOXICITY

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It has been suggested that potentially harmful aldehydes are detoxicated via multiple oxidoreductive pathways. We have employed two hepatoma cell lines to study the correlation between the metabolism and the cytotoxicity of four aldehydes, namely benzaldehyde (BA), acetaldehyde (ACA), propionaldehyde (PA) and valeraldehyde (VA). The rat hepatoma cell line HTC possesses very high levels of aldehyde dehydrogenase (ALDH), particularly with BA as a substrate, and less marked levels of alcohol dehydrogenases (ADH) and aldehyde reductase (ALRED). The mouse hepatoma cell line Hepalclc7 has barely detectable levels of the above enzyme activities, with all substrates. The cytotoxicity of the four aldehydes was assessed by two "in vitro" assays: the colony forming efficiency, and the detachment of dead cells from the monolayer. The HTC cell line was shown to be highly sensitive to BA in the two assays, and less sensitive to ACA. On the contrary,the poorly metabolizing Hepalclc7 cell line was much less influenced. Moreover, the two assay systems were found to be differently sensitive in detecting the toxicity of aldehydes. Inhibition of BA metabolism reversed the effects, thus ALDHs do not play a detoxicating role in this model, but other mechanisms could be involved in the cell toxicity.

4.14 FREE RADICAL MECHANISMS OF 1,2-BENZOQUINONE DERIVATIVES CYTOTOXIC ACTION

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Experiments have been performed with HeLa cells and Ehrlich ascite tumor cells to study the mechanisms of 1,2-orthobenzoquinone (OBQ) derivatives cytotoxic action. The incubation of cancer cells in the presence of lipophilic derivatives induces a significant decrease of cellular viability. Meanwhile polar quinones have no influence. The OBQs cytotoxicity correlates with quinone ability to penetrate cellular membranes. The cytotoxic effect of OBQ derivatives is oxygen-dependent and involves the formation of active oxygen species (AOS). By means of AOS quenchers has been shown that the main mediators of quinone toxic action on the tumor cells are superoxide anion radical and hydrogen peroxide. An elimination of the AOS by impermeable acceptors protects cells from the OBQs cytotoxicity. The appearence of AOS in extracellular medium is supposed to be the result of transplasma membrane electron transfer owing to OBQ derivatives redox cycling reactions in cytoplasmic membrane. The results obtained show that the generation of AOS in extracellular medium mediated by transplasma membrane electron transport is the major mechanisms of OBQ derivatives-induced tumor cells damage.

4.15 THE NADPH ROLE IN THE REGULATING GSH-MIXED DISULFIDES AFTER OXIDATIVE STRESS.

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It is reported that during oxidative stress there is an increase in mixed disulfides (MDS) between GSH and proteins. It is also known that thioltransferase could regulate the equilibrium of MDS formation: GSH+PSSG===GSSG+PSH. Data are presented of in vitro experiments, carried out in the cytosolic fraction of rat liver which confirm the existence of the equilibrium in model systems much more complex than those previously studied and indicate that the NADPH supply is important in regulating the MDS formation. Rat liver homogenates were treated with increasing doses of H_2O_2 and tert-butyl hydroperoxide; kinetic studies of GSH and GSSG were carried out and the total GSH (GSHt), sum of GSH and GSSG, was calculated. The GSHt decrease was a clear index of MDS formation; this index increased with increased doses of peroxides. Direct measurements of MDS concentration confirmed the MDS formation. During oxidative stress with 1-2 mM of peroxides, GSH was extensively transformed to GSSG and about a 30% of MDS was formed. The addition of NADPH was able to regenerate all GSH and decrease MDS to their initial values.

4.16 HYDROGEN PEROXIDE AND HYDROXYL RADICAL GENERATION BY RAT LIVER NUCLEI

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Rat liver nuclei contain cytochrome P_{450}, NADPH- and NADH- cytochrome c reductase. The effect on OH and H_2O_2 generation, of either inducing the rat liver nuclear mixed-function oxidase system by phenobarbital (PB) or 3-methylcholanthrene (MC) or iron overload, was studied. PB and MC treatments increased by two-fold nuclei cytochrome P_{450} content and Fe-dextran dose reduced the levels to undetectable values. To evaluate the effects of the treatments on the generation of OH, the ability of the nuclei to oxidize chemical scavengers to known products was assayed. In the NADPH-dependent system the generation of formaldehyde from DMSO in the presence of Fe-EDTA, was increased by 92% and 63% after PB and MC treatments, respectively and decreased by 60%, 6 h after iron overload. NADH-dependent DMSO oxidation by rat liver nuclei obtained after PB or MC exposure, showed an increase of 54% and 22%, respectively, but iron dextran single injection decreased OH generation by 56% after 6 h. The rate of H_2O_2 generation in the presence of Fe- EDTA was higher with NADH than with NADPH. Treatment of the rats with PB and MC stimulated H_2O_2 production in the presence of NADPH by 104% and 68%, respectively. Hydrogen peroxide generation after iron overload was decreased by 22% in the absence of added iron and 40% in the presence of Fe-EDTA, using NADPH as cofactor. The data presented here suggest that cytochrome P_{450} activity is involved in oxygen radical generation by isolated rat liver nuclei and that this production is affected by inducing agents or iron overload in vivo.

4.17 NEUTROPHIL ACTIVATION OF HYDRAZINE DERIVATIVES TO ALKYL RADICALS

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Neutrophil (PMN) induced xenobiotic derived free radicals could have a role in the significant correlation observed between chronic inflamatory sites and tumor induction. Here we show that the metabolism of the carcinogenic hydrazine derivatives (HD): methylhydrazine (MeH), dimethylhydrazine (DMH), phenylethyl hydrazine (PEH) or procarbazine (PCZ), by PMN from rat peritoneal exudates leads to the formation of alkyl radicals, as determined by spin-trapping with POBN. Mono-subst-HD oxidation by phorbol ester or Zymocel activated PMNs generates 11.6-13.0 uM POBN-alkyl adducts, while di-subst- HD produce 1.4-3.5 uM. Radical formation from supernatant of sonicated PMN generates radicals to a similar extent which for mono-subst-HD are inhibited by the myeloperoxidase (MPO) inhibitor, azide. The active oxygen sequesters, SOD and catalase also inhibit radical formation by PMN/PMA: 30% in MeH and 60% in DMH metabolism to alkyl radicals. These results show that activated PMN metabolise HD to alkyl radicals mediated by both MPO and active oxygen species in different degrees, depending on hydrazine substitution.

4.18 ALLOXAN CYTOTOXICITY MAY INVOLVE LYSOSOMAL DAMAGE

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The diabetogenic effect of alloxan is not understood in detail, although it is thought to involve reactions mediated by oxygen and alloxan radicals. These reactive species may form extra- or intracellularly and cause cell damage through a variety of complex interactions with several macromolecules. Plasma and lysosomal membranes may be important targets. A model system of J-774 cells were studied in culture. Fluorescein diacetate and propidium iodide double staining technique was used to investigate the plasma membrane permeability. Acridine orange was used to study lysosomal membrane integrity. Fluorescence intensities were recorded by a Leitz MPV III microscope fluorometer connected to a personal computer. Exposure of J-774 macrophages to 2 mM alloxan and reducing substances (1 mM ascorbic acid or cysteine) in the surrouning medium rapidly resulted in plasma and lysosomal membrane damage and ensuing cell death. Separate pre-addition of catalase, desferrioxamine or superoxide dismutase resulted in evident, slight and no protection, respectively. The combined pre-addition of catalase, and desferrioxamine resulted in a pronounced inhibition of cell damage, while catalase desferrioxamine and superoxide dismutase together totally inhibited cell damage. Cells preincubated with 30 µM $FeCl_3$ in F-10 medium with 10% fetal calf serum for 24 h showed a much enhanced sensitivity to alloxan and reducing agents. The results are interpreted as indicating cell damage mainly due to the extracellular formation of H_2O_2 and $OH^{\cdot}$. The latter agent forming in close proximity to the cells with effect on the plasma membrane while the former, after diffusing into the cell, may have several intracellular targets, including lysosomes. Secondary lysosomes normally contain trace amounts of iron some of which may occur in reduced, reactive form allowing $OH^{\cdot}$ forming Fenton reactions to take place. $OH^{\cdot}$ may initiate peroxidation resulting in lysosomal membrane damage.

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4.19 BIOTRANSFORMATION REACTIONS AND LIPID PEROXIDATION IN MICROSOMES FROM CIRRHOTIC RAT LIVER

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Microsomes from thioacetamide(TAA)-induced macronodularly cirrhotic livers exhibited a reduced cytochrome P450 content and lowered activities of ethylmorphine N-demethylase, ethoxycoumarine O-deethylase and epoxide hydrolase. The malondialdehyde (MDA) production of microsomes from TAA-treated rats was decreased either when stimulated by ascorbate-iron or by ADP-iron-NADPH. Contrarily, in isolated hepatocytes from cirrhotic livers MDA formation was increased in both systems. The microsomal phospholipid fatty acid pattern was significantly changed in the cirrhotic livers. The 18:2/20:4 ratio of phospholipid fatty acids was markedly elevated. Increased free radical formation due to the inhibition of mitochondrial respiration might account for the differences in lipid peroxidation in microsomes and hepatocytes from cirrhotic livers.

4.20 THE EFFECT OF SOME ANTICOAGULANT RODENTICIDES ON THE ACTIVITY OF ANTIOXIDANT ENZYMES IN THE BLOOD OF RATS

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The effect of hemosterilant (α-chlorhidrin) and anticoagulants (bromadiolon, chlorofacinon and flokumafen) in vivo under the chronic treatment on the activity of antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) in the blood and glutathione-S-transferase (GST) in plasma in DA strain of male rats were studied. All examined supstances increase the SOD activity in the blood after 7 days of treatment. Long term treatment with α-chlorhidrin reaching the SOD activity on the control level, until all anticoagulants decreased SOD activity under the control level 14th day. After 21 day activity of SOD was increase in groups with bromodiolon and chlorofacinon. All examined anticoagulants decreasing the activity of catalase after 21 day of treatment. Activity of GSH-Px in all groups was increase after 14 and 21 day of treatment. The greatest changes was found in the activity of GST. After 7 days of treatment of rats GST activity was decrease in all studied groups.Long term exposure reaching the GST activity on the control level. After 21 day GST activity again decreased characteristically in animals treated with α-chlorhidrin.

4.21 PHOTOSENSIBILIZATOR-ANTIBODY CONJUGATES USAGE AS FREE RADICALS GENERATORS FOR SELECTIVE DESTRUCTION OF VIRUSES AND CELLS

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The usage of conjugates of photosensitive molecules with specific antibodies (AB) has some advantages in localizing drug effect: enhanced specificity because of AB, space selectivity because of limitary irradiation range, and ability to control the preparation action time in organism. The conjugates of influenza virus AB with porphyrine demonstrated the preserving of AB antigen activity and porphyrine nuclei ability to generate active oxygen forms. During neutralization reaction in dark the conjugates decreased virus culture infectious grade as well as AB alone. Being subsequently exposed to visible light virus culture was led to the additional significant loss of the infectivity. This way is suitable not in the case of viruses only, but also for the whole cells. There are some convincing results as for the selective destruction of the B-lymphocytes (the T-lymphocytes are kept alive) by the conjugates of B-lymphocyte specific AB with diiodofluorescein. These data proved the undoubted perspectivity of this way of the struggle against virus infections and tumors.

4.22 EFFECT OF ALCOHOL ADMINISTRATION ON ATP, CELL ENERGY POTENTIAL, OXYGEN FREE RADICALS FORMATION AND CELL INJURY IN RAT HEPATOCYTES.

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The effects of alcohol (ETOH) were studied in hepatocytes isolated from fed or fasted (24 hrs) rats. Freshly isolated hepatocytes were inbedded in agarose gel threads and perfused with KHB. Intracellular ATP (IATP) and the cell energy potential (CEP) were measured by 31P-NMR spectroscopy Oxygen free radicals formation was assessed by chemiluminescence. In fasted rats, IATP was depressed 48% compared with fed controls ($p<0.05$). The CEP was depressed even more 50% in the fasted group ($p<0.05$). ETOH decreased IATP by 20% in the fed group and 95% in the fasted group ($p<0.01$) and CEP was depressed 15% and 90% respectively ($p<0.001$). ETOH did not increase oxygen free radicals formation in either group, but LDH release increased 3 fold in the fed group and 20 fold in the fasted group ($p<0.001$). These data indicate that fasting decreases the IATP concentration, and the hepatocyte energy potential and that the nutritional status is an important determinant of damage to liver cells due to alcohol.

4.23 SHIFT OF DBE METABOLISM IN THE LIVER BY SIMOULTANEOUS TREATMENT WITH CCl_4.

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Hepatocytes isolated from rats were exposed to combined treatment of carbon tetrachloride (CCl_4) and 1,2-dibromoethane (DBE) up to five hours. A significant potentiation of lipid peroxidation and plasma membrane damage was observed in presence of the mixture CCl_4 plus DBE. This enhanced toxicity could be due to the reduced levels of GSH-S-transferase by CCl_4, following a shift of DBE metabolism versus the oxidative dehalogenation by microsomal enzyme system. The microsomal metabolism of CCl_4 is not affected by the contemporary presence of DBE, while the covalent binding of DBE reactive metabolites to hepatocyte protein was significantly enhanced in the presence of CCl_4. As a consequence, CCl_4 could impair the DBE detoxification pathway by increasing the microsomal transformation of DBE into highly reactive chemical species. To support these conclusions, recent studies were performed on acute treatment of rats with ethanol. The obtained results in this experimental condition suggest that ethanol could reduce either GSH-transferase and aldehydic DBE metabolic pathways leading to an excess of bromoacetaldehyde toxicity.

4.25 GSH MODIFICATION INDUCED BY XENOBIOTICS IN ISOLATED HEPATOCYTES. EFFECT OF OXYGEN CONCENTRATION.

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1,2-dibromoethane (DBE), diethylmaleate (DEM) and phorone are well known glutathione (GSH) depleting agents, in rat liver in vivo. In contrast, carbon tetrachloride (CCl_4) poisoning is known to increase liver GSH in vivo. We have also previously found that these changes of GSH concentration, are inversely correlated to changes of the liver Vit C concentration.

We have investigated the in vitro effects of DBE, DEM, phorone and CCl_4 treatment on GSH concentration in isolated rat hepatocytes. Hepatocytes incubated at 37°C under aerobic conditions showed a significant decrease of GSH content in the presence of DBE, DEM or phorone. Maximum effect was seen at 1 h for DEM and DBE and 30 min for phorone. The extent of GSH depletion was similar to that observed in vivo. However, in contrast with the effect observed in vivo, CCl_4 produced a decrease of GSH in the hepatocytes, being the lowest concentration at 4 h. Experiments carried out with hepatocytes incubated under anaerobic conditions indicated that oxygen plays a determinant role in modulating the in vitro effects of CCl_4 on GSH concentration.

In addition, the possibility that the different effects elicited by CCl_4 in vivo and in isolated hepatocytes on GSH concentration can be explained by a modulating effect of Vit C, which concentration seems to be markedly decreased in isolated hepatocytes, is under current investigation.

4.26 DAUNOMYCIN TOXICITY IN DIFFERENTIATING ERYTHROLEUKEMIA CELLS

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The production of oxygen radicals by the antineoplastic agent Daunomycin can be triggered by oxyhemoglobin, which can act as a reductant of the anthracycline thereby giving rise to at least 50% of the hydrogen peroxide detectable in human erythrocytes. The relative role of hemoglobin in the reductive activation of Daunomycin was investigated during differentiation of Friend erythroleukemia cells, a process during which an induction of hemoglobin synthesis occurs. 75-150 μM Daunomycin as well as 2-20 mM hydrogen peroxide proved to be more toxic to differentiated Friend cells, which had a 20-fold increased hemoglobin content, than to undifferentiated cells. However, Daunomycin-dependent, cyanide-insensitive oxygen-consumption of homogenates, derived from control or induced cells did not differ significantly, indicating similar activation rates of the drug in both cell types. Moreover, we did not notice any appreciable differences, between differentiated and undifferentiated Friend cells, in the specific activities of a series of oxygen-free radicals detoxifying enzymes. Thus, the observed increased susceptibility of differentiated Friend cells toward Daunomycin was apparently not related to increased cellular levels of oxygen free radicals. On the other hand, no altered Daunomycin uptake rates or differences in drug metabolism were detectable when the two cell types were compared. These findings are indicative of a minor role of hemoglobin in Daunomycin activation in nucleated cells. They suggest as well that the cytotoxicity of this drug in differentiated erythroleukemia cells may be due to alterations in the physiology of the cells, closely linked to the differentiation process, which could make some target (membranes and/or DNA) more susceptible to the action of Daunomycin. Experiments are in progress to investigate this possibility.

4.27 DAMAGE OF RAT LIVER MICROSOMAL MIXED FUNCTION OXIDASE BY CARBON TETRACHLORIDE THE ROLE OF LIPID PEROXIDATION

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Two kinds of free radical reactions can be involved in the liver injury by carbon tetrachloride. The first process is lipid peroxidation (LPO). The second one is covalent binding of products of metabolic cleavage of CCl_4 to proteins and lipids. In this paper we try to clarify the role of LPO in injury of rat liver microsomal mixed function oxidase. It was found that administration of a single dose of CCl_4 resulted in an increase of conjugated dienes (CD) level in liver to 160.2±30.7 nmol/g tissue whereas lipid hydroperoxide level increased only to 10.9±1.8 nmol/g tissue and the amount of TBA reactive substances practically did not change. The hydroperoxy groups of the unsaturated fatty acids of phospholipids were found can be changed to hydroxy groups as analyzed by HPLC. We also found that the injury action of carbon tetrachloride on liver monooxygenase of female rats was substantially stronger than that of the chemical on the enzyme of male rats, but the level of products of LPO (CD) was practically the same in both male and female rats. Pretreatment of rats with 4-[4-N-sodium-N-(5-ethyl-1-thia-3,4-diazol-2-yl) sulfophenylamino]-5-methoxy-1,2-benzoquinone (Q) before CCl_4 inhibited lipid peroxidation by 85% but did not prevent cytochrome P-450 destruction, decrease of hydroxylase activity, and loss of the capability to bioactivate carbon tetrachloride in rat liver microsomes. Thus, we propose that initiation of LPO is not crucial factor of cells injury by CCl_4 because antioxidant defence system convert lipid hydroperoxides into appropriate alcohols, which include conjugated dienes, but which are rather stable and less dangerous to cells.

4.28 OXYGEN FREE RADICAL (O-FR) PRODUCTION BY HUMAN SPERMATOZOA

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Recent research has shown that human spermatozoa produce Oxygen-Free Radicals (O-FR), and it is probable that these are involved in sperm-oocyte interaction.

The aim of this work was to evaluate O-FR generation in: a) 15 fertile normospermic subjects; b) 25 male partners of infertile couples (semen profile: total sperm count > 20 x 10^6; >30% progressive motility at 2 h.; leucocytes < 1 x 10^6/ml). The spermatozoa were washed twice in modified Tyrode's solution and finally resuspended at a concentration of 20 x 10^6/ml.

O-FR production was assayed, both in basal conditions and in response to treatment with ionophore A 23187, using luminol as the luminescent probe. In addition, the following tests were carried out in all the subjects: a) sperm motility evaluation at 30 min, 2 h, 6 h, and 24 h; b) the Swelling Test (SW-T) as a marker of the functional integrity of the sperm membrane.

A more significant O-FR production ($p < 0.01$) was observed in infertile subjects than in normal subjects. Furthermore, there was an inverse correlation between O-FR values and residual motility at 6 and 24 h. There was no correlation between O-FR values and the SW-T. However, it was observed that in all cases in which the SW-T values showed alteration, also O-FR values were elevated.

The increase in O-FR generation could be one of the pathogenic aspects in some cases of infertility.

4.29 FURTHER STUDIES ON OXIDATIVE STRESS-INDUCED ALTERATIONS OF CYTOSKELETON

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Cytoskeleton is a preferential target in oxidative stress-induced cell injury and previous biochemical studies have demonstrated marked alterations in microfilament as well as microtubule proteins (Bellomo et al. J.Cell.Physiol. 143, 118, 1990). Using immunocytochemical techniques, the localization of some cytoskeletal structures was investigated in T3T fibroblasts exposed to the redox-cycling quinone menadione. Microfilaments. As a consequence of menadione metabolism, actin stress fibers were progressively destroyed, and a perinuclear accumulation of aggregates occurred, with a concomitant loss of plasma membrane anchorages. One of the major actin-binding protein α-actinin co-distributed with actin fibers in control cells. Following treatment with menadione, clusters of α-actinin were early detected in the perinuclear region and subsequently in discrete regions scattered over the whole cell. Vinculin is a cytoskeletal protein involved in the attachment of actin microfilaments to the inner face of the plasma membrane and, in control cells it was localized in small patches whose immunoreactivity to monoclonal antibodies disappeared during menadione metabolism. Microtubules. Two major alterations in microtubule organization have been detected: (i) an early depolymerization and (ii) the appearance of tubulin-devoided vacuoles surrounded by retracted bundles of tubulin structures. At later time-points tubulin was concentrated in spots whivh coincided with the regions in the plasma membrane where small protrusions (blebs) appeared.

In conclusion, these data demonstrate that a disruption of microfilament and microtubule organization occurs during oxidative cell injury.

4.30 OXIDATION OF POLYCYCLIC NITROARENES WITH POTASSIUM SUPEROXIDE

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Superoxide anion radical plays a crucial role in a vast spectrum of metabolic processes. Several nitroarenes are known as environmental carcinogens and/or mutagens and the aromatic ring oxidation as well as the nitro reduction is involved in their metabolic activation pathways. To clarify the action of superoxide anion radical on the oxidation of polycyclic nitroarenes, the reaction of potassium superoxide with a series of nitroarenes was examined.

Oxidation of nitrated polycyclic aromatic hydrocarbons such as nitrated pyrenes with KO_2/crown-ether system afforded ring hydroxylated products. Hydroxylation proceeded by two different manners. One was the direct oxidation of the aromatic ring and the other was the oxidative replacement of nitro group by hydroxyl group. In the case of the oxidation of 1-nitropyrene, the former reaction was predominant and the hydroxylation proceeded at 5, 6, 8 and 9 positions. On the other hand, the oxidation of 1,6- and 1,8-dinitropyrenes mainly gave 1-nitropyren-6-ol and 1-nitropyren-8-ol, respectively. The pattern of the oxidation greatly depends on the multiplicity of nitro substituent, the substituted position and the number of fused aromatic rings. The details and the possible reaction mechanisms will be also discussed.

4.31 FREE RADICAL ACTIVATION OF ALKYLHYDRAZINES BY CYTOCHROME P-4502E1.

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Spin trapping experiments performed using liver microsomes prepared from chronic ethanol-fed (EtOH), phenobarbital (PB), β-naphtoflavon (β-NF) or acetone (S/A) pretreated rats and incubated with methyl-, ethyl-, isopropyl- and 1,2 dimethyl-hydrazine show that only EtOH and S/A microsomes actualy display a two fold rise in the ESR signal intensities expressed by nmoles of cytochrome P-450. Furthermore, EtOH microsomes have Km values for ethyl-hydrazine about ten time lower than PB microsomes. The possible role of ethanol-inducible cytochrome P-4502E1 (CYP2E1) in catalyzing the free radical activation of hydrazines is supported by the observation that the addition of specific substrates of CYP2E1, such as diethyldithiocarbammate and p-nitrophenol, or of antibodies against CYP2E1 selectively interfere with the free radical formation in control, EtOH or S/A microsomes, while are without effect in PB microsomes.

In conclusion the results obtained indicate that the isoenzyme CYP2E1 is responsible for the metabolism of alkylhydrazines to free radical intermediates and that this effect might be important in modulating the toxic as well as the carcinogenic properties of these compounds.

4.32 Reactivity of catalase towards L-Dopa and derivatives: ESR spin stabilization approach.

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Catalase is a redox hemoprotein which functions as defence against oxidative damage due to endogenously produced hydrogen peroxide. L-Dopa and derivatives are widely used as antihypertensive agents and stimulate H_2O_2 production, causing chronic active liver desease. In particular some authors give evidence of α-methyldopa inducing spectral variations (catalase Soret band)in a purified catalase and isolated hepatocytes ,using the traditional optical spectroscopic method (1). Our aim is to verify the catalase redox mechanism towards this kind of substrates at different substrate oxidation steps.
This is possible using an ESR spin stabilization approach to individuate o-semiquinone free radicals complexed with diamagnetic metal ions (first step of substrate oxidation mechanism) (2), combined with electronic method in order to characterize the second step of this redox cycle (o-quinone formation).
The experiments are done on purified catalase, isolated hepatocytes and liver microsomes from rats.

(1): D.P.Jones, D.B.Meyer, B.Andersson and S.Orrenius, Mol. Pharmacol. 20, 159 (1981).
(2): R.P.Ferrari, E.Laurenti, L.Casella and S.Poli, Spectrochim. Acta, in press.

4.33 Mechanism of $CBrCl_3$ toxicity: influence on energy-dependent processes in isolated rat hepatocytes

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The haloalkane $CBrCl_3$ causes cellular injury due to its radical inducing metabolism. The central mechanisms mediating the cytotoxic effects are discussed controversially. A potential target of $CBrCl_3$ toxicity might be the cellular energy state. Thus, in isolated rat hepatocytes total ATP, mitochondrial metabolism of MTT (a tetrazolium dye) and ATP-dependent processes like lysosomal uptake of neutral red and intracellular K^+ were investigated in the presence of 0.5, 1.0, and 1.5 mmol/l $CBrCl_3$.
A rapid decrease in total ATP content was accompanied by a moderate decline in MTT reduction, a dose-dependent decreased K^+ accumulation and a dose-dependent marked inhibition of neutral red uptake, which was almost complete at the highest concentration levels.
The rapid initial decline of all parameters , which paralleled the metabolic activation of $CBrCl_3$ was followed by a plateau phase indicating a lower cellular steady state of energy consumption and production (K^+, ATP, MTT) and a profound inhibition of other energy-dependent processes like neutral red uptake, which appeared to be irreversible.

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Session 5

Free Radicals in Medicine I (Lung, Kidney)

5.1 MOLECULAR TARGETS OF BIOLOGICAL DAMAGE BY INHALED ENVIRONMENTAL OXIDANTS

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In vitro exposures of human plasma (whose antioxidant defenses have been well characterized and which serves as a preliminary model for the respiratory tract lining fluids) to gas phase cigarette smoke (CS) and to ozone (O_3) were performed. Both of these important environmental pollutants are believed to damage the lung via oxidative mechanisms. We measured the temporal relationships between antioxidant depletions in relationship to the occurrence of oxidative damage to proteins and to lipids. CS exposure caused rapid ascorbic acid depletion followed by oxidative damage to lipids (Frei et al. Biochem J 227:133-138, 1991) and induced oxidative protein damage as monitored by loss of -SH groups and increases in protein carbonyls. Ascorbate appeared to protect against peroxidation (Frei et al.) but did not protect against protein carbonyl formation. This was, however, partially prevented by GSH. CS exposure did not deplete plasma uric acid. By contrast, when plasma was exposed to O_3, uric acid and ascorbate were oxidized quickly. Oxidative protein damage was readily detected by decreases in protein thiols, increases in carbonyls and changes in fluorescence. Only submicromolar concentrations of lipid hydroperoxides could be detected. The results demonstrate that quantitative differences exist in the spectrum of oxidative damage caused by different air pollutants. Hence there is a need for different antioxidants to protect the various molecular targets.

5.2 ROLE OF GLUTATHIONE IN OXYGEN-INDUCED LUNG INJURY OF THE NEWBORN.

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Glutathione (GSH) is an important component of the pulmonary antioxidant defence network. We have previously shown that lung GSH increases 2-fold over late fetal and early neonatal life. We have used the cytosolic GSH depleting agent diethylmaleate (DEM) to lower lung GSH concentration to determine if, and how, this influences hyperoxia-induced lung injury in the newborn.
A GSH depletion protocol was followed, which led to a 75% depletion of GSH in the lung over 6-12 hours, followed by a period of enhanced GSH status from 12-18hrs. 48hrs of hyperoxic exposure (95% O_2) significantly increased mortality of newborn guinea pigs. Markers of lung injury (microvascular permeability and inflammatory cell numbers) were increased in these pups, even though alveolar and intracellular GSH were elevated relative to controls by 24hrs, and were normal by 48hrs. These results suggest that GSH is important in the protection of the lung during the early period of hyperoxic exposure. Interventions which lead to increased pulmonary GSH in the newborn may provide protection against hyperoxic stress.

5.3 PULMONARY ANTIOXIDANTS IN THE FETUS AT TERM. INFLUENCE OF MATERNAL ADRENAL STEROIDS

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It has been previously shown that rat antioxidant enzymes increase in the fetal lung during the final days of gestation, probably preparing the tissue for the exposure to relatively higher O_2 concentrations at birth. This change is stimulated by dexamethasone treatment and delayed by glucocorticoid blockade with metyrapone. In this study, the effect of early maternal adrenalectomy on levels of CAT, GPx, GR, cytochrome oxidase, GSH, ascorbate and uric acid (HPLC) in the lung of the fetus at term and on corticosterone levels in maternal and fetal blood was investigated. Adrenalectomy profoundly depressed maternal, but not fetal blood corticosterone. No changes for any antioxidant were found when fetuses from control or adrenalectomized mothers were compared. It is concluded that maternal corticosterone is not required for the normal development of the fetal antioxidant capacity in the lung.

5.4 THE GLOMERULUS AS A SOURCE OF REACTIVE OXYGEN METABOLITES (ROM)

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There is considerable evidence suggesting that ROM (superoxide anion, hydrogen peroxide, hydroxyl radical, hypochlorous acid) are implicated in the pathogenesis of toxic, ischemic, and immunologically-mediated glomerular injury. The capacity of glomerular cells, especially mesangial cells, to generate ROM in response to several stimuli suggests that these autacoids may play a role in the models of glomerular injury that are independent of infiltrating polymorphonuclear leukocytes and monocytes. The mechanisms whereby ROM formation results in morphologic lesions and in modifications of glomerular permeability, blood flow, and filtration rate have been inferred from in vitro studies. They involve direct and indirect injury to resident cells (mesangiolysis) and glomerular basement membrane (in concert with metalloproteases), and alteration of both release and binding of vasoactive substances such as bioactive lipids (e.g. prostaglandin E_2, prostacyclin, thromboxane), cytokines (e.g. tumor necrosis factor alpha), and possibly endothelium-derived relaxing factor. The importance of such processes appears to be modulated by the intrinsic antioxidant defenses of glomeruli. Further studies are needed to address the role of ROM in human glomerular diseases.

5.5 FREE RADICALS PRODUCTION DURING IN VIVO ISCHEMIA/REPERFUSION OF RABBIT KIDNEY

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In vivo ischemia of 60 minutes in rabbit kidney followed by subsequent reperfusion of 10 minutes provokes a significant fall (48%) in vitamin E content of renal cortex, when compared to kidney not subjected to occlusion. The decrease in this major antioxidant substance is associated with : 1) a free radical production within the first minutes after reperfusion as evidenced by esr spectroscopy (with PBN as spin trap agent) in the venous blood arising from the ischemic kidney ; 2) a decrease of 29 and 49% in the preclamping value of renal blood flow (Doppler Laser), respectively after 5 and 10 minutes of reperfusion; 3) a significant increase of 249% after 120 minutes reperfusion in the initial level of lacticodeshydrogenase (LDH) measured in the renal venous blood.
The involvement of free radical production in our rabbit model is also confirmed by the following observations : 1) an ischemia of 15 minutes followed by 10 minutes of reperfusion does not modify the vitamin E content of cortex, produces two fold less free radical than after 60 minutes of ischemia, does not alter the blood flow after the reperfusion and only increases the LDH concentration up to 102% of the preclamping value; 2) the administration of desferrioxamine (i.v.; 50 mg/kg), as iron chelator, before inducing a 60 minutes ischemia significantly reduces the fall in vitamin E (16% vs 48%), restores a normal blood flow after reperfusion, and decreases the release of LDH (53% of the pre-clamping value) in the venous blood.

5.6 COMPARITIVE ACTIVITY OF REDOX ENZYMES IN COLD RENAL ISCHEMIA UTILYZING EURO-COLLINS(EC) VS. UNIVERSITY OF WISCONSIN(UW) PRESERVATION SOLUTION IN A WHOLE ORGAN MODEL.

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Exogenous addition of free radical scavengers has been of limited success in extending the viability threshold of transplanted organs after long hypothermic preservation. To understand the possible mechanism of this limited success, we have studied the effect of cold flush hypothermic perfusion with EC vs UW preservative solutions on endogenous redox enzyme activity. The kidneys of Sprague -Dawley rats were flushed in situ with either EC or UW Solution, surgically removed and preserved at 4°C for various times in each solution. Controls were cold flush perfused only. Kidneys in each group were analyzed morphologically by electronmicrograpy and part homogenized for enzymatic and western blot analysis. Catalase(CAT), Superoxide Dismutase(SOD) and Glutathione Peroxidase(GPX) activities are reported as percent control:

PRESERVATION	CAT		SOD		GPX	
TIME (hrs)	EC	UW	EC	UW	EC	UW
0	100	100	100	100	100	100
24	78	130	84	85	86	122
48	93	133	79	87	103	169
72	83	84	77	81	104	129
96	57	96	79	87	65	140

Western blot demonstrated approximately the same levels of CAT, SOD and GPX at all time intervals for both EC and UW solution,indicating that the decrease in enzymatic activity is secondary to inactivation rather than degradation. UW had a significant protective effect on CAT and GPX. The inability of both solutions to preserve SOD activity may be the rate limiting factor in organ preservation. Supported by DCI.

5.7 PLATELET-ACTIVATING FACTOR (PAF) ANTAGONIST WEB2086 BLUNTS HYPEROXIC-INDUCED LUNG INJURY IN THE PRETERM GUINEA PIG

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PAF has been implicated in the development of chronic lung disease (CLD) in the preterm infant, however, its precise role remains unclear. In the present study we assessed the effects of WEB2086, a PAF antagonist in the preterm guinea pig (GP) model of oxygen-induced pulmonary injury. Preterm GP's were delivered by Caesarian section three days prior to term (68 days) and were randomly allocated to receive either 95% O_2 or 21% O_2 for 72h. Half of the pups in each group were given an I.P. injection of WEB2086 (5mg/Kg) or saline every 12h. Bronchoalveolar lavage (BAL) was then performed after 72h.

Treatment	BAL PMN (10^4cells/ml)	BAL Protein (mg/ml)
Saline	2.05±1.53	1.57±1.07
WEB2086	0.46±0.62*	0.88±0.41

n=6-9 animals/group. WEB2086 < Saline, *p<0.05. These data suggest that PAF may play an important role in oxidative lung injury in the immature lung.

5.8 INHALED TAURINE AND AIRWAY HYPERRESPONSIVENESS.

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Recently it has been suggested that Taurine (2-aminoethane sulfonic acid) has antioxidant and membrane stabilizing properties.
We evaluated the ability of Taurine (T) administered by aerosol route to prevent the bronchoconstriction induced in man by ultrasonic nebulized distilled water (UNDW) bronchial challenge and Methacoline (M) challenge. Taurine pretreatment (250 mg.) was compared to placebo (P) and Disodium cromoglycate (20mg.) (DSCG), in 12 asthmatic subjects. Results showed that inhaled T prevents bronchoconstriction induced by UNDW challange similarly to DSCG. The protective effect of T, as compared with those of P, was found to be statistically significant (p less than 0.01). Furthermore there were no significant differences in changes in PC20 Methacoline between T and DSCG pretreatment. The ability of T to inhibit aiway hyperresponsiveness may lead to a new and more large therapeutic application of this aminoacid in prventing lung injury generated by oxidant air pollutants.

5.9 N-ACETYL CYSTEINE (NAC) AMELIORATES HYPEROXIC LUNG INJURY IN THE PREMATURE GUINEA PIG.

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The efficacy of NAC as a protective agent against hyperoxic lung injury was assessed in a guinea pig model of prematurity. Day 65 gestation, 3 days preterm guinea pig pups were exposed to normoxic (21% O_2), or hyperoxic (95% O_2) conditions for 72 hours and injected with 400 mg/kg body weight NAC or saline twice daily. Pups exposed to 95% O_2 showed elevated levels of GSH in both lung and liver compared to normoxic controls. Hyperoxic exposure resulted in an increase in bronchoalveolar lavage fluid (BALF) protein content (0.66±0.07 vs 0.33±0.07 mg/ml, $P<0.05$). NAC administration did not alter tissue, blood or BALF GSH content, either in normoxic or hyperoxic conditions. NAC did, however, reduce the BALF protein content in hyperoxia-exposed pups (0.32±0.05 vs 0.66±0.07 mg/ml, $P<0.05$). The activities of the antioxidant enzymes catalase and glutathione peroxidase in the lung were unchanged by NAC treatment.

The benificial effect of NAC on lung injury cannot be ascribed to an improved antioxidant status.

5.10 DIETARY VITAMIN E (VE) SUPPLEMENTS INCREASE LUNG VITAMIN E CONCENTRATIONS BUT FAIL TO PREVENT OXIDATIVE INJURY.

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Exposure of preterm guinea pigs to hyperoxic conditions provokes lung injury similar to that seen in premature infants. Preterm guinea pigs have low circulating levels and tissue stores of the antioxidant vitamin E (VE). 3 day preterm guinea pig pups were exposed to 85% O_2 or 21% O_2. The animals were fed either a standard formula milk (NM) (6.4 mg/l vitamin E), or a VE supplemented milk (100 mg/l) for up to 7 days. After 3 days VE supplementation, plasma but not erythrocyte VE concentrations were elevated, while following 7 days both plasma and erythrocyte VE concentrations were significantly increased. Lung and liver VE concentrations were elevated at both 3 and 7 days. At 3 days the increase in lung VE was oxygen-dependent, suggesting that the lung increases uptake of VE in response to oxidative stress.

Despite an increase in the VE concentration of the lungs of preterm guinea pigs, no amelioration of the lung injury was observed. These results suggest that VE is unable to adequately protect the lungs from hyperoxic injury.

5.11 FASTING AND HYPEROXIC LUNG INJURY IN THE PRETERM GUINEA PIG.

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Undernutrition may exacerbate hyperoxia-induced lung injury. 72 hours food restriction in guinea pig pups delivered 3 days preterm increased mortality rates among pups exposed to 95% oxygen (8/18), yet had no effect on 21% oxygen (air) exposed pups (0/10). Reduced tolerance of hyperoxic conditions was not, however, associated with increased lung injury.

	BAL Protein mg/ml	BAL Neutrophils 10^4/ml
Air Fed	0.30±0.04	1.4±0.7
Air Fasted	0.29±0.04	0.8±0.2
O_2 Fed	0.68±0.11	2.3±0.5
O_2 Fasted	0.66±0.14	1.6±0.5

Pulmonary antioxidant enzyme activities (Cu/Zn-SOD, MnSOD, GPx, CAT) were unaltered by starvation or hyperoxia. Lung glutathione concentration was slightly decreased following food restriction, whereas hyperoxic exposure did not change either lung or bronchoalveolar lavage fluid glutathione concentrations. Increased susceptibility to the lethal effects of oxygen in the starved, preterm guinea pig pup could not be attributed to a deficiency of pulmonary antioxidant defences.

5.12 THE SUPEROXIDE ANION CAN TRIGGER HUMAN SPERM HYPERACTIVATED AND CAPACITATION.

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Capacitation (CA) of mammalian spermatozoa is essential for fertilization and is visually characterized by hyperactivated motility (HA). Since reactive oxygen species (ROS) can induce in cells some of the changes observed during CA (calcium influx, changes in enzyme activities, etc.), we investigated whether low levels of exogenously added ROS could induce HA and CA in human spermatozoa. Percoll-washed spermatozoa were incubated, at 37°C, in Ham's F-10 medium supplemented or not with sources of ROS. The combination xanthine (0.6 mM) + xanthine oxidase (0.05 U/ml) + catalase (50 µg/ml) (X+XO+Cat) induced levels of HA that were higher (15.4 ± 1.6%, mean ± SEM, n=14) than those observed with foetal cord serum (FCS, 7.5%) (8 ± 1%, n=17), a known inducer of HA and with Ham's medium alone (5.4 ± 0.7%, n=15). The HA measured were part of the CA process: ROS-treated spermatozoa showed higher levels of spontaneous (7.9 ± 0.9%) and lysophosphatidylcholine-induced acrosome reaction (27 ± 3%) than spermatozoa incubated in Ham's medium alone (3.1 ± 0.3% and 12 ± 2% respectively). Spermatozoa that were added to the X+XO+Cat mixture after the $\cdot O_2^-$ generation had ended, reached similar levels of HA (5.6 ± 0.5%, n=7) than spermatozoa incubated in Ham's medium alone. The presence of superoxide dismutase (SOD) prevented the HA induced by X+XO+Cat and FCS as well as the spontaneous HA observed with spermatozoa incubated at pH ≥ 8. These results suggest that $\cdot O_2^-$ is involved in human sperm HA and CA.

Supported by the Medical Research Council of Canada.

5.13 ATP DEPLETION AND INSUFFICIENT AXONEMAL PHOSPHORYLATION IN SPERMATOZOA IMMOBILIZED WITH REACTIVE OXYGEN SPECIES.

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Reactive oxygen species (ROS) can be detected in 25% of semen from infertile men. Furthermore, spermatozoa incubated with ROS generating systems become immotile. To investigate how ROS affect sperm function, Percoll washed spermatozoa were treated with H_2O_2 (0.5 mM) or xanthine (0.6 mM) + xanthine oxidase (0.05 U/ml). The decrease in beat frequency observed within the first hour of treatment was associated with a rapid loss of intracellular ATP. Motility of intact spermatozoa stopped when their ATP was reduced by 85 ± 5%. Damage to the axoneme, the microtubule structure responsible for sperm movement, was confirmed when ROS treated spermatozoa could not reactivate motility after demembranation in a medium containing Mg.ATP. However, in conditions allowing rephosphorylation of the axonemes (addition of cAMP and protein kinase or sperm extract to the medium), the motility could reactivate. ROS immobilized spermatozoa, supplemented with pyruvate, reinitiate motility in parallel with an increase in their ATP level. The effects of rotenone, an ATP depleting agent, were similar to ROS effects and could also be reversed by pyruvate. These data suggest that ROS treatment produced axonemal damage, which results in sperm immobilization, mostly as a result of ATP depletion.

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5.14 OXYGEN RADICAL PRODUCTION OF BRONCHO-ALVEOLAR LAVAGE FLUID (BALF) FROM SUBJECTS WITH CHRONIC BRONCHITIS

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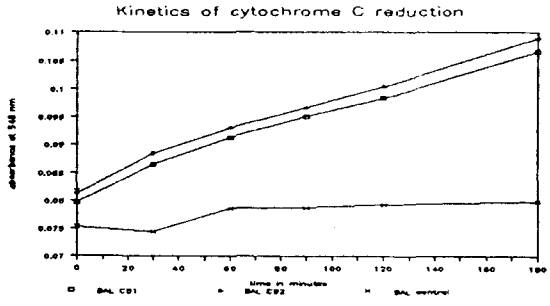
In order to investigate dioxide (1-) (superoxide or $O_2^{\cdot -}$) production of broncho-alveolar lavage fluids (BALF), we compared the kinetics of cytochrome c reduction in presence of BALF obtained from patients with chronic bronchitis (CB) with those from control healthy subjects. Cytochrome concentration in the experiments was 1.5 x 10^{-5} M and pH 5.5 - 6. Kinetics were monitored with a UVIKON 860 spectrophotometer. Results are shown here.



The results clearly show the production of superoxide by CB BALF. The speed of cytochrome c reduction increased upon adding 0.2 units of xanthine oxidase to CB BALF, but not when added to control BALF. Upon comparison of spectra of BALF and purines, it was possible to exclude that $O_2^{\cdot -}$ production could be due to nucleic acid degradation caused by BALF handling.

5.15 PROTECTIVE EFFECT OF GLUTATHIONE IN GENTAMICIN NEPHROTOXICITY IN RATS

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Aminoglycosides represent an important clinical tool used to combact serious infections involving bacteria and micobacteria. It has been established that aminoglycoside use is associated with nephrotoxicity. Since gentamicin nephrotoxicity appears to be correlated with the production of reactive oxygen metabolites, reduced glutathione (GSH), a substance able to restore intracellular antioxidant potential, was used in an attempt to limit such toxicity.
Rats were injected daily with gentamicin s.c.(100 mg/Kg), or with gentamicin s.c. plus GSH (1200 mg/Kg) i.p. for 8 consecutive days. 24 h after the last dose the following were determined: blood creatinine and urea, renal cortical gentamicin and GSH, renal cortical TBARS production (thiobarbituric acid reactive substances). Gentamicin nephrotoxicity was accompanied by an increased TBARS production and a reduction in renal GSH. Nephrotoxicity was significantly attenuated when renal cortical content of gentamicin was significantly reduced.

5.16 REDUCTION OF PARAQUAT TOXICITY BY A POLYAMINE-CHELATOR COMBINATION

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Paraquat (PQ) uptake in alveolar type two cells (ATTC) is mediated via the polyamine receptor. The anti-PQ effect for ATTC of the lipid soluble iron chelator 1-$(CH_2)_2CH_3$-2-methyl-3-hydroxypyridine-4-one (CP22) with spermidine attached was investigated. Two analogues, SSC359 and SSC460, were compared with the anti-PQ activity of deferoxamine (DF), which inhibits PQ uptake because of its polyamine-like structure. At 100 μM both compounds inhibited (45%) ATTC lysis (25.6±6.8%) by 100 μM PQ. DF, and CP22 without spermidine attached were as effective as SSC359 and SSC460. Saturation with iron of DF and CP22 completely blocked their anti-PQ activity, whereas iron-saturated SSC359 and SSC460 still significantly inhibited ATTC lysis. In agreement with the iron-dependent toxicity of PQ, SSC359 and SSC460 at 1 mM reduced ·OH generation by 50%. It is concluded that attachment of spermidine to CP22 combines two anti-PQ activities that act separately in one molecule.

5.17 TARGET SPECIFICITY OF THE FERRIC NITRILOTRI-ACETATE-INDUCED LIPID PEROXIDATION IN MICE

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We have reported that ferric nitrilotriacetate (Fe-NTA) is a nephrotoxin which causes lipid peroxidation (LP) in the proximal tubules (PT), and carcinogenic to the kidney. The present study was undertaken, using mice, to see: (1) the difference of fatty acid composition between renal cortex (rich in PT) and medulla; (2) if the sex differences seen in Fe-NTA-induced LP could be explained in terms of γ-GTP (a key enzyme for an iron-reductant cysteine on degradation of GSH) activity and localization.

The result showed unsaturated fatty acids are more abundant in the cortex than in the medulla. Mice treated either with Acivicin (a γ-GTP inhibitor), or BSO (γ-glutamylcysteine synthetase inhibitor) showed inhibition of Fe-NTA induced LP. Histochemical demonstration of in vitro lipid peroxidation showed LP localization and γ-GTP activity were well correlated.in 10-week-old mice. γ-GTP activity in the kidney is known to increase after birth. In males TBARS became significantly high from 6 weeks, reaching maximum at 10 weeks. In females, TBARS began to rise also from 6 weeks, but no peak was observed thereafter.

The results is consistent with our assumption that target specificity of Fe-NTA-induced LP is determined by the presence of iron-reductant.

5.18 ARGIMESNA, A NEW DRUG WITH UROPROTECTIVE ACTION

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Mesna (M) sodium salt of 2-mercaptoethansulfonic acid (MES) is an uroprotective thiol agent used to prevent oxazaphosphorine-induced hemorragic cystitis.

Argimesna (AR) is a new salt of MES in which sodium is replaced by arginine, with consequent advantage, over M, of avoiding sodium overload when high doses are used.

In this study the urinary excretion of the two salts has been compared after single oral equimolar dose administration to 5 healthy volunteers.

Following a cross-over design, 800 mg of M and 1800 mg of AR (equivalent to 920 mg of M) have been administered.

In spite of a total (free thiol plus disulfide) higher urinary excretion after Argimesna, the recovery of the reactive thiol groups was almost comparable with the two salts : 0-12 hrs recovery were 14.8% and 13.2% of the dose after M and AR respectively, the higher concentration being excreted within 0-4 hrs (3.9 and 3.3 μmol ml^{-1} for AR and M).

According to Burkert et al. (Arzneim Forsch. Drug Res. 34, 1597, 1984), 0.61 μmol ml^{-1} is presumed to be the minimum protective concentration : both salts mantain higher level until 8 hrs after administration.

We conclude that AR ensures an uroprotective activity equivalent to that of M and can represent an advantageous sobstitute of M when oral treatment is requested.

5.19 THE PROTECTIVE EFFECT OF RECOMBINANT HUMAN COPPER-ZINC SUPEROXIDE DISMUTASE (R-HCUZNSOD) IN ACUTE HYPEROXIC LUNG INJURY IN NEONATE PIGLETS

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Nearly 50% of premature infants develop respiratory distress syndrome (RDS), with a subsequent development of bronchopulmonary dysplasia (BPD) in up to 40% of these patients. The recent introduction of surfactant replacement therapy effectively reduced mortality but did not alter the incidence of BPD among these RDS patients.

Pre-term infants are routinely treated with oxygen and mechanical ventilation. This therapeutic intervention may in fact initiate acute lung injury which could lead to BPD. In newborn piglets, 48h of hyperoxia and hyperventilation (HO/HV) causes acute lung injury which is not ameliorated by surfactants. We hypothesized that the injury is mediated by superoxide radicals and that r-hCuZnSOD therapy may attenuate the insult. Newborn piglets were hyperventilated with 100% oxygen (HO/HV) for 48h and compared to piglets treated with intratracheal (IT) h-rCuZnSOD at time zero. Sham control piglets were normally ventilated with room air for 48h. In the HO/HV piglet group there was a significant decrease in lung compliance and an increase in tracheal aspirate cell counts, number of neutrophils, elastase activity, protein concentration and chemotactic activity over 48h. Morphologic examination revealed moderate atelectasis, inflammation and pronouced cell necrosis. HO/HV piglets administered IT r-hCuZnSOD showed a significant reduction in the severity of all assessed indices, and were comparable to the sham controls.

These results suggest that acute lung damage from 48h of hyperoxia and hyperventilation can be significantly ameliorated by a single dose of SOD. Clinical studies are underway to determine if SOD can prevent acute lung injury and BPD in pre-term infants.

5.20 FLUORESCENT PROBES FOR INTRACELLULAR H_2O_2 PRODUCTION IN ENDOTHELIAL CELLS

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Dichlorofluorescin diacetate (DCFH-DA) and dihydrorhodamine 123 (DHR) have been used to quantify oxidant production in neutrophils and other cells. Both are proposed to be membrane permeable, oxidized by H_2O_2-dependent mechanisms to fluorescent compounds [dichlorofluorescein (DCF) and rhodamine (RH)], and become trapped intracellularly (by esterase-depended deacetylation of DCFH-DA to DCFH and localization of RH within mitochodria). We evaluated these agents as fluorescent probes for intracellular H_2O_2 production in bovine aortic endothelial cells (BAEC).

Spontaneous oxidation in media alone was low; 0.5% for DCFH-DA and 0% for DHR over 1 hr. Both agents achieved stable intracellular levels by 15 min and remained stable for 1 hr. After 1 hr, 16% of DCFH was oxidized to DCF and 4.5% of DHR to RH. Loss of intracellular DCFH, DCF, and DHR was significant (92% ↓ DCFH, 99% ↓ DCF, 94% ↓ DHR after 1 hr) while RH was well maintained (↓ 13% after 1 hr). Exposure of BAEC to 100 μM H_2O_2 for 1 hr resulted in a 2 fold increase in DHR oxidation but no change in DCFH oxidation. In vitro studies showed that DCFH and DHR were not oxidized by H_2O_2 alone. Both probes were oxidized by H_2O_2 + Fe^{+2} and by H_2O_2 + hemeprotein. The oxidation of DHR by H_2O_2 + cytochrome c was inhibited urate but not by DMSO or SOD. Xanthine + xanthine oxidase oxidized DHR only in the presence of Fe-EDTA and oxidation was inhibited by hydroxyl radical scavengers.

Oxidation of DHR and DCFH reflect changes in intracellular H_2O_2 production due to secondary reactions. Unlike DCF, the oxidized product of DHR, RH, has stable intracellular levels. In vivo studies with BAEC indicated DHR is a more sensitive indicator of changes in intracellular H_2O_2 than is DCFH-DA.

5.21 ARGIMESNA INTERACTION WITH SOME CARCINOGENIC TRYPTOPHAN METABOLITES: EXPERIMENTAL DATA AND POSTULATED CLINICAL INVOLVMENT

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Argimesna (L-arginine mono 2-mercaptoethansulfonate; AR) is a sulphur-containing nucleophile under study as bladder chemoprotectant against electrophiles arising from compounds like the activated species of alkylating anticancer agent or other compounds which are postulated playing a role in bladder carcinogenesis as some tryptophan (T) metabolites (anthranilic acid and its derivatives) or aromatic amines and other occupational carcinogens.Our attention was focused on the urinary T metabolic profile in bladder cancer patients and on AR activity in binding the T metabolites anthranilic acid (AA) and 3-hydroxyanthranilic acid (3OHAA). In vitro experiments documented their interaction with AR both in physiological solution and in urine samples obtained from bladder cancer patients (almost complete disappearance of AA and 3OHAA after incubation at 37°C for 6 hrs). The in vivo study on 24 bladder ca patients treated with AR (four 450 mg cps), showed a 61% reduction of AA and 71% of 3OHAA. Assuming that recurrences of superficial bladder ca could be stimulated by the constant oncogenic stimulus exerted by AA and its derivatives and on the basis of our previous preliminary positive results with Mesna (sodium mercapto-ethansulfonic acid; oral treatment using the commercially available i.v. vials, the only one at that time available) in prevention of bladder ca recurrences, together with the recent published data on in vitro grow inhibition of 2/4 bladder cancer lines by Mesna,we started a multicentre study with AR in order to evaluate its efficacy in prevention of recurrences in patients completely resected (TUR) for superficial bladder cancer.

5.22 SULFITE ACTIVATES OXIDATIVE METABOLISM IN HUMAN NEUTROPHILS.

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Long-term exposure of dogs to SO_2/sulfite induced inflammatory reactions in the lungs accompanied by an influx of neutrophils (PMN). In their defensive role PMN may deleteriously affect the lung by releasing large amounts of oxygen radicals when the oxidative metabolism is stimulated. We therefore studied the effect of sulfite on the oxidative metabolism of PMN in vitro. Human PMN were incubated with sulfite for 20 min at pH 7 and 37°C and O_2^- production was determined by lucigenin-dependent chemiluminescence (CL). The CL of resting PMN increased by 595±22% (mean±SEM, n=12) with 1 mM sulfite. A 1.6-fold CL-increase was already found with 0.01 mM sulfite. PMN preincubated with the same sulfite concentrations and then stimulated with zymosan or phorbol myristate acetate (PMA) exhibited a two-fold higher CL than control cells with zymosan or PMA. The activity of NADPH oxidase, measured in membranes of sulfite-treated PMN by CL, increased by a factor of 2.6 compared to membranes of resting control cells ($7.7\pm1.3\times10^7$ CL counts/mg protein (n=5) versus $3.0\pm0.3\times10^7$ CL counts/mg protein in resting PMN). PMN from patients with chronic granulomatous disease with deficiency in the components gp91-phox and p47-phox of NADPH oxidase did not show any increase in CL by sulfite. The inhibitor of protein kinase C, H7, as well as the inhibitors of Ca^{2+} and calmodulin-dependent processes, W7 and Calmidazolium, completely inhibited the increased CL of sulfite-treated PMN. These results indicate, that sulfite stimulates PMN to produce O_2^- by activation of NADPH oxidase through a signal transduction pathway involving protein kinase C and Ca^{2+}/calmodulin.

5.23 HIGH LEVELS OF Mn-SUPEROXIDE DISMUTASE IN SERUM OF PATIENTS WITH NEUROBLASTOMA AND IN HUMAN NEUROBLASTOMA CELL LINE

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Levels of serum manganese superoxide dismutase (Mn-SOD) in normal children and children with various hematological and malignant diseases were determined by enzyme-linked immunosorbent assay. Assuming the upper limit of normal Mn-SOD level in serum to be the mean value ± 2SD of children at each age, high serum levels of Mn-SOD were found for 8 of 12patients with neuroblastoma, three of four patients with Wilms tumor, and four of five patients with acute myeloid leukemia. The patients with neuroblastoma exhibited a transient increase in Mn-SOD following chemotherapy, but after 1 week the levels decreased markedly to the control levels. The changes in serum Mn-SOD levels in the patients with neuroblastoma correlated with the levels of neuron-specific enolase. High levels of Mn-SOD were also found in cultured human neuroblastoma cells. These data indicate that Mn-SOD is expressed in neuroblastoma cells, may serve as one of the diagnostic and prognostic markers for the neuroblastoma, and may be useful to predict the effectiveness of chemotherapy for neuroblastoma and the recurrence of this disease.

5.24 MODULATION OF THE RESPIRATORY BURST OF CANINE NEUTROPHILS BY SURFACTANT PROTEIN A

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Pulmonary surfactant is essential for normal lung function. The major surfactant associated protein, SP-A, was shown to affect the host defence system of the lung. We were interested in the influence of SP-A on the respiratory burst of neutrophils (PMN) under various conditions. Canine PMN (1.2×10^5/ml) were incubated in the absence or presence of canine SP-A (12 µg/ml) and the formation of reactive oxygen metabolites was measured by lucigenin dependent chemiluminescence (CL) for O_2^- production and by oxidation of extracellularly added methionine to methionine sulfoxide. Spontaneous release of O_2^- by PMN was inhibited in presence of SP-A in both PBS-buffer or RPMI-medium (Table), whereas O_2^- release was increased upon stimulation by opsonized zymosan as compared with control cells. O_2^- generation of PMA-stimulated cells pretreated with SP-A was decreased in PBS-buffer but increased in RPMI-medium. Similar results were obtained measuring methionine sulfoxide formation. It is hypothesized that spontaneous release of reactive oxygen species by PMN as a result of unspecific activation is prevented by SP-A to avoid unwanted oxygen burden. This inhibitory potency of SP-A is reduced or abolished when cells are activated by specific stimuli.

	O_2^- production of PMN in presence of SP-A*		
	spontaneous	with op. zymosan	with PMA
PBS/Glc	35±5 (n=9)	112±13 (n=6)	44±9 (n=8)
RPMI/Glc	39±6 (n=7)	112±17 (n=5)	134±13 (n=7)

*Values are given as % CL of control without SP-A (mean±SEM).

5.25 DEVELOPMENT OF MARKERS FOR THE REACTION OF OZONE WITH BIOLOGICAL MATERIALS

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Ozone (O_3) is an important toxic component of photochemical air pollution. It is a powerful oxidant, capable of oxidising several biological molecules directly and it reacts slowly with water at physiological pH to yield the highly reactive hydroxyl radicals, ·OH.

In an "In Vitro" study the reactions of O_3 with Uric acid, DL-Phenylalanine and Salicylic acid were studied. The principal products of oxidation were measured by HPLC, high performance liquid chromatography. Uric acid yields Allantoin as the major product, DL-Phenylalanine yields o-, m- and p- Tyrosines and Salicylic acid yields 2, 3 and 2, 5 Dihydroxybenzoic acids. O_3 probably oxidises Uric acid directly, whereas hydroxylation of Salicylate and Phenylalanine is probably mediated by ·OH.

Uric acid has been shown to be the most important scavenger of O_3 in human blood plasma and it may play a significant role in removing inhaled ozone in the upper respiratory tract lining fluids. Ascorbic acid and protein -SH groups were also oxidised, whereas there was no loss of bilirubin or α-tocopherol. There was little formation of lipid hydroperoxides and no detectable formation of 4-hydroxynonenal, hexanal or nonanal, or changes in lipoprotein electrophoretic mobility.

Oxidative damage to lipids must not be assumed to be the mechanism of respiratory tract O_3 toxicity.

ARGIMESNA : NEW THIOL FREE-RADICAL SCAVENGER 5.26

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In the recent years there has been growing evidence that thiol compounds interact very closely with free radicals, reactive oxygen compounds and other cytotoxic agents, and consequentely defend cells against these toxic agents damage. Argimesna (AR) is a new thiol derivative, arginine salt of mercaptoethansulphonic acid, recentely synthetized in order to overcome the possible osmotic imbalance consequent to sodium overload resulting from high dose administration of Mesna (M). M is a site-selective chemoprotective agent clinically used (i.v. use) to prevent the oxazaphosphorines urotoxicity, mainly hemorrhagic cystitis.

Moreover, preliminary experiences with M were suggestive for new indications : prevention of superficial bladder ca recurrences and cystinic calculosis therapy. These indications require long term treatments and an oral administration is mandatory.

Interestingly, the salification of mercaptoethansulfonic acid with the aminoacid arginine, made possible a stable oral formulation (capsules containing 450 mg AR).

Indeed, the salification with arginine was choosed for its lack of toxicity and for its potentially positive effects of enhancing non specific immune response and host responces to tumors.

A review of preclinical and clinical experiences of AR vs M will be presented.

5.27 EFFECT OF DESFERRIOXAMINE AND Mn-DESFERRIOXAMINE COMPLEXES ON O_2-MEDIATED LUNG CELL INJURY *IN VITRO*

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A chronic lung injury, bronchopulmonary dysplasia, is a common outcome in preterm infants requiring prolonged respiratory support and may be due, at least in part, to pulmonary O_2 toxicity. Protection against oxidant lung injury by desferrioxamine (DES) has been reported in *in vitro* and *in vivo* models, presumably by binding Fe^{3+} to prevent hydroxyl radical formation from the superoxide anion and hydrogen peroxide. Complexes prepared from DES and manganese, either in the absence (GD) or presence (PD) of ascorbate, can enhance cellular superoxide dismutase activity in non-mammalian eukaryotic cells. Based on their potential for clinical use we investigated whether DES and DES-Mn complexes could protect fetal lung fibroblasts and epithelial cells against hyperoxic injury *in vitro*. Cells were maintained at a fetal PO_2 of 20 mm Hg and made hyperoxic by exposure to 95% O_2 for 48 h ± 1-100 μM DES, GD or PD. Cyto-toxicity was assessed by release of preincorporated [^{14}C]adenine and DNA synthesis by [^{3}H]thymidine incorporation into DNA. At a PO_2 of 20 mm Hg cytotoxicity and inhibition of DNA synthesis were not seen at ≤1 μM DES, GD or PD, but were evident at ≥10 μM. In 95% O_2 1 μM DES and GD enhanced O_2 cytotoxicity. PD resulted in a minimal, but not significant ($P>0.05$) inhibition, of O_2-mediated injury. O_2-mediated inhibition of DNA synthesis was enhanced by the addition of DES=GD>>PD. These data suggest that the beneficial effects reported for DES *in vivo* derive from actions on cell types not present in these *in vitro* studies e.g. phagocytes, nor were we able to show a significant protective effect from either manganese-desferrioxamine complex, though different responses to DES and PD suggested some amelioration of DES effect by PD. [Supported by MRC Canada]

INHIBITION OF CYCLOSPORIN A-INDUCED NEPHROTOXICITY IN RATS BY COADMINISTRATION OF SUPEROXIDE DISMUTASE AND CATALASE 5.28

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The main adverse effect of Cyclosporin A (CsA, Sandimmun®) immunosuppression is nephrotoxicity. The mechanisms underlying this adverse reaction are still unclear. We recently found that CsA induces an oxidant stress in rat kidneys both in vivo and in vitro. The goal of this study was to determine whether free reactive oxygen participates in the pathomechanism of the drug in vivo. The specific free reactive oxygen detoxifying enzymes superoxide dismutase (SOD) and catalase (CAT) were therefore coadministered with the drug in the rat, and the side effects determined by histology and clinical chemistry.

CsA administered orally once daily at a dose-level of 50 mg/kg for 15 days caused distinct histological changes in rat kidneys, such as tubular regeneration, formation of inclusion bodies, calcification and tubular vacuolization. The simultaneous s.c. injection of both 10 mg/kg/day SOD (5000 U/mg) and 10 mg/kg/day CAT (10000 U/mg) one hour before each CsA application led to significant reductions of CsA-induced kidney damage in the range of 34 to 89 %. Similar results were obtained as regards creatinine clearance, a clinical chemical parameter of kidney function also used for drug monitoring in man. SOD and CAT given together significantly inhibited the CsA-induced decrease of creatinine clearance by 78 %. SOD and CAT also significantly inhibited the decrease in protein content in kidneys of CsA treated rats by 64 %. SOD and CAT did not, however, change the plasma levels or the immunosuppressive properties of the drug.

Our results suggest that the free reactive oxygen species superoxide anion and hydrogen peroxide contribute to the CsA-induced renal side effects. We could also show that it is possible to inhibit these adverse effects by means of specific free radical scavengers without affecting the pharmacological properties of the drug.

5.29 PLASMA OF PRETERM BABIES STIMULATES PEROXIDATION OF PULMONARY SURFACTANT.

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Respiratory distress syndrome, a major cause of mortality in preterm babies, is produced by deficient surfactant activity and leakage of plasma across the alveolar wall. We hypothesized that these two mechanisms may be linked by lipid peroxidation of surfactant due to non-protein-bound iron in the plasma. We investigated, in vitro, the ability of plasma from babies (cord blood, n=8 preterm and n=9 term) and adults (n=8) to inhibit intrinsic iron induced lipid peroxidation of porcine surfactant liposomes. Inhibition of peroxidation by the adults was significantly greater than by the babies ($p<0.005$). The plasma of the term babies inhibited peroxidation, in contrast to that of the preterm babies, which actually stimulated peroxidation ($p<0.005$). In 71% of the preterm babies, 33% of the term babies, but none of the adults non-protein-bound iron was present. In the babies the inability of plasma to inhibit surfactant peroxidation was related to the presence of non-protein-bound iron ($p<0.05$) and addition of increasing concentrations of apo-transferrin increased inhibition to 100%. Similar results were obtained by incorporation of α-tocopherol into the surfactant liposomes. Measurements of plasma hemoglobin and heme and studies in which they were added to plasma did not provide evidence that these compounds contributed to surfactant peroxidation. The presence of non-protein-bound iron in plasma may play a role in the pathogenesis of respiratory distress syndrome in the preterm baby. Addition of primary or secondary antioxidants to exogenous surfactant may improve the effectiveness of this new therapy.

5.30 ANTIOXIDANT DEFICIENCY IN PREMATURE NEONATES.

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A new method for the measurement of total plasma antioxidant activity has been used to investigate the antioxidant status of premature and normal term infants. While it is known that premature babies are α-tocopherol deficient, and that oxygen therapy carries with it the dangers of harmful side effects, the retinopathy of prematurity remains a problem, and such babies are highly likely to develop other complications associated with inability to de-toxify free radicals. This work has shown that the levels of water soluble antioxidants in premature infants rapidly fall after birth, and that phototherapy to remove bilirubin from the circulation may depress the plasma level of an important antioxidant at a time that is highly significant for the neonate. A case can therefore be made for further augmentation of the antioxidant intake of premature infants, and for not removing bilirubin from the circulation unless there is a strong chance of the development of kernicterus.

5.31 MACROPHAGE-PRODUCED OXYGEN RADICALS AS AN ORIGIN OF BLEOMYCIN-INDUCED LUNG FIBROSIS

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It is usually supposed that anticancer effect of bleomycin (BLM) is due to DNA destruction by the activated Fe(III)BLM complex. BLM also exhibits strong side effects inducing the development of lung fibrosis. Earlier, we have shown that BLM efficiently stimulates oxygen radical production by macrophages. Therefore, one may propose that the induction of lung fibrosis by BLM is a consequence of the overproduction of oxygen radicals by BLM-stimulated macrophages. Indeed, it was found that bioflavonoid rutin, which is a free radical scavenger and a chelator, suppressed BLM-induced lung fibrosis in rats.
The mechanism of BLM stimulation of oxygen radical production by rat peritoneal macrophages has been studied. Using corresponding enzyme inhibitors, taurin, indomethacin, and NDGA, we showed that BLM does not activate myeloperoxidase, cyclooxigenase, and lypoxigenase, and that there is no non-reception stimulation. Therefore, we proposed that BLM activates NADPH oxidase supposedly via the interaction with cytochrome b_3.

5.32 CELLULAR PROTEASES AND OXIDANTS IN PATHOGENESIS OF PULMONARY DISEASES

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The role of cellular proteases, namely the role of protease/antiprotease inbalance is known in the pathogenesis of chronic obstructive pulmonary diseases. The inherited molecular defect of α_1-antiprotease inhibitor (α_1-PI) might be a primarily cause of pulmonary emphysema.
Recently toxicity caused by oxygen and its active species has received much atenttion in connection with variety of pathological events. "Respiratory burst" in neutrophils after microorganisms ingestion generates a series of oXidants. These might be contributors on tissue damage at site of inflammation. On this way modified structural components of lung tissue might be of enhanced susceptibility to proteolitic digestion. On the other hand the major antiprotease , α_1-PI , may be oxidative destructed,too.The similar reactions might be generated by oxidants from cigarette smoke. In this work the activity of proteases, protease inhibitors and indices of antioxidant status, namely antioxidant enzymes and small organic compounds were investigated in healths nonsmokers and smokers, as well as in a group patients suffering of chronic obstructive pulmonary diseases. On the basis of results obtained the importance of oxidative tissue degradation in pathogenesis of pulmonary emphysema was discussed.

5.33 REDUCED GLUTATHIONE MAY IMPROVE THE IMMUNOLOGICAL SURVEILLANCE IN ALVEOLAR ENVIRONMENT.

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The control of redox balance is important for the maintenance of normal pulmonary cellular function. To confirm this assumption we have been administering aerosolic reduced Glutathione (600 mg bid for 6 days) to patients affected with chronic obstructive pulmonary disease in steady state. Before and after administering Glutathione we valuated the CD3, CD4, CD8 lymphocyte subsets (monoclonal antibodies and flow cytometry) and CD4/CD8 ratio in bronchoalveolar lavage (50 ml of normal saline solution at 37°C for 4 times in middle lobe) and the alveolar macrophages phagocytosis (superoxid anion production after phagocytosis of opsonized zymosan). The 10 patients studied (7 males, 3 females) showed an increase in CD4 lynphocyte subset (32.1 vs 39.6; $p < 0.03$) and in CD4/CD8 ratio (which nevertheless does not reach any statistical significance) and an improvement of alveolar macrophages phagocytosis (33.2 vs 50.7; $p < 0.04$). We think there is a possible connection between the increase of helper lymphocite subsets and the improvement of alveolar macrophages phagocytosis. Though our study deserves further confirmations to get to any decisive conclusion, our data already show an increase in immunological surveillance in alveolar environment, after administering aerosolic reduced Glutathione.

5.34 EVIDENCE OF LIPID PEROXIDATION IN CHRONIC RENAL FAILURE

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Although there have been several reports of increases in lipid peroxidation products in the plasma of patients with chronic renal failure, both the methodology and the interpretation of these results in vivo is still under considerable debate. We have used second derivative spectrophotometry as a sensitive method for detecting conjugated dienes in the plasma and adipose tissue of patients in different stages of chronic renal failure (CRF) including dialyzed subjects. The study was carried out on 111 patients affected by CRF and 28 healthy controls. A significant increase in conjugated dienes was observed, both in the plasma and adipose tissue, exclusively in end-stage CRF patients (glomerular filtration rate <10ml/min). Deloused patients showed values similar to those of the controls. No significant correlation was observed between conjugated dienes level and the lipid profile of patients and controls. This study suggests that the evolution of CRF is accompanied by an increase in lipid peroxidation at its terminal stage. After dialysis the level of conjugated dienes return to the same levels as compared with controls.

Plenary Session on Free Radicals and Cancer

B.1 CELLULAR RESPONSE TO OXIDATIVE STRESS

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Growth promotion by oxidants is observed with cultured human- and mouse fibroblasts as well as epidermal cells. It is expected to play a role in inflammation, fibrosis and tumorigenesis. Indeed, oxidants trigger (patho)physiological reactions which resemble those induced by growth- and differentiation factors. They activate protein kinases, cause DNA breakage and induce the growth competence related protooncogenes c-fos and c-myc.

Mechanistic studies indicate that protein-phosphorylation and -polyADP-ribosylation are required for the transcriptional induction of c-fos by oxidants and the synthesis of protein factors, including FOS- and JUN-proteins, which bind to the fos-AP1 enhancer element. Poly ADPR participates in the efficient repair of DNA breaks which otherwise may retard or block transcriptional elongation. A fine balance of the multiple components of the cellular antioxidant defence determines the growth response of cells to oxidative stress. Transfectants of mouse epidermal cells which overproduce Cu,Zn-SOD were sensitized to the toxic effects of an oxidant while overproducers of catalase (CAT) were protected.

B. Trump & P. Cerutti-Cancer Cells 3:1-7(1991).
P. Cerutti-Eur. J. Clin. Invest. 21:1-5(1991).

FREE RADICALS AND CANCER B.2

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Classic concepts of tumor initiation and promotion have been useful tools to explore the roles of oxidants and antioxidants in carcinogenesis. However, the conceptual basis for the investigation of the role of free radicals and oxidizing agents in human cancer has been greatly extended by the confirmation, using molecular biological techniques, of the multi-step nature of carcinogenesis. It is clear that for certain human cancers there are at least six discrete steps in the progression between a normal cell and a clinically overt cancer. In such cases it is not unreasonable to assume that there are at least six different targets for the action of free radicals. Moreover, it is apparent that in a single step, such as at the p53 tumour suppressor gene, there are different patterns of genetic damage caused by different carcinogenic agents, thus further magnifying the possible loci of activity for free radicals and active oxygen species. It is suggested that the focus of research in this field should not be on whether or not human cancers have a free radical basis, but rather on what extent the causative role in any one of the many steps leading to cancer can be attributed to free radicals and active states of oxygen. This will require a careful evaluation of the potential role of free radical mechanisms in the development of different cancers. As each of the steps appears to be necessary but not sufficient for cancer, each in which a free radical reaction is involved represents a target for anticarcinogenic action through antioxidant agents.

B.3 CURRENT AND FUTURE EPIDEMIOLOGIC PERSPECTIVES ON VITAMIN E AND BETA-CAROTENE IN PREVENTION OF CARDIOVASCULAR DISEASE

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In basic research, the properties of antioxidants, including the possible inhibition of oxidation of low density lipoprotein cholesterol, have raised the possibility of their role in reducing risks of cardiovascular disease. In epidemiologic studies, descriptive, cross-sectional, case-control and observational cohort, the data are somewhat, but not entirely, consistent with this possibility. We addressed this question in two large, prospective studies which assessed dietary intake at baseline and followed apparently healthy individuals for the development of cardiovascular disease outcomes. In the Nurses' Health Study, over 87,000 women completed dietary questionnaires in 1980 and 927 developed cardiovascular disease outcomes during eight years of follow-up. Those consuming diets high in beta-carotene as well as vitamin E had lowered risks of cardiovascular disease, even after controlling for a large number of confounding variables. In a cohort study of 1299 Massachusetts elderly, 161 died of cardiovascular disease after 4.75 years of follow-up. Those consuming diets high in beta-carotene had significantly lower risks of cardiovascular disease. In the Physicians' Health Study, among 333 men with chronic stable angina there was a significantly reduced risk of a subsequent cardiovascular disease events among those assigned at random to beta-carotene supplementation. At present, dietary antioxidants represent a promising but unproven means to reduce risks of cardiovascular disease. Large-scale randomized trials, such as the Physicians' Health Study of over 22,000 men and the Women's Health Study of over 44,000 women, are necessary to provide reliable data about whether antioxidants themselves reduce the risk of cardiovascular disease.

LIPID PEROXIDATION AND CELL DIVISION B.4

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For many years it has been established that tumour tissue exhibits a striking resistance to lipid peroxidation, a damaging process to which most other cells and cell membranes are highly susceptible. Other rapidly dividing cells were also shown to be resistant to lipid peroxidation and it was suggested that low lipid peroxidation activity is a common feature of cell division and that there is actually a biochemical connection between the two processes. The crux of this hypothesis was that possibly lipid peroxidation is a normal cellular process that somehow acts as a coarse regulator of cell division. The resulting research that tested that hypothesis has been extremely valuable. It has led to a greater understanding of the fundamentals of lipid peroxidation in biological systems and to exciting developments, such as the study of the previously obscure hydroxyalkenal products of lipid peroxidation that are so well characterised today. This presentation will review this area of research from the standpoint of the work done by our group in recent years. We have studied the mechanisms of resistance of tumours to lipid peroxidation and have attempted to establish the relative roles of pro-oxidant factors such as cytochrome P-450 and antioxidant factors such as α-tocopherol. The tumour work has led into the study of rapidly dividing non-transformed liver cells using the regenerating liver model and has provided interesting observations on the tight temporal connection between cell division and resistance to lipid peroxidation, and the role of α-tocopherol in this phenomenon. The mechanisms of resistance of foetal liver and intestinal epithelial cells have also been investigated and the overall impression from all these cell types is that there is no common mechanism of resistance. Finally, the association between antioxidants and cancer has been extended to the human situation and recent observations on changes in plasma antioxidants in cancer patients will be discussed, as will the story of how investigations into low free radical levels in uterin cervix cancer have led in the direction of a biochemical method of diagnosing malignancy.

Session 6

Redox Chemistry and Antioxidants

6.1 REACTIVITY OF THIOLS TOWARDS THE HIGH OXIDATION STATE OF MYOGLOBIN, FERRYLMYOGLOBIN. IMPORTANCE OF ELECTRON TRANSFER AND ALKYLATION REACTIONS

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The reactivity of several thiols, including glutathione, dihydrolipoic acid, cysteine, *N*-acetylcysteine, and ergothioneine, as well as several disulfides, toward different redox states of myoglobin, mainly met- and ferryl-myoglobin, was evaluated by optical spectral analysis, product formation, and thiyl free radical generation.

All thiols interacted to different extent with the high oxidation state of myoglobin, *i.e.*, ferrylmyoglobin, via two processes. First, direct electron transfer to the heme iron in ferrylmyoglobin with formation of metmyoglobin or oxy-myoglobin; the former transition was effected by all thiols except dihydrolipoate, which facilitated the latter, *i.e.*, the two-electron reduction of ferrylmyoglobin. Second, nucleophilic addition onto a pyrrole in ferrylmyoglobin with subsequent formation of sulfmyoglobin. The contribution of either direct electron transfer to the heme iron or nucleophilic addition depended on the physicochemical properties of the thiol involved and the availability of H_2O_2 to reoxidize metmyoglobin to ferrylmyoglobin. The thiyl radicals of glutathione, cysteine, and *N*-acetylcysteine were formed during the interaction of the corresponding thiols with ferrylmyoglobin and detected by EPR in conjunction with the spin trap 5,5'-dimethyl-1-pyrroline-*N*-oxide. The intensity of the EPR signal was insensitive to superoxide dismutase and it was decreased, but not suppressed by catalase.

The disulfides of glutathione and cysteine did not react with ferrylmyoglobin, but the disulfide bridge in lipoic acid interacted efficiently with the ferryl species by either reducing directly the heme iron to form metmyoglobin or adding onto the pyrrole ring to form sulfmyoglobin; either process depended on the presence or absence of catalase (to eliminate the excess of H_2O_2) in the reaction mixture, respectively.

RECENT ASPECTS IN PEROXYL AND PERTHIYL RADICAL CHEMISTRY 6.2

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Halogenated peroxyl radicals, R(Hal)OO$^{\bullet}$, are generally known as one-electron oxidants and as such readily deplete, e.g., vitamines C and E, phenothiazine drugs and many other substrates of biological significance. Recent investigations on the R(Hal)OO$^{\bullet}$ induced oxidation of, for example, organic sulfides has revealed that these peroxyl radicals are also capable of overall two-electron oxidations. The mechanism involves an adduct radical as key intermediate and an intramolecular electron transfer within this adduct. It resembles certain features of two-electron oxidations in electrochemical systems. An efficient oxidation of methionine to methionine sulfoxide, initiated by hydroxyl radicals, is observed in oxygen containing solutions of methionine and the vitamine E analogue Trolox C. Evidence will be presented and discussed that this oxidation operates also via the above two-electron mechanism and involves Trolox-derived peroxyl radicals.

The peroxyl analogue perthiyl radicals, RSS$^{\bullet}$, (generated, e.g., by means of photolysis and radiolysis of organic trisulfides) have so far been investigated to evaluate their reactivity in relation to thiyl radicals, RS$^{\bullet}$. They appear to be moderately good oxidants, e.g. towards ascorbate (e-transfer) or PUFA (H-atom abstraction). Perthiyl radicals also readily add to molecular oxygen. The respective rate constants for these processes are, generally, lower than for the corresponding reactions of thiyl radicals. A most interesting finding is the formation of inorganic sulfate as a result of the perthiyl reaction with molecular oxygen. The proposed mechanism of sulfate formation involves oxygen-adduct radicals, rearrangements and characteristic features of general peroxyl chemistry. The possible role of the hydroperoxide analogue RSSH as a potential protective agent will also be discussed.

6.3 PROPERTIES OF THE ∝-TOCOPHEROXYL RADICAL IN PHOSPHOLIPID BILAYER MEMBRANES

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The ∝-tocopheroxyl radical has been generated within phospholipid bilayer membranes by photoionisation of ∝-tocopherol using a nanosecond XeCl excimer laser pulse at 308nm. The radical located in the dimyristoylphosphatidylcholine bilayers reacts with ascorbate in solution with a rate constant of ~$2x10^5$ M^{-1} s^{-1} at room temperature and an activation energy of 26 kJ/mol. The rate of reaction is significantly decreased by addition of an acidic lipid, dipalmitoylphosphatidic acid, to the bilayer membrane. The results show that the reaction is not particularly impeded by the membrane interface, except when the membrane carries an excess negative charge. The resonance Raman spectrum of the tocopheroxyl radical has also been obtained and shows that the radical has significant semiquinone-like character.

LIPID AND WATER SOLUBLE AZOINITIATORS AS A TOOL FOR THE STUDY OF THE ANTIOXIDANT PROPERTIES OF UBIQUINOLS WITH DIFFERENT CHAIN LENGTH 6.4

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We have recently determined the antioxidant effectiveness of ubiquinol (QH_2) by studying the autoxidation of egg phosphatidylcholine initiated by lipid-soluble azocompound both in homogeneous solution and in liposomes. Our results showed that Q_3H_2 behaves as a chain-breaking antioxidant, its inhibition rate constant being about one half that α-tocopherol (α-T). In organic solvents the stoichiometric factor was found ca. 2 and in liposomes ca. 0.5. In a similar study on the antioxidant activity of $Q_{10}H_2$, Frei *et al.* (1) estimated a value of 1.1. This work was undertaken to investigate whether the difference between the stoichiometric factors of the two homologues could be ascribed to their different localization in the lipid bilayer or to a difference in their physical association in the model membrane. To this purpose we used both water-soluble and lipid-soluble radical initiators, which generate radicals at a specific site and at a convenient, known and constant rate. In liposomes containing either α-T or Q_3H_2 or Q_7H_2 oxidation initiated by both lipid-soluble or water-soluble azocompound was inhibited and the rates of oxidation during the inhibition period were similar. It is to be pointed out that the inhibition time was strikingly different for equal amounts of the three antioxidants and these differences could be related to the azocompound used. These preliminary results suggest that the longer side chain quinol is embedded deeper into the membrane with little availability at the surface, and that it might be partially accomodated in the lipid bilayer as a separate phase. The short chain quinol, instead, can be intercalated among the acyl chains, exposing the quinol ring to the water environment.

1. Frei, B., Kim, M.C. and Ames, B.N. (1990) Proc. Natl. Acad. Sci. USA, 87, 4879-4883.

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6.5 FORMATION BY EPINEPHRINE OF AN IRON-DEPENDENT REACTIVE SPECIES THAT IS NOT HO• BUT IS CAPABLE OF EFFICIENT HYDROGEN ATOM ABSTRACTION.

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We have observed that epinephrine and other catecholamines markedly potentiate free radical production in systems containing ferritin and a source of superoxide anion. In particular, it was demonstrated that the generation of 1-OH-ethyl radicals from ethanol was enhanced very significantly by the catecholamines, but this effect was not observed with adrenergic agonists lacking the catechol function. When xanthine oxidase was incubated with hypoxanthine, ferritin, and ethanol, in the presence of the spin trap, DMPO, there was moderate production of both hydroxyl and 1-OH-ethyl radicals at an intensity ration of 2:1. But when epinephrine was added to this system there was up to a 15-fold increase in the intensity of the 1-OH-radical signal with no increase in the HO• radical generation. These results suggest that epinephrine and related catecholamine compounds interact with superoxide anion and ferritin to form a reactive species that is capable of efficient hydrogen atom abstraction, but is clearly not the hydroxyl radical itself. In an effort to understand the role of epinephrine in this reaction, a simpler system containing ethanol and Fe^{2+} was incubated with DMPO. No EPR signal was observed in this system. When epinephrine was added, a strong signal for the 1-HO-ethyl radical appeared, but no evidence of the hydroxyl radical was detected. Further studies with structurally similar compounds lacking the hydroxyl groups in the ortho position on the benzene ring were not active. Other studies indicated that iron chelation by the catechol functional group was critical for the formation of the reactive species. This property of catecholamines may be responsible for some their known toxicity of these compounds. (Supported by NIH Grant T32 GM08237).

6.6 A NOVEL ANTIOXIDANT FLAVONOID (IdB 1031) AFFECTING MOLECULAR MECHANISMS OF CELLULAR ACTIVATION

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By screening several flavonoids for antioxidant capacity we identified the compound 3-hydroxyfarrerol (IdB 1031), which scavenges DPPH and inhibits microsomal lipid peroxidation. The reactivity with hydroperoxyl radicals, measured as inhibition of crocin bleaching in the presence of a generator of hydroperoxyl radicals, gives an account for the observed antioxidant capacity, which is in the same range of quercetin. When tested on human neutrophils activated by fMLP, IdB 1031 inhibits "respiratory burst", measured as superoxide production (ID_{50}: 20 uM). This effect could be due to the inhibition effect of IdB 1031 on PKC (ID_{50}: 50 uM). This seems rather specific since IdB 1031 does not inhibit tyr-kinases and casein-kinase-2, while quercetin inhibits all these enzymes. In HeLa and smooth muscle cells, IdB 1031 inhibits the release of calcium from intracellular stores (IP_3 sensitive) induced by an oxidative stress. The effect of IdB 1031 on different mechanisms of cellular activation, possibly related to the free radical scavenging capability, candidates IdB 1031 as a drug addressed to diseases in which peroxidative damage is associated to the activation of cellular responses such as "respiratory burst" or smooth muscle contraction.

6.7 Trolox C Enhances Hydroxyl Induced Oxidation of Methionine to Methioninesulfoxide

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A survey will be given on the •OH induced reaction of the vitamin E anlogue trolox C with methionine to yield methionine sulfoxide. γ-Irradiation of an aqueous solution of methionine (10^{-3} M Met-S) results in the formation of methionine sulfoxide (Met-SO) with G=0.35 corresponding to 7% of the •OH radicals which are available for the oxidation of methionine. If the same experiment on Met-S oxidation is carried out in the presence of trolox C (5×10^{-3} M) the yield of Met-SO increases to G=2.1, i.e. to ca 40% of the initial •OH radical concentration. A similary high yield of Met-SO (G=2.3) is also found when the concentration of trolox C is diminished by a factor of ten. When trolox C is added to an aqueous solution of Met-S immediately after irradiation the sulfoxide yield is considerably reduced to G=0.4. This indicates that Met-SO does not result from a stable •OH-induced degradation product of trolox C, thereby excluding, e.g., epoxides, peroxides or hydroperoxides. The sulfoxide formation must be associated though with the •OH + trolox C reaction and is suggested to involve carbon centered peroxyl radicals. Chain reactions are indicated by different sulfoxide yields obtained upon variation of the steady state radical concentration. A possible mechanism will be presented and discussed.

6.8 IRON-THIOLATE INDUCED OXIDATION OF METHIONINE TO METHIONINE SULFOXIDE IN PEPTIDES. INTRAMOLECULAR CATALYSIS BY HISTIDINE

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In biological systems the oxidation of methionine to methionine sulfoxide is observed in several proteins under various conditions of oxidative stress. Similiar mechanisms might be responsible for the pronounced liability of methionine towards oxidation under conditions of synthesis, isolation and storage of pharmaceutical relevant proteins which are nowadays produced in large scale quantities through the recombinant DNA technology. Thus a detailed mechanistic study was undertaken to investigate the influence of sequence and structure on the stability of methionine in proteins towards oxidizing systems such as, for example, iron-thiolate-oxygen. Formation kinetics and yields of methionine sulfoxide were found to depend strongly on concentration of peptide, iron, and thiol, on the pH, and on neighboring amino acids. Effective oxidation of methionine, for example, was observed whenever histidine was present in the same peptide skeleton in His–$(Gly)_n$–Met peptides with n=0–4. Kinetics, but not yields, of the latter process could be suppressed by addition of methanol. In contrast histidine did not catalyze methionine oxidation intermolecularly. Mechanistic conclusions derived from the above experiments are of use for the design of peptides more stable towards oxidation and therefore of greater use for therapeutical purposes.

6.9 NADPH-DEPENDENT OXIDATION OF REDUCED EBSELEN AND OF 2-(METHYLSELENO)BENZANILIDE CATALYZED BY PIG LIVER FLAVIN-CONTAINING MONOOXYGENASE

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The selenazole ring-opened metabolites of ebselen, 2-(hydroxyseleno)benzanilide and 2-(methylseleno)benzanilide, are substrates for flavin-containing monooxygenase from pig liver. The K_m values were 25 and 3 uM,respectively, measured at 37 °C, pH 7.4, in the presence of 1 mM GSH. The V_{max} values were 390 mU/mg of protein, similar to those obtained with methimazole or other substrates for FMO1. Although ebselen also appears to be a substrate in the absence of GSH, it progressively inactivates the enzyme, apparently by binding covalently to essential enzyme thiols. The oxidation products of the selenol and methylseleno derivatives are rapidly reduced by GSH, regenerating the parent substrates. Rapid reduction of the selenide oxide by GSH was unexpected and suggests that, unlike S-oxidation of sulfides, Se-oxidation of selenides may be a route for bioactivation. The data show that in the presence of FMO1 micromolar amounts of either of these ring-opened metabolites establish a futile cycle catalyzing the oxidation of GSH to GSSG by NADPH and oxygen.

INHIBITION OF FREE RADICAL REACTIONS BY 4-(4-R-PHENYLAMINO)-5-METHOXY-1,2-BENZOQUINONES *IN VITRO AND IN VIVO* 6.10

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Free radical reactions including lipid peroxidation are widely discussed as a possible mechanism of tissues injury during ischemia-reperfusion. If so, use of free radical scavengers would be important for prevention of degradative events in these conditions. In this paper, we describe the inhibition of free radical reactions by 4-(4-R-phenylamino)-5-methoxy-1,2-benzoquinones. We found that these substances can be easily reduced enzymatically in mitochondria, microsomes and cytosol. Their reduced forms - dioxybenzoles - appeared to be powerful antioxidants that effectively inhibited NADPH-dependent lipid peroxidation and free radical processes initiated by CCl_4 *in liver* microsomes and *in vivo*. Recently, was shown that the free radical injury to endothelial cells of the vascular wall can be initial event of the reperfusion injury. We investigated the inhibitory effect of o-benzoquinones on lipid hydroperoxide-induced injury of cultured endothelial cells. It was found that the addition of 4-[4-N-sodium-N-(5-ethyl-1-thia-3,4-diazol-2-yl) sulfophenylamino]-5-methoxy- 1,2-benzoquinone (Q) significantly inhibited the increase in cellular lipid peroxide level and the release of LDH activity. The inhibitory potency of Q was slightly stronger as that of butylhydroxytoluene (BHT). IC_{50} of Q and BHT for LDH release was found to be 20 μM and 25 μM, respectively. In the following experiments, protection action of Q was studied in conditions of ischemia-reperfusion *in vivo*. It was shown that Q at a dose 50 mg/kg increased the time of reversible circulatory ischemia of rabbit brain from 5 up to 15 minutes. Thus, we expecting such antioxidants would be important for a clinical use.

6.11 A TWO-PHASE MODEL FOR THE STUDY OF ANTI-OXIDANT RECYCLING MECHANISMS

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A new method has been developed, which allows direct observation of antioxidant regenerating processes during stimulated autoxidation of unsaturated fatty acids. The assay utilises a two-phase system comprising an aqueous buffer and chlorobenzene, wherein the level of conjugated dienes can be continuously measured by an automated HPLC system. The method further facilitates sampling of the status of the antoxidant and co-antioxidant. Importantly, in this system hydrophilic supporting co-antioxidants such as ascorbate or thiols do not interfere with the peroxidation process. The model thus allows convenient assessment of antioxidant properties in a physiologically relevant situation.

MITOCHONDRIAL INNER MEMBRANE PERMEABILITY CHANGES INDUCED BY OCTADECADIENOIC ACID HYDROPEROXIDE. ROLE OF MITOCHONDRIAL GSH POOL. 6.12

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It has been studied the effect of exogenous octadecadienoic acid hydroperoxide (HPODE) on the functional properties of inner membrane of isolated rat liver mitochondria, as evaluated by the measurement of the membrane potential ($\Delta\Psi$). Very low concentrations of HPODE (1.5-4.5 nmol/mg prot) do not appreciably modify the $\Delta\Psi$ of control mitochondria while it brings about the drop of $\Delta\Psi$, in a concentration dependent mode, in mitochondria with a GSH level diminished by approx 60%. Mitochondrial GSH depletion has been obtained by intraperitoneal administration to rats of buthionine sulfoximine a specific inhibitor of GSH synthesis. The presence in the incubation system of GSH-methyl ester which normalizes mitochondrial GSH,fully prevents $\Delta\Psi$ drop induced by HPODE. The same protective effect has been presented by EGTA, which chelates the available Ca^{++}. Neither an antioxidant nor a specific inhibitor of mitochondrial phospholipase A_2 are able to prevent the HPODE effect. From the results obtained we can assume that HPODE itself, at the concentrations here used, induces permeability changes in the inner membrane with the loss of coupled functions, when the GSH mitochondrial level is below a critical value.

6.13 CYTOTOXICITY OF H_2O_2 ON E. COLI

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H_2O_2 lethality on E.coli has been characterized by two modes of killing occurring at two different concentrations, one below 2 mM and the second at concentrations higher than 10 mM, and indicating different toxic species and/or sites of damage(1). Actively growing cells in nutrient broth, when exposed to 20 mM H_2O_2 in the presence of hydroxyl radical scavenger DMSO were protected from the toxic effect of H_2O_2. α-tocopherol also significantly increased the surviving fraction of the cells. However, percentage survival was not significantly effected when cells were treated at high concentrations of H_2O_2 at lag, log, or stationary phases. Further studies on the identification of the toxic species and/or sites of damage are in progress.

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6.14 SOD-LIKE ACTIVITY OF COPPER (II) COMPLEXES

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Superoxide anions and related free-radicals are involved in a number of diseases, including inflammation .In many cases, SOD enzymes or sinthetic SOD mimiking compounds are an actual therapeutic approach. In this context we have synthesized ß-cyclodextrins with one or two coordinanting residues (1) and have investigated their copper(II) complexes, a potential SOD-mimicking systems.

SOD activity has been determined by the indirect method originally developed by Beauchamp and Fridovicth (2). Our results suggest a correlation between the structure of the complexes in solution and SOD activity, and point out a synergistic effect due the contemporary presence of cyclodextrin hydrophobic cavity and coordinanting moieties. Putative therapeutic effects of these complexes will be investigated by *in vitro* and *in vivo* experiments.

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2) C.Beauchamp and I.Fridovich (1971) Analytical Biochemistry, 44, 276.

6.15 GLUTATHIONE, CAPTOPRIL AND OTHER DRUGS AS FREE RADICALS SCAVENGER

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The interest in radicals shown by the biologists and clinicians has been raised recently by the discovery of the importance of radical reactions in normal body chemistry and in the mode of action of several toxins involved in different pathological conditions. Protection against oxidants in biological systems is obtained in various modes: by enzymes, by small molecules, by sequestration of metal ions, by repair systems.

In this paper we examined the ability of some molecules: glutathione, captopril (ACE inhibitor) and some new substances (aldoso reductase inhibitors). The technique that was used to asses the scavenging ability of these drugs was described by Misra and Fridovich (1977). In this assay free radicals are generated by photo-oxidation of dianisidine sensitised by riboflavin.

The obtained results show that glutathione, captopril, AR_4, AR_5, AR_6 have a value for IC_{50} equalto 10^{-5} M.

6.16 FREE RADICAL CHEMISTRY OF HETEROCYCLIC THIOLS

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The mechanisms of radioprotective and antimutagenic effects of thiols are based on their ability to scavenge and inactivate damaging free radicals. Aliphatic thiols, such as glutathione, are moderately good H-atom and poor electron donors. Aromatic thiols are better H-atom and excellent electron donors. The antioxidant mechanism of heterocyclic thiols has not been fully established yet. To better understand the protective properties of heterocyclic thiols, we investigated the efficiency of imidazolethiols as free radical quenchers in the reactions with various free radicals.

Free radicals were generated in aqueous solutions by radiation processes. Their reactions and kinetics were investigated by pulse and gamma radiolysis. Some imidazolethiol derivatives were found to efficiently inactivate superoxide radicals by one-electron donation, as opposed to glutathione, which was unreactive. Tyrosine phenoxy radicals rapidly oxidize ovothiol derivatives, $k = 6.6 \times 10^6 M^{-1}s^{-1}$, whereby tyrosine is restored. Thiyl radicals may be removed in the reaction with ascorbate, $k = 1.1 \times 10^7 M^{-1}s^{-1}$. On the basis of these data, the ability of heterocyclic thiols to act as antioxidants - electron donors is compared with that of aromatic and aliphatic thiols, on the one hand, and phenols, on the other.

6.17 STUDIES ON THE MECHANISM OF PHOSPHATIDIC ACID INFLUENCE ON Fe^{2+} OXIDATION AND LIPID PEROXIDATION

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The phospholipid composition of biological membranes is important in determining the susceptibility of the membranes to the oxidative stress. Not only the hydrophobic but also the hydrophilic components of the membrane affect lipid peroxidation catalyzed by iron. We have shown that the presence of 50% phosphatidic acid (PA) in phosphatidyl choline liposomes causes: rapid Fe^{2+} oxidation and oxygen consumption; increased generation of lipid hydroperoxides (LOOH); decreased generation of thiobarbituric acid reactive materials; very low inhibition of Fe^{2+} oxidation and LOOH generation by BHT; inhibition of the termination phase of lipid peroxidation at high $FeCl_2$ concentrations. However, at the present, the mechanism by which iron interaction with negatively charged unsaturated liposomes can exert such effects, is not known.

Studies conducted in suitable experimental conditions have shown that PA causes rapid Fe^{2+} oxidation and oxygen consumption also when present in saturated liposomes. A rapid reduction of nitro blue tetrazolium is observed during Fe^{2+} oxidation both in the presence of saturated and unsaturated PA containing liposomes. These and other data suggest that the polar head of this phospholipid is able to interact with the metal and increase its susceptibility to autoxidation.

However, when the two types of PA containing liposomes are studied, the parameters measured are differently affected by the addition of various cations. Also the addition of BHT to the unsaturated liposomes does not abolish the observed differences. Other physical and chemical properties of the two types of liposomes, among which the ability to differently interact with cations, appear to be responsible for the results observed.

6.18 THE GENERATION OF INORGANIC SULPHATE FROM PERTHIYL RADICALS (RSS•).

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Redox processes of di- and trisulphides induced by both photochemical and radiation chemical methods have been shown to be a convenient means to generate perthiyl radicals. Perthiyl radicals are moderately good oxidants, although weaker than the corresponding thiyl radicals, ($RS^•$) and readily react with molecular oxygen in an addition process as indicated by the following absolute rate constants: $k(RSS^• + \text{ascorbate}) = (4.1\pm1.0)\times10^6\ M^{-1}s^{-1}$ and $k(RSS^• + O_2) = (5.1\pm1.0)\times10^6 M^{-1}s^{-1}$ respectively.

Probably the most interesting chemistry of the perthiyl radicals is that inorganic sulphate SO_4^{2-} is formed in their reaction with molecular oxygen.

The quantitative measurement of SO_4^{2-} ions thus represents an appropriate means to probe free radical processes in which perthiyl radicals are believed to be involved. For example, the 'chemical repair' of radical damaged sites on biomolecules by perthiols, (RSSH).

The proposed mechanism for sulphate generation from perthiyl radicals in the presence of molecular oxygen will be presented.

6.19 EFFECT OF N-ACETYLCYSTEINE ON THE OXIDATIVE METABOLISM OF HUMAN PHAGOCYTES

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The oxygen metabolism of phagocytic cells gives rise to various reactive oxygen species (ROS). The balance between the release of ROS and their inactivation by antioxidants could be critical to the prevention of tissue damage during the inflammatory response.

The effect of the antioxidant N-acetylcysteine (NAC), concentration range 0.688mM - 137.5mM, on the ROS released by inflammatory cells was studied in vitro. The oxygen metabolism of starch grain-activated phagocytes in heparinized blood, buffy coat, and isolated polymorphonuclear leucocytes (PMNL) was quantified by the method of chemiluminescence (CL).

Both luminol-and lucigenin-amplified CL of PMNL was suppressed in a dose-dependent manner by serial dilutions of NAC. The reduced ROS formation by PMNL in the presence of NAC proves its role as an antioxidant. On the other hand, in blood and buffy coat the antioxidant effects were observed using the highest concentration (137.5mM) of NAC only.

These findings show that it is advisable to observe the effects of antioxidants in blood and buffy coat too, as the joint presence of the tested agent and plasmatic factors can lead to changing its effect.

6.20 THE NATURE OF BOUND REDUCING MOIETIES ON RADICAL DAMAGED PROTEINS

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We have shown that cytochrome c and free copper ions are significant reduced by proteins previously exposed to hydroxyl radicals, (Simpson *et al*, 1992). The observed reductive capacity can be generated by gamma radiolysis, UV irradiation and Fenton type chemistry systems. A major product of hydroxyl radical addition to tyrosine is the catechol 3,4-dihydroxyphenylalanine (DOPA, Karem *et al*, 1984). Catechols like DOPA are well characterised reducing agents. DOPA is important for dopaminergic neuron transmission as well as a direct precursor for tyrosinase-catalysed generation of melanin pigments. Ito *et al* (1984) have demonstrated that mushroom tyrosinase can generate DOPA on proteins.

We have shown that free commercial DOPA can reduce cytochrome c and free copper ions. Incubations of free tyrosine with tyrosinase can also reduce cytochrome c. Conversion of the reductive capacity to DOPA equivalents by reference to a standard curve compares very well with the HPLC-detectable DOPA yields. Nearly all of the reductive capacity is accounted for as DOPA.

Incubation of BSA or Insulin with tyrosinase also generates significant TCA precipitable reductive capacity. Yields were some 2-3 times greater with the insulin. Reductive capacity as DOPA equivalents compares very closely with the HPLC DOPA yields of Ito *et al* (1984). This provides good indirect evidence that the protein bound reducing moitieties are DOPA.

Acid hydrolysis followed by HPLC analysis using C_{18} reverse phase chromatography and flurometric detection has shown that gamma irradiated proteins do contain significant amounts of protein bound DOPA (PB-DOPA) which may account for the observed cytochrome c reduction.

This data shows that the obsereved reductive activity on the radical damaged protein is probably PB-DOPA. This suggests that PB-DOPA may have a possible role in the replenishment of reduced transition metal ions involved in metal-dependent free radical generating systems.

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6.21 Antioxidant Activity of Lutein & Related Carotenoids in Vitro

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Lutein is a most common xanthophyll carotenoid in green & yellow vegetables and is found in large amounts in blood. It is specifically associated with lipid in the low density lipoproteins (LDL) & is also the most abundant carotenoid in high density lipoproteins. One of its biological role may therefore be protection of lipids against peroxidation. We have found that Lutein at final concentration of 2μM inhibits $CuCl_2$ induced oxidation of LDL. The extent of inhibition varied between LDL preparations from different individuals. We examined the effect of Lutein on azo initiated peroxidation of methyl linoleic acid by measuring the oxygen uptake. It inhibited the uptake of oxygen at concentrations as low as 2μM. When compared with other carotenoids order of inhibition of oxygen consumption was β-cryptoxanthin› lutein› lycopene› β-carotene. Trolox failed to show any effect in above system at concentrations ‹10μM. Above 10μM it exhibited a lag period when oxidation was fully suppressed. After the lag phase peroxidation proceeded at a rate similar to that of control. No lag period was observed in the presence of carotenoids. They inhibited the peroxidation at a constant rate which remained unchanged after complete bleaching of carotenoid. These results will be discussed in the light of relative importance of carotenoids in protecting lipids from oxidation.

6.22 THE PROTECTIVE POTENTIAL OF INTRA- AND EXTRACELLULAR FREE RADICAL SCAVENGERS

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In free radical based pathologies, the relative importance of intra- and extracellular production and scavenging of free radical intermediates has never been clearly defined. Yet, great efforts are invested in extracellular therapies, notably exogenous superoxide dismutase (SOD) and catalase. Nitroxide radicals, which disproportionate O_2^- and oxidize the reduced metal ions required for •OH formation, are novel therapeutic alternatives. They dramatically protect cells and organs against free radical injury. In this study, Chinese hamster ovary cells were exposed to O_2^- (from hypoxanthine/xanthine oxidase) or H_2O_2. The effects of two nitroxides (TEMPO and CAT1) and SOD, all provided at identical SOD activity, as well as catalase were investigated by monitoring cell survival (colony forming ability), membrane integrity (trypan blue exclusion and lactate dehydrogenase release) and •OH production (dihydroxybenzoate production from salicylate). A free radical challenge which resulted in 90 - 95% mortality did not affect membrane integrity, suggesting that the critical damage induced by the extracellular challenge was intracellular. The addition of SOD did not increase cell survival, whereas catalase protected strongly. Thus, O_2^- itself was not toxic to the cells, and the membrane penetrating species was H_2O_2, rather than HO_2•. TEMPO, which equilibrates freely across membranes, reduced mortality to 20% and the strictly extracellular CAT1 to 60%. The protection was not due to their SOD-mimic activity, since SOD itself did not protect. The nitroxides do not react directly with H_2O_2. Mortality correlated well with the production of •OH, suggesting that the nitroxides protected by decreasing free radical production (probably by metal oxidation), rather than by affecting cell metabolism. A practical implication is that extracellular therapy is useful only if targeted to the appropriate active oxygen species.

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6.23 TOCOPHEROLQUINONE-MEDIATED FORMATION OF OXYGEN RADICALS BY NADPH-CYTOCHROME P-450 RERUCTASE SYSTEM.

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∝-Tocopherolquinone (TQ) found in animal tissues is a metabolite of ∝-tocopherol (Toc). It has been reported that TQ served as an effective antioxidant like Toc in cell cultures, suggesting the presence of TQ reducing system. NADPH-cytochrome P-450 reductase which catalyzes an one-electron reduction of quinones, may be a plausible candidate. We investigated the reduction mechanism of TQ by rat liver NADPH-cytochrome P-450 reductase and microsomes, and observed the oxygen radical formation using the ESR spin trap, DMPO. NADPH oxidase activity of the enzyme was increased by the addition of TQ. During the NADPH-TQ reductase reaction, the ESR spectrum of the spin trap adduct DMPO-OOH changed to a combination of one characteristic of DMPO-OOH and one of DMPO-OH. Typical DMPO-OOH and DMPO-OH spectra were observed in the presence of catalase or SOD, respectively. The formation of DMPO-OH spectra was enhanced by the addition of 100μM H_2O_2 in the presence of SOD. No signal was seen in the presence of both catalase and SOD. It is concluded that H_2O_2 is necessary for the reaction with TQ radicals to form OH· and O_2^{-}· is the precursor of H_2O_2. Superoxide formation was continued when the enzyme was replaced by microsomes.

6.24 SPIN TRAPPING AND CHEMILUMINESCENT STUDY OF FREE RADICALS, GENERATED IN THE REACTION OF FE(II)+HYPOCHLORITE

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The main radical-producing reactions mediated by iron ions are known as follows:

$Fe(II) + O_2$ -----> $Fe(III) + O_2^{-}$· (1)

$Fe(II) + H_2O_2$ -----> $Fe(III) + OH^- + OH$· (2)

$Fe(II) + LOOH$ -----> $Fe(III) + OH^- + LO$· (3)

These reactions have been investigated by means of spin trapping and chemiluminescence. It was found that using PBN spin trap it is possible to detect and identify free radicals generated not only in Fenton's reaction (2), but also in the reactions of lipid peroxidation (3). In living organisms free radicals can also be produced in different reactions with ferrous iron. Hypochlorous acid is an example of such reagents. It was found that interaction of ferrous iron with hypochlorite in the presence of spin trap produces spin adducts with parameters identical to those of hydroxyl radicals, generated in Fenton's reagent. Chemiluminescent measurements showed that interaction of ferrous iron with hypochlorite produces an intensive flash with amplitude proportional both to the concentrations of iron and hypochlorite. Spectral characteristics of this flash were very close to those of chemiluminescent flash in Fenton's reagent. These findings show that interaction of hypochlorite with iron is accompanied by the formation of hydroxyl radicals.

6.25 IDENTIFICATION AND REACTIONS OF THE PROTEIN RADICAL FROM HAEMOGLOBIN

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Reaction of Methaemoglobin (MetHb) with H_2O_2 generates a ferryl [iron(IV)-oxo] species which is one oxidising equivalent above the initial level. The second oxidising equivalent from the peroxide is believed to be rapidly transferred from the haem centre into the globin generating a protein radical. Previous studies have suggested that this radical is centred on an aromatic residue, but this species has not been unambiguously assigned. This system has been reexamined using EPR spectroscopy. Reaction of equimolar MetHb with H_2O_2 using a stopped-flow system gives rise to a transient EPR signal ($t_{½}$ ca. 100 secs); the lifetime, g value, and partially resolved hyperfine couplings are consistent with the formation of a tyrosine phenoxyl radical. Similar signals are observed with EtOOH and other oxidants confirming that this is a globin-derived radical. This radical is not observed if the haem centre is blocked (CN^-, F^-, N_3^-), if the apoprotein is used, if OxyHb is employed, or if the tyrosine residues in MetHb are modified by acetylation. These observations are consistent with electron-transfer from a tyrosine to an initial iron(IV)-oxo porphyrin radical-cation. This phenoxyl radical reacts with a number of oxidants and reducing agents suggesting that it partially exposed on the surface of the protein. Spin trapping studies have demonstrated the formation of a globin-derived radical probably arising from further reaction of the phenoxyl radical.

6.26 POTASSIUM *TERT*-BUTOXIDE-CATALYZED OXYGENATION OF VITAMIN E AND ITS MODEL COMPOUND

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The importance of vitamin E (mainly α-tocopherol) as a biological antioxidant is widely recognized. Its antioxidant mechanisms have, however, been understood only in part. Extensive studies of its oxidation reactions are necessary for the elucidation of vitamin E functions.

We report here that the *tert*-butoxide-catalyzed oxygenation of α-tocopherol and its model compound, 2,2,5,7,8-pentamethylchroman-6-ol, in aprotic solvents under an oxygen atmosphere give three novel types of oxidation products. We determined the structures of these products by ^{1}H- and ^{13}C-NMR, Mass, UV, and IR spectroscopies, and X-ray crystallographic analysis. The oxidation products from α-tocopherol are 5,6,7,8-tetrahydro-5,7-dihydroxy-2,5,7-trimethyl-8-methylene - 2- (4',8',12'- trimethyltridecyl)chroman -6- one, 5,6,7,8-tetrahydro -6,8- dihydroxy -2,6,8- trimethyl - 7- methylene-2-(4',8',12'-trimethyltridecyl)chroman-5-one, and 7,8-dihydro-2,5,7-trimethyl-8-methylene - 2 - (4',8',12' - trimethyltridecyl) - 6 (5*H*) - oxachroman-5,7-carbolactone. The oxidation products corresponding to the above compounds are obtained from the model compound. The structures of these oxidation products are quite unique and very interesting.

We propose possible reaction pathways for the *tert*-butoxide-catalyzed oxygenations of α-tocopherol and its model compound, and discuss the effects of basicity on the oxygenations. These results reveal the mechanism of molecular oxygen trapping by vitamin E under basic conditions and the novel reactivity of vitamin E in its antioxidant function.

6.27 REACTION OF VITAMIN E WITH OZONE

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α-Tocopherol (vitamin E) protects ozone-induced damage in biological systems. In order to see its defense mechanism against ozone, we attempted to investigate details of ozonization of α-tocopherol.

α-Tocopherol and its model compound, 2,2,5,7,8-pentamethyl-chroman-6-ol, were oxidized with ozone in acetonitrile at ambient temperature. While the model compound was completely consumed, a new unique spiro compound was produced. The product was characterized by UV, IR, NMR, and mass spectroscopies and deduced to be 10-acetyl-3,4,7,7,-tetramethyl-2-oxo-1,6-dioxaspiro[4.5]deca-3,9-diene. *RRR*-α-Tocopherol gave rise to a pair of isomeric 10-acetyl-7-(4', 8', 12' - trimethyltridecyl) - 3, 4, 7 - trimethyl - 2 - oxo-1,6-dioxaspiro[4.5]deca-3,9-diene in a combined yield of 35 %. The HPLC monitoring of the reaction revealed that α-tocopherolquinone and its possible precursor, 8a-hydroxytocopherone, in addition to the above spiro compounds, were formed, before α-tocophrol was consumed. These observations indicate that ozone attacks both 5,6-double bond and 8a-carbon atom in the chroman ring. The ozonization at 5,6-double bond may be followed by cleavage of the aromatic ring which may lead to the formation of the spiro compounds. When ozone attacks the 8a-position, α-tocopherolquinone may be formed. Because of the structural uniqueness of the spiro compounds, they might serve as biochemical markers for ozone exposure.

6.28 ANTIOXIDANT ACTIVITIES OF UBIQUINOL HOMOLOGUES IN LIPOSOME

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Antioxidant activity of ubiquinol has received renewed attention. We reported that ubiquinol-10 could scavenge lipid peroxyl radical almost as fast as α-tocopherol (VE) in the bilayer [J Nutr Sci Vitaminol (1990) 36, 505]. Kagan et al. reported that antioxidant activities of ubiquinol homologues (UQ-n) increased with decreasing number of isoprenoid chain length in the oxidation of biomembranes induced with iron/ascorbate or iron/NADPH [Free Rad Biol Med (1990) 9, 117]. The differences were suggested to result from differences in partitioning into membranes, intramembrane mobility, and non-uniform distribution of UQ-n. In order to distinguish these parameters, we have studied the antioxidant activities of UQ-n in the oxidation of soybean PC multilamellar liposome at 37°C in air. When the oxidation was initiated with oil-soluble initiator, a distinct induction period was observed by the addition of UQ-n. The length of induction period decreased with increasing n although the same amount of UQ-n was added. At the end of induction period, all UQ-1 and UQ-3 were consumed but some of UQ-7 and UQ-10 still remained, suggesting UQ-7 and UQ-10 formed their clusters in the bilayer. The plot of rate of oxidation during the induction period against the reciprocal of the UQ-n concentration consumed by the end of induction period gave linear correlations. The ratio of the slopes for UQ-1:UQ-3:UQ-5:UQ-7:UQ-10 was about 1:2:2.5:4:4, suggesting that UQ-1 can trap PC peroxyl radical about 4 times faster than UQ-10 in the bilayer. Furthermore, UQ-10 spared VE as well as UQ-1 in this system. When the oxidation was initiated with water-soluble initiator, stronger effect of the tail was observed on the antioxidant activities of UQ-n. UQ-1 gave a clear induction period, while UQ-10 not. UQ-10 could not spare VE, but UQ-1 did. These data suggest that there is big difference among UQ-n in intermembrane mobility, but not in intramembrane mobility.

6.29 EFFECTS OF CHELATING AGENTS IN THE OXIDATION OF LIPIDS INDUCED BY COPPER AND IRON

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The oxidations of soybean phosphatidylcholine (PC) liposomes or methyl linoleate (MeLH) micelles in aqueous dispersions induced by copper and iron have been studied aiming specifically at elucidating the action of the metal ions in the chain initiation. In micelle system, sodium dodecyl sulfate (SDS) and tetradecyltrimethylammonium bromide (TTAB) were used as anionic and cationic surfactants, respectively, in order to see the effect of electric charge of the micelle surface.

Both cupric and ferric ions induced steady oxidations and the addition of *tert*-butyl hydroperoxide enhanced the rate of oxidation. In both cases of liposome and micelle systems, the rate of oxidation induced by cupric ion was proportional to the first power of substrate concentration and to the half power of both cupric ion and *tert*-butyl hydroperoxide concentrations, suggesting that the oxidation was initiated by the oxygen-centered radicals formed in the decomposition of hydroperoxide by cupric ion.

The effects of such metal chelators as ethylenediaminetetraacetic acid disodium salt (EDTA), nitolirotriacetic acid (NTA), and adenosine-5'-diphosphate disodium salt (ADP) were also studied. EDTA and NTA suppressed the copper-induced oxidations, whereas they enhanced the oxidations induced by iron. ADP had little effect, while penicillamine and triethylenetetramine had accelerating effect for both metal ions. These results were supported by the experiments using a chemiluminescence probe luminol.

ACTION OF ANTIOXIDANTS IN LIPOSOMAL MEMBRANES 6.30

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Chain-breaking antioxidants play a very important role to prevent lipid peroxidation in biomembranes and lipoproteins in our body. However, the action of antioxidants has been studied mostly in homogeneous solution. As a site of reaction between lipid peroxyl radicals and antioxidants, liposomal membrane should be very different from homogeneous solution in terms of mobility and orientation of lipids, and position of antioxidants. Therefore, we have tried to see the effect of antioxidants on the products of lipid peroxidation in liposomal membranes. Linoleic acid was chosen as a substrate since its oxidation mechanism is well-understood in homogeneous solution. Four different hydroperoxides (13-cis, trans-, 13-trans, trans-, 9-trans, cis-, and 9-trans, trans-octadecadienoic acid) are the major products of the oxidation and the ratio of the products gives us useful information on the action of antioxidants. The oxidation of linoleic acid (7.1 mM) incorporated in dimyristoylphosphatidylcholine (14.8 mM) liposome was carried out at 37°C in air in the absence or presence of antioxidants. The four hydroperoxides were analyzed by a reversed phase HPLC after mixing a portion of liposomal suspension with methanol and triphenylphosphine. In the absence of antioxidants, the amounts of hydroperoxides produced were 9-trans, trans- = 13-trans, trans- > 9-trans, cis- = 13-cis, trans-. On the other hand, in the presence of 200 μM of α-tocopherol or 2,2,5,7,8-pentamethyl-6-chromanol, the product distribution changed drastically as 9-trans, cis- > 13-cis, trans- >> 13-trans, trans- ≥ 9-trans, trans-.

6.31 ACUTE RAT TREATMENT WITH DEHYDROEPIANDROSTERONE (DHEA): POSSIBLE ANTIOXIDANT ACTION.

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DHEA is a 17-ketosteroid secreted by the adrenal gland. Studies report that DHEA is able to inhibit the development of tumors in different organs of rat and mice and a long-term administration of the steroid alters the energy utilization in such a way as to lower the body weight. Recently a possible "in vitro" antioxidant action of DHEA has been postulated. The data here reported suggest the "in vivo" antioxidant effect of a single i.p. dose of DHEA (0.23 μmol/ kg), given 17 hrs before the intoxication with CCl_4. The DHEA pretreatment blocks totally the increase of MDA production by liver homogenates from CCl_4 poisoned rats. The serum levels of SDH, GOT and GPT, 24 hrs after CCl_4, indicate that the DHEA pretreatment is able to give a 30-40% reduction of serum release of enzymes if compared with the CCl_4 treated group. More, DHEA pretreatment prevents the decrease of the cytosolic GSH-S-transferase activity induced by CCl_4. Further, the hormone does not interfere with the microsomal monoxygenase system functionality. These results, supported by lipophilic property of DHEA suggest that DHEA protection against CCl_4 toxicity is due to the antioxidant action of the ketosteroid.

EPR STUDIES OF THE REACTION OF H_2O_2 WITH IRON-CONTAINING HEART MITOCHONDRIA F_1 ATP ASE. 6.32

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Metal-catalyzed oxidation of proteins has been implicated in a variety of biological processes, particularly in the marking of proteins for subsequent proteolytic degradation. We have recently presented evidence that purified bovine heart mitochondria F_1 ATPase ligates Fe(III) ions, which appeare not-tightly bound in the protein and redox-active as they mediate the enzyme inactivation by H_2O_2, probably leading to site-directed generation of more deleterious oxygen radical species. In the present study we have used the EPR technique at liquid helium temperature to directly investigate the possibility that protein-derived radicals are involved in the reaction of isolated F_1 ATPase with H_2O_2, at the same time characterizing the Fe(III)-protein adduct. The spectrum of F_1 ATPase which contains Fe(III) in a ratio of 1 - 2 mol/mol exhibits an EPR signal at g 4.3. Reaction of the protein with equimolar concentration of H_2O_2, followed by rapid freezing, gives a radical type signal with line width of 16 G and g 2.03. The intensity of such radical signal drastically decreases upon 2 min incubation at room temperature and disappears after 4 min. Studies are in progress which are investigating the temperature dependence and microwave saturation behaviour of this radical to characterize its nature. Such reactions may be relevant from a physiopathological point of view, as the importance of mitochondria in the cellular homeostasis of iron has been well documented, while the exact chemical nature of mitochondrial "non-heme non-FeS" iron-binding ligands is not known; in addition overproduction of H_2O_2 by heart mitochondria is likely to take place in free radical-associated cardiopathies.

Session 7

Gene Expressions and Oxidative Damage

7.1 REGULATION OF GENE EXPRESSION IN OXIDATIVE STRESS

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Some antioxidant enzymes and some oxidant repair enzymes may be classified as oxidative stress proteins; in other words, oxidative stress can cause the overexpression of the genes which encode such proteins. Exposure to mild oxidative stress initiates a series of adaptive responses that provide increased protection against more severe stress. Such adaptation is accompanied by increased expression of the oxidative stress genes. Inhibitors of RNA synthesis or protein synthesis both prevent the production of new stress proteins and preclude successful adaptive responses: in contrast, inhibitors of DNA synthesis have no effect on short-term adaptation or stress protein synthesis. We have conducted such studies in *Escherichia coli*, mammalian cells (HA1 cells), isolated muscles, and exercising animals.

In *E. coli* we have recently discovered a novel regulatory element (or regulon) which we have called *oxoR*. The *oxoR* gene controls the overexpression of several other stress genes which encode the "late proteins" seen in *E. coli* following adaptation to hydrogen peroxide: expression of the "early proteins" is controlled by a previously known regulon called *oxyR*. In HA1 cells adaptation to H_2O_2 occurs over an 18 hour time course and involves the overexpression of multiple gene products. Intact muscles (studied *in vitro*) also exhibit altered patterns of gene expression following H_2O_2 exposure, and parallel studies involving heat shock reveal only limited cross-induction between the oxidative stress proteins and the heat shock proteins. During exercise (rats) we observe overexpression of oxidative stress proteins, heat shock proteins, and glucose regulated proteins For example, levels of HSP70 and GRP78 mRNA, and HSP70 and GRP78 protein, increase several fold in skeletal muscle, heart, and liver from exercised rats. A possible mechanism linking the regulation of oxidative stress genes and heat shock genes will be discussed.

7.2 OXIDANT-INDUCIBLE EXPRESSION OF THE HUMAN HEME OXYGENASE GENE

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The human heme oxygenase 1 gene is induced as a general response to a variety of stress conditions (including oxidants) in most cell types derived from all mammalian species tested. The gene is transcriptionally activated and analysis of the region of the gene upstream of the mRNA cap site has revealed *cis*-acting regions involved in transcriptional activation and a short DNA element within the promoter which binds to a protein that is active only in extracts from induced cell populations. In many studies, we have used UVA (320-380 nm) radiation as a model agent since it is a natural oxidant which induces several active intermediates. Experiments with various radical scavengers have demonstrated that generation of singlet oxygen by UVA radiation may be a key event that occurs early in the signal transduction pathway leading to transcriptional activation. However, the final result of this stimulation may be more general and involve the strengthening of cellular protection against oxidative reactions involving free iron (see Vile and Tyrrell, abstract, this volume).

7.3 CHARACTERIZATION OF THE soi28 GENE PRODUCT IN *E. coli*

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The *soi28* (superoxide inducible) gene is induced by paraquat under control of *soxRS* in *E.coli* (Kogoma *et al*, 1988). A 8 kb *soi28::lacZ* fusion cloned in a pBR derivative plasmid resulted in a 9 fold paraquat inducibility of β–galatosidase activity in the strain carrying this plasmid. A 1.5 kb deletion between KpnI and SmaI restriction sites resulted in a 2 fold paraquat inducibility. The region responsible for the paraquat response is located in this 1.5 kb fragment. Analysis of the sequence within this region allowed us to identify probable -35, -10 and SD sequences. The region donwstream of Sma1 site was then subcloned and sequenced, by a nested deletion strategy. Sequence analysis reveals strong homology with *K. pneumoniae* gene encoding a pyruvate-flavodoxin oxidoreductase. This gene contains a 3516 bp open-reading frame (Arnold *et al*, 1988). The product of *soi 28* gene has a molecular weight of approximately120 kDa and the estimated open-reading frame is around 3300 bp, which is close to the size of *K. pneumoniae* gene. The main role of the pyruvate-flavodoxin oxidoreductase appears to be the reduction of flavodoxin and ferredoxin. We will discuss the role of pyruvate-flavodoxin oxidoreductase during oxidative stress. We will also discuss the possibility that O_2^- *per se* may not induce the *soxRS* response in *E. coli*.

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7.4 REGULATION OF MANGANESE SUPEROXIDE DISMUTASE EXPRESSION IN NORMAL AND TUMOR CELLS

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Tumor cells are characterized by an altered antioxidant capability, particularly a low Mn-superoxide dismutase activity, but the reason for this peculiarity has not yet been elucidated. We investigated on the regulation of the expression of Mn-superoxide dismutase in rat liver and Morris hepatomas 9618A and 3924A (different growth rate and differentiation degree) and in different normal tissues of the rat. In the experimental tumors we observed a decrease in the activity of the enzyme and in the content of its messenger RNA with respect to liver tissue with both diminutions more pronounced in the fast growing hepatoma. The rat tissues studied showed different enzymatic activities and corresponding different levels of messenger RNA. These observations suggest the existence of a pre-translational mechanism of gene expression. Transition metals are now thought to be main factor in the transcriptional regulation of oxidative-stress inducible genes such as those of SODs protein and some proofs supporting this hypothesis have been obtained in prokariotic cells. We investigated on tumor cells and different rat tissues and the results obtained clearly demonstrate a very good correlation between metal content, message and activity of the Mn-superoxide dismutase, one exception being the heart. For this tissue additional mechanisms could be invoked such as an active mitochondrial e^- transport generating high level of oxy-radical species. Supp. by AIRC

7.5 REGULATION OF CELL GROWTH BY α-TOCOPHEROL

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α-Tocopherol, the major lipid-soluble chain-breaking antioxidant in cells, responsible for the protection against free radical damage, possesses the additional function to retard cell growth. This property is not directly related to its radical scavenging features. Specific inhibition of aorta smooth muscle cell proliferation can be observed at physiological concentrations of α-tocopherol. Endothelial cells, but not Balb-3T3, sarcoma or neuroblastoma cells are equally sensitive. Other water and lipid soluble antioxidants are inactive, including β-tocopherol. Proliferation induced by PDGF or endothelin is inhibited while growth induced by other factors is insensitive to α-tocopherol. The site of cell regulation by α-tocopherol appears to be in the late G_1 phase of the cycle. α-Tocopherol inhibition of VSMC proliferation by α-tocopherol correlates with inhibition of cellular protein kinase C activity, of its translocation, of EGF transmodulation and of the phosphorylation of its specific substrates. The regulation of cell growth by α-tocopherol, may represent a physiological mechanism, relevant to the onset of diseased states such as arteriosclerosis and cancer.

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7.6 GLUTATHIONE-S-TRANSFERASE SUBUNITS IN CELLS OF EPITHELIAL ORIGIN ISOLATED FROM RAT LIVER. CONSTITUTIVE AND INDUCIBLE ISOZYME PROFILE.

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Cytosolic glutathione transferases (GSTs) are a family of multifunctional enzymes involved in phase II metabolism of xenobiotics. In this paper we present a complete analysis of GST subunits in cytosolic fractions obtained directly from hepatocytes and biliary epithelial cells (BEC) isolated from rat liver. This approach, that avoids contamination by GSTs from liver cells of mesenchymal origin, gives the following major information: a) hepatocytes exhibit the complete pattern of subunits belonging to alpha and mu classes but almost undetectable levels of subunit 7 (pi class); b) BEC have a distinctive constitutive pattern comprising high levels of subunit 7 (pi class), subunits 2 (alpha class) as well as 3 and 4 (mu class); c) hepatocytes show a clear induction profile of alpha and mu class subunits after phenobarbital and β-naphthoflavone; induction is much less evident in BEC; d) BEC and hepatocytes show a clear induction of all constitutive subunits after dietary ethoxyquin supplementation with special reference to subunit 7.

7.7 ANALYSIS OF SODA TRANSCRIPTION IN E. COLI USING A SET OF SODA::LACZ OPERON FUSIONS DELETED IN THE PROMOTER REGION.

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The sodA gene of E.coli, coding for the Manganese superoxide dismutase is expressed in response to numerous stimuli. Genetic analysis have shown that sodA is part of the regulons soxRS, soxQ, fur, arc, fnr and that its expression is modulated by the IHF factor. Under oxidative stress conditions SoxRS acts as an activator of sodA expression. SoxQ is also an activator but the inducer stimulus in unknown. Fur associated to Fe^{2+} acts as a repressor in response to intracellular iron concentration. Arc and Fnr repress sodA expression in anaerobiosis.
A sodA::lacZ operon fusion was constructed in vitro and a set of unidirectional deletions upstream the promoter region was obtained. The intact and deleted fusions were transferred on the chromosome of a ΔsodA strain.
Analysis of fusions expression was done in aerobiosis and anaerobiosis, in different genetic backgrounds. It suggests that the effectors SoxS and SoxQ bind in a 40pb region located upstream the -35 promoter box. SoxS and SoxQ activate sodA transcription in an independent way. It indicates that Arc binding site is located downstream the -35 box and confirms that Fur binds to the -35 region. Repression by IHF factor and by Fnr is observed only when repression by Fur is totally relieved. The overlaps of the binding sites of these multiple effectors may indicate alternative regulation depending on the oxidative threat.

7.8 Oxidation of Proteins during Aging.

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Metal-catalyzed oxidation of proteins is implicated in a number of physiologic and pathologic processes, including the aging process. Oxidation is mediated by free-radicals, but the reaction is constrained to specific sites within the protein. This site-specificity is conferred by metal-binding sites normally present in the proteins. These oxidized proteins (1) are more susceptible to proteolytic degradation; (2) contain carbonyl groups in their side chains, providing a marker for the detection and quantitation of modified proteins; and, (3) accumulate during aging, causing decreased specific activity in many key enzymes. In mammalian cells, the multicatalytic proteinase degrades oxidized proteins while sparing their native counterparts. Studies of aging rodent liver and brain demonstrate dramatic decreases in the activity of the multicatalytic proteinase. These results suggest that the regulatory mechanisms which control synthesis of new proteins are able to monitor the presence of a particular protein, but they are unable to assess its functional integrity. The role of the specialized proteinase is to detect modified proteins and degrade them. This will cause a drop in total protein, that is, immunoreactive protein. The drop in protein can be sensed, leading to transcriptional or translational activation and then synthesis of new protein. Thus, proteinases can be intimately involved in gene regulation. The accumulation of oxidized, dysfunctional proteins during aging may therefore be the result of a deficiency of an essential proteinase.

7.9 REGULATION OF HSP72 EXPRESSION FOLLOWING PORPHYRIN PHOTODYNAMIC THERAPY

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Photodynamic therapy (PDT), an experimental cancer treatment modality, utilizes a photosensitizer (Photofrin II) in combination with laser generated light for the site specific erradication of tumor tissue. Singlet molecular oxygen may be the primary agent responsible for the cytotoxicity of PDT. Murine tumor cells were incubated with Photofrin II (25 µg/ml) for 16 hr or 1 hr, localizing the drug in organelle membranes or plasma membranes, respectively. Following drug removal, cultures were irradiated with red light, and then pulse labeled 4-5 hrs post treatment with ^{35}S methionine. The PDT treatments increased the expression of a number of stress proteins, including high molecular weight heat shock proteins (HSP72) as measured by SDS-PAGE and Western immunoblot analysis. Furthermore, mammalian cells treated *in vitro* with PDT developed transient thermotolerance. Gel shift assays using a mouse HSP72 heat shock element (HSE) were positive for specific HSE binding activity in nuclear extracts from murine cells subjected to heat treatment, but negative from cells exposed to PDT. Likewise, an HSP72 promoter-CAT fusion produced heat inducible CAT activity when transfected into K-12 cells but failed to respond to PDT. These results suggest that increases in steady state levels of HSP72 mRNA and protein following Photofrin II mediated PDT may be regulated by post transcriptional mechanisms. The role of *de novo* mRNA synthesis in PDT mediated HSP72 expression will be evaluated using nuclear run off transcription assays.

7.10 REPRESSION OF THE EXPRESSION OF MnSOD IN *E. COLI* BY THE FUR AND ARC REGULATORY PROTEINS : FOOTPRINTING ANALYSIS

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The MnSOD expression is regulated in *E. coli* in response to several environmental stimuli. Both global regulators Fur (ferric uptake regulation) and Arc (aerobic respiration control) control transcriptionally the MnSOD expression (1). DNase I footprinting analysis of the MnSOD promoter with the purified Fur protein and crude extracts containing the overproduced ARcA protein, allowed us to determine the overlapping footprints of the two protiens. The Fur binding to *sodA* promoter is metal dependent; the footprint covers about 50 pb, it overlaps with the -35 promoter region and the two potential Fur consensus sequences ("iron box") of the MnSOD promoter. The ArcA footprint covers a large region (about 65 pb) downstream from the T35 promoter region. Binding of the ArcA protein to DNA is obtained with preparations made in anaerobiosis or aerobiosis as well, although there is no *in vivo* repression of MnSOD by ArcA in aerobiosis (2). Hydroxyl radical footprinting assays showed that the ArcA protein interacts wiht the DNA at only one face of the double helix. Purification of the protein is in progress. The results support the finding made *in vivo*, that one effector alone is enough to repress the MnSOD in anaerobiosis.

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7.11 INTERACTION OF A DNA-BINDING PROTEIN, DISTINCT OF SoxRS, WITH 5'-FLANKING REGION OF 3 PARAQUAT-INDUCIBLE GENES IN *E.coli*.

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Around 40 polypeptides are induced in *E.coli* in response to superoxide generating compounds. The expression of nine genes seems to be under *soxRS* control, among them are *micF*, *nfo*, *rink*, *sodA*, *soi17*, *soi28* and *zwf*. In order to identify a possible concensus superoxide responsive element, the 5'-flanking regions of *micF*, *nfo*, and *zwf* were compared. A region of strong homology was found between 100 to 50 bp upstream to -35. Similar homology was also found upstream of *ndh*, the gene encoding for NADH dehydrogenase II in *E. coli*, although paraquat induction of this gene remains to be clearly established. Gel mobility shift assay were performed with each promotor region to characterize DNA-protein complexes, and to determine whether or not SoxRS was involved in the complex formation. A clear shift was observed due to a protein of approximately 50 kDa. Each 5'-flanking region competes with the others for this DNA-binding factor, suggesting that they all share a consensus sequence recognised by this factor. Genetic analysis demonstrated that this factor was neither SoxRS nor SoxQ. Another complex migrating faster was also identified in SoxRS overproducing strains and could be due to SoxRS. Paraquat treatment of cells provoked the release of the main factor we observed. This suggests that this factor is more likely acting as a repressor. We further observed that generation of superoxide *in vitro* provoked the release of this DNA-binding protein, while hydrogen peroxide was inefficient, thus confirming the *in vivo* effect of paraquat. It would therefore appear that we have identified a second factor regulating the expression of many genes regulated by SoxRS. Foot-printing experiments have been performed to further characterise the binding region. The consensus sequence for this novel regulon, as well its physiological role will be presented.

7.12 INHIBITION OF c-myc EXPRESSION IN HL-60 CELLS INDUCED TO DIFFERENTIATE BY 4-HYDROXYNONENAL, A PRODUCT OF LIPID PEROXIDATION

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Repeated treatments with 1 µM 4-hydroxynonenal (HNE) are able to induce differentiation in HL-60 human leukemic cells. Differentiation process has been estimated by acquisition of phagocytic ability, production of chemiluminescence after soluble or corpuscolate stimulation, changes in enzymatic activities.
Since several studies demonstrated that the induction of differentiation is related to a modulation of c-myc expression, we analized the level of c-myc mRNA at different times after the beginning of HNE treatments. Our results demonstrate a strong and progressive inhibition of c-myc expression during the incubation with HNE. At the end of the treatments and for the following 4-5 hours c-myc mRNA was almost undetectable; then the transcript amount increases progressively and returns to the control levels 24 hours after the beginning of HNE treatments.
These data seem to indicate that HNE, like other differentiation inducers, may act through modulation of c-myc expression.

Session 8

Oxidant and Antioxidant Reactions in Plants

8.1 MICROLOCALIZATION OF SCAVENGING ENZYMES FOR ACTIVE OXYGEN IN CHLOROPLASTS

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Chloroplasts photoproduce superoxide in the autooxidation of the membrane-bound electron carriers in PSI at a rate of 0.2 mM s^{-1}. O_2^- and H_2O_2 are estimated to be lowered to 1 nM and 100 nM, respectively, by SOD and ascorbate peroxidase (APX) assuming their uniform distribution in whole chloroplasts. However, when H_2O_2 was added to chloroplasts through the envelope at only 10 nM s^{-1}, the photosynthetic activity was lost, indicating that chloroplasts can effectively scavenge the active oxygen produced in the thylakoids but not that administered through the envelope. In accordance with these observations, APX occurs in a thylakoid-bound form in addition to that localized in the stroma. Thylakoid-bound APX is enriched in the stroma thylakoids where PSI is localized, and functions as the primary scavenging system. The stroma APX would scavenge the H_2O_2 which is failed to scavenge in the primary system. Molecular properties of the two APXs in chloroplasts and the regeneration systems of ascorbate from monodehydroascorbate radicals in the two scavenging systems are presented.

8.2 A REVIEW OF OXYGEN RADICAL GENERATION DURING THE RESPONSE OF PLANTS TO PATHOGENIC CHALLENGE

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Knowledege of the means by which plant resistance genes are activated to produce the wide range of host responses identified with disease resistance is limited. The most widely held hypothesis suggests that gene- specific products from invading pathogen races to which the host is resistant are recognised at specific sites on the host cell membrane. Recognition at these sites, which are coded for by host resistance genes, then leads to the activation of host responce aimed at inhibiting further growth of the pathogen in the host tissue. Evidence from several laboratories points to significant increases in extracellular production of reactive oxygen species during these host defensive reactions. Initially interest in these oxidants has centred on their potential to act as antimicrobial agents or as a cause of host cells necrosis. More recently it has been suggested that oxygen species may be involved in the signal transduction mechanisms which trigger plant defense responses. Should the production of reactive oxygen species prove to be significant in plant disease in vivo, then obvious parallels exist between mechanisms of pathogen control in plants and the respiratory burst of mammalian phagocytic cells. However, there are some fundamental differences between these systems which must be considered when such comparisons are made.

8.3 DETECTION OF A GLOBIN-DERIVED RADICAL FROM LEGHEMOGLOBIN : POSSIBLE PROTECTIVE EFFECT OF GLUTATHIONE.

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Metleghemoglobin (MetLb), the iron (III) form of the myoglobin-like oxygen carrying protein from the root nodules of leguminous plants, is known to react with H_2O_2 to generate Lb (IV). This intermediate is one oxidising equivalent above the initial level. We have shown that the second oxidising equivalent from the peroxide is rapidly transferred into the surrounding protein generating a protein radical in a reaction analogous to that observed with metmyoglobin ; this species has been detected by ESR spectroscopy. It appears to be formed by electron-transfer within the protein rather than by the generation and subsequent reaction of hydroxyl radical. The ESR signal of this species is consistent with the formation of a tyrosine-derived phenoxyl radical.
Similar reaction systems containing intact metLb have been shown to stimulate lipid peroxidation in the peribacteroid membrane which surrounds the microsymbiont in functioning nodules. This stimulation has been shown however not to be mediated by the Lb (IV) ferryl species ; it is therefore suggested that this may be occuring via a reaction of the protein radical. Such a process is consistent with the inhibition of both radical formation and membrane peroxidation by salicylate and thiourea.
These processes can also be ameliorated by the presence of glutathione (GSH), which is present at high concentrations *in vivo*. This compound has been shown to reduce Lb(IV) to metLb, with the concomitant oxidation of GSH. This process is comparable to the previously described pseudoperoxidatic activity of Lb. Furthermore, GSH has been shown to significantly inhibit the formation of the globin-derived radical in metLb treated with H_2O_2. These reactions indicate that this hydrophilic molecule can interact with both the protein radical and the heme group ; the latter process is probably favoured by the large, open, heme pocket of Lb. These processes may constitute a protective mechanism for the nitrogen-fixing system in legume root nodules.

8.4 METHYL VIOLOGEN - INDUCED FORMATION OF FREE RADICALS IN CHLOROPLASTS

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The generation of free radicals in chloroplasts of wheat and lettuce was investigated under various conditions in the presence of methyl viologen (MV^{2+}) by means of electron spin resonance and spin trapping using 5,5-dimethyl-1-pyrroline-1-oxide (DMPO). An increase in the formation of different radical species, including oxygen free radicals, was observed depending on the concentration of MV^{2+}, the light intensity and the amount of chloroplasts. The monocation radical of MV^{2+} ($MV^{\bullet +}$) accumulated in the light under conditions of exhaustion of oxygen and vanished in the dark. The disappearance of this ESR signal was strongly dependent on the amount of chloroplasts both for wheat and lettuce, indicating the transfer of electrons from $MV^{\bullet +}$ to acceptors within the chloroplasts. NADPH stimulated and $NADP^+$ decreased the formation of reactive oxygen species. The addition of the NADPH-specific enzyme glutathione reductase diminished the DMPO-OH signal both in the absence and in the presence of MV^{2+}. This is explained by the restoration of the $NADP^+$-pool by the action of the enzyme.

8.5 EFFECTS OF 7,8-DIHYDROXYFLAVONE ON GLUTATHIONE DEPLETION AND LIPID PEROXIDATION IN BROMOBENZENE INTOXICATION

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7,8-Dihydroxyflavone is a phenolic compound exerting antioxidant effects *in vitro* and able to protect isolated hepatocytes against xenobiotic toxicity (1). We have investigated the *in vivo* anti-hepatotoxic effects of this flavonoid in bromobenzene treated mice (2). Test compounds were administered p.o. 4 hr after bromobenzene (9 mmoles/kg). Blood samples were taken and animals were killed 17 hr later. Bromobenzene-induced toxicity was counteracted by the flavonoid in a dose-related manner with a better potency than silymarin and it was able at the highest dose tested (200 mg/kg) to bring serum glutamate-pyruvate transaminase levels close to those measured in non-intoxicated animals. Only those mice treated with silymarin (400 mg/kg) or 7,8-dihydroxyflavone (200 mg/kg) showed a significant reduction in the values of thiobarbituric acid reactive substances. This dose of the latter flavonoid nearly abolished lipid peroxidation. Bromobenzene induced a depletion in both reduced and total glutahione levels by 8 and 5 fold, respectively. In silymarin-treated mice, only those receiving the highest dose presented significant increases in both parameters, while for 7,8-dihydroxyflavone there was a dose-related increase in reduced glutathione (9.2 ± 1.9 nmoles/mg protein at 200 mg/kg, against 3.7 ± 0.5 in bromobenzene control and 30.1 ± 1.8 in non-intoxicated control). The inhibition of bromobenzene-induced toxicity by 7,8-dihydroxyflavone is likely related to its effect on glutathione levels as well as to a direct action as antioxidant, affording protection against oxidative stress.

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8.6 FREE RADICAL SCAVENGING, ANTIOXIDANT AND ANTI-HEPATOTOXIC ACTIVITY OF SILIPIDE

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The Sylibum Marianum flavanolignans are considered for therapeutical approach of human liver diseases. We report here on the hepatoprotective effects of the silybin-phosphatidilcholine complex Silipide (IdB 1016). In liver microsomes prepared from rats treated intragastrically with Silipide, the MDA production induced by NADPH, CCl_4 and cumene hydroperoxide as well as the ESR signals detected after addition of ADP/iron, methyl hydrazine and CCl_4 were significantly decreased compared to controls. We have also evaluated whether the antioxidant and free radicals scavenging properties of Silipide were associated with a protective action against oxidative injury to the liver. Lipid peroxidation and cell damage induced in isolated hepatocytes by cumene hydroperoxide, allyl alcohol and $CBrCl_3$ were partly or completely prevented following both *in vivo* or *in vitro* administration of Silipide without interfering with the mechanisms of toxicity of these compounds. This confirmed that the antioxidant and free radical scavenging capability of Silipide can counteract the hepatotoxic effect of different compounds and indicated the potential therapeutical uselfulness of Silipide.

8.7 DECREASE OF OXYRADICALS-RELATED ENZYMES ACTIVITIES IN PEARS DURING STORAGE OF THE FRUITS AT DIFFERENT TEMPERATURES

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The formation of oxygen radicals mediated reactions during fruit repening and storage is an event involved in the deterioration of the fruits.
We studied the behaviour of catalase (CAT), glutathione peroxidase (GPD) and superoxide dismutase (SOD) activities in mature pears stored at 0°C and 21°C for 7 and 14 days. In comparison to the fresh fruits, the more accentuated reduction of all enzymatic activities was observed after 7 and 14 days of storage at 21°C, respectively. Also at 0°C there was a reduction of SOD and GPD activities after 7 and 14 days of storage while less evident was the decrement of CAT activity; moreover in the pears after the 14 days of storage at both temperatures there was a significant increase in the level of TBARS. These data confirm that the formation of oxygen dependent damages are probably caused by a reduction of the enzymatic oxyradicals-scavenger activities.

8.8 ANTI-OXIDANT ACTIVITY OF CHINESE HERBS

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The anti-oxidant activity of 9 compounds isolated from Schisandraceae, a traditional Chinese tonic, were studied in vitro and vivo. 7 out of 9 dibenzocyclooctene lignans were shown to significantly inhibit lipid peroxidation of liver microsomes induced by iron/cysteine and Vit C/NADPH as well as the generation of superoxide anion in xanthine oxidase system. The lignans also inhibited lipid peroxidation of freshly isolated rat hepatocytes. Scanning electromicrograph showed that the damages of the surfaces of hepatocytes were markedly protected. In addition, the hemolysis of rat erythrocytes, depletion of -SH group and formation of high molecular aggregate of ghost membrane were all counteracted. Of the 7 effective lignans, schisanhenol (Sal) was the most active and even more than vitamin E. Sal was further found to antagonize adriamycin-induced rat heart mitochondria injury. But Sal has no influence on the anti-tumor activity of adriamycin. Sal and severa other lignans have the ability to scavenge oxygen free radicals detected by ESR.

8.9 FLAVONOIDS ISOLATED FROM *CLERODENDRUM INDICUM* L. AS INHIBITORS OF LIPID PEROXIDATION

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The leaves of *Clerodendrum Indicum* L. are traditionally used in Indian folk medicine against inflammatory disorders. Since the species of the same genus are rich in flavonoids, phenolic compounds endowed with anti-inflammatory and antioxidant properties, we have considered of interest to study the influence of flavonoids isolated from the above species on microsomal lipid peroxidation. Lipid peroxidation was induced in rat liver microsomes by $FeSO_4$ /ascorbate. Products of peroxidation were measured by the thiobarbituric acid method, using 1,1,3,3,-tetramethoxypropane as external standard. All compounds were active at the initial screening concentration of 100 μM and they were tested further at a range of concentrations to calculate the half maximal inhibitory concentration (IC_{50}). Some other commercially available flavonoids have also been tested to permit the establishment of structure-activity relationships. Nepetin was the most potent compound with IC_{50} =7 μM, which is in the range of synthetic antioxidants such as propyl gallate. Hispidulin, scutellarein and scutellarein-7-*O*-glucuronide showed IC_{50} between 40 and 60 μM. All the active compounds were polihydroxylated at positions 5 and 7, with one or more hydroxyl groups in ring B. As observed in previous work, the presence of a catechol group in ring B increases the activity (1).

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8.10 INHIBITION OF LIPID PEROXIDATION BY LAUDANOSOLINE AND OTHER BENZYLISOQUINOLINE ALKALOIDS

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It has been demonstrated that some biscoclaurine alkaloids behave as superoxide ion scavengers in free radical generation by polymorphonuclear leukocytes (1). Besides, other types of benzylisoquinoline alkalaloids can inhibit microsomal lipid peroxidation induced by several methods and superoxide generation in a non-enzymic system (2). Thus, we have studied further the antioxidant and free radical scavenging properties of benzylisoquinoline alkaloids. Lipid peroxidation was induced by $FeSO_4$/ascorbate in rat liver microsomes and the products of peroxidation were estimated as thiobarbituric acid-reactive substances. The evolution of the peroxidative process with time and the influence of the tested compounds were also studied. The superoxide scavenging ability of compounds was tested using the phenazine methosulfate-NADH-initiated nitroblue tetrazolium reduction. The simple benzylisoquinoline laudanosoline, was the most potent inhibitor on both test, it behaved as a superoxide scavenger with IC_{50}=40μ M and as an inhibitor of microsomal lipid peroxidation with IC_{50}=7 μM. The structurally related compound protopapaverine, exerted lower effects. All the aporphines assayed showed antiperoxidative effects with IC_{50} in the range of 20 μM. In this respect apomorphine was the most active compound, with a behaviour similar to laudanosoline. Nevertheles they showed a very weak scavenging action. The main structural feature of active compounds is the presence of phenolic hydroxyl groups .

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8.11 A COMPARISON OF THE ANTI-HEPATOTOXIC ACTIVITY OF GOSSYPETIN DERIVATIVES IN MICE

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Since flavonoids are known to possess free radical scavenging properties as well as they inhibit drug-induced lipid peroxidation and show a membrane-stabilizing action, we have investigated the effect of different flavonoids, gossypetin, its glucoside gossypin and its glucuronide hibifolin, on the enhanced lipid peroxidation following glutathione depletion induced by bromobenzene in mice (1). Animals were starved overnight and bromobenzene was administered intragastrically at the dose of 9 mmol/kg. Control mice received vehicle alone. Flavonoids were given orally 4 hr after bromobenzene poisoning at doses between 150 and 50 mg/kg. Liver damage was assessed by measuring serum glutamate-pyruvate transaminase (SGPT) levels. Lipid peroxidation was estimated in liver homogenates as tissue content of malondialdehyde. Marked differences were seen among these 3 flavonoids concerning their inhibitory action. The treatment of mice with gossypin after bromobenzene-intoxication completely prevented liver necrosis in the animals sacrificed 17 hr after poisoning (97% inhibition of SGPT level at the dose of 50 mg/kg) and it caused an inhibition of lipid peroxidation over 50% at the dose of 150 mg/kg. However, hibifolin did not show any anti-hepatotoxic effect when it was administered orally after bromobenzene intoxication. Based in these results it seems that the introduction of glucuronic acid highly decreases the protective activity on liver damage as we observed previously when comparing their anti-inflammatory properties (2).

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8.12 FLAVONOIDS AS INHIBITORS OF CYTOCHROME P-450 ACTIVITY IN RAT MICROSOMES.

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Flavonoids are natural compounds with a wide range of biological activities.They are present in the diet and can exert a modulating role in the metabolism of xenobiotics acting as inducers or inhibitors of cytochrome P-450 mediated reactions (1), an enzymatic system of great interest in the bioactivation of different toxins and carcinogens. Flavonoids can inhibit lipid peroxidation (2), which has been related to their free radical scavenging or chelating properties. On the other hand, their interference with cytochrome P-450 activity can also contribute to this action. We have used rat liver microsomes to test a series of flavonoids on aminopyrine demethylase activity. The IC_{50} of the most potent compounds were: flavones chrysin (40μM) and luteolin (64μM); biflavone amentoflavone (42μM) and flavanone eriodictyol (85μM). We have also established some structure-activity relationships. It comfirms the importance of 5,7 -dihydroxylation in ring A; flavones were more active than their respective flavonols; O-glycosylation abolishes the activity. Flavonoids inhibit CCl_4-induced lipid peroxidation (2), which is related to free radical generation due to its bioactivation by the NADPH-cytochrome P-450 system. Our results suggest that flavonoids effects can be related to the inhibition of cytochrome P-450 activity.

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8.13 INTERACTION OF THE NEW SILYBIN COMPLEX IdB 1016 WITH ETHANOL-DERIVED FREE RADICALS.

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Ethanol metabolism by CYP2E1 produces free radical intermediates, identified as hydroxyethyl radicals. We have observed that the oral pretreatment of rats with IdB 1016 (Silipide), a new 1:1 complex of silybin with phosphatidyl-choline, is able to decrease by about 40% the spin trapping of hydroxyethyl radicals in microsomes from both control and chronic ethanol-fed rats. This effect is not due to an interference with cytochrome CYP2E1 functions or with the metabolism of ethanol by CYP2E1, but is rather related to the capacity of silybin molecule to scavenge hydroxyethyl radicals. Further experiments *in vivo* have shown that IdB 1016 administration is also effective in decreasing the amounts of hydroxyethyl radicals detectable in the bile of rats acutely treated with ethanol. Such an effect is, however, lost when pure silybin in amounts comparable to those present in IdB 1016 is administered instead, probably because of the low bioavailability of the uncomplexed flavonoid. The ability of IdB 1016 to scavenge ethanol-derived radicals along with its antioxidant activity makes this drug a potentialy usefull agent to counteract the oxidative damages caused by ethanol.

Session 9

Ischemia-Reperfusion

9.1 CELLULAR CHANGES LEADING TO CELL DEATH FROM HYPOXIA AND OXIDATIVE STRESS.

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Acute hypoxic and toxic injury initiates a sequence of cellular events culminating in cell death. In hepatocytes, one of the earliest structural changes which can be observed is the formation of cell surface blebs. These blebs, which seem to represent the detachment of the plasma membrane from the underlying cortical cytoskeleton, grow and coalesce until 1 to a few large terminal blebs remain. Cell death is precipitated by rupture of a terminal bleb with loss of cytosolic enzymes and metabolic intermediates and collapse of all ion and electrical gradients across the plasma membrane. In hypoxic hepatocytes and neonatal myocytes, plasma membrane breakdown also marks the transition from reversible to irreversible injury. Inhibition of mitochondrial ATP production is a common feature of both hypoxia and oxidative stress. In both injuries, mitochondrial membrane potential is preserved for 15-40 minutes, but then is abruptly lost, possibly because of a mitochondrial permeability transition. Following mitochondrial depolarization is breakdown of lysosomes. The latter event is closely followed by plasma membrane lysis and loss of cell viability. Acidotic intracellular pH retards the onset of cell death during hypoxia and oxidative stress, an event associated with suppression of phospholipase activity but not with prevention of bleb formation. During reperfusion of ischemic cells, pH rises sharply and precipitates lethal injury independently of reoxygenation. This 'pH paradox' may be a mayor causative factor in reperfusion injury to heart, liver and other organs.

9.2 OXIDATIVE STRESS DURING ISCHAEMIA/ANOXIA AND RE-OXYGENATION: SIMILARITIES AND DIVERGENCIES DEPENDING ON THE EXPERIMENTAL MODEL

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Oxygen deficiency and reoxygenation initiate dramatic perturbation in energy metabolism, membrane constituents, ionic composition and they initiate or are accompanied by oxygen activation. The cause and sequence of such variations are highly important with respect to basic principles of metabolic regulation and cellular integrity as well as to medical implications in diagnostics and therapy.

Rat liver, hepatocytes and small intestine are susceptible against oxidative stress. The response of these systems on anoxia/ischaemia and reoxygenation will be discussed with respect to

- Xanthine oxidase mediated purine breakdown and superoxide radical generation
- Lipid peroxidation and metabolism of aldehydes
- Glutathione system and intracellular calcium
- Protective actions of allopurinol, oxipurinol and superoxide dismutase.

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9.3 INVOLVEMENT OF FREE RADICALS IN THE REPERFUSION INJURY: PREVENTION BY INFUSION OF SPIN TRAPS.

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The causal role of free radical generation during ischemia and/or reperfusion was evaluated by observing the effects of spin traps, because of their selective anti-radical properties. α-Phenyl-t-butyl nitrone (PBN) was used for the following reasons: a) it reacts with many oxygen- and carbon-centered radicals; b) it was shown to be devoid of cardiotoxic effects up to 2.5 mM in dogs and up to 5 mM in rats; c) it is taken up by myocardial cells and partitions to subcellular organelles including mitochondria, which is a major site of radical production. Langendorff isolated rat hearts were subjected to low flow ischemia (reduction of the individual coronary flow to 10%; perfusion solution N_2-saturated); after 1 hr ischemia oxygenated solution was infused at the normal flow rate. During ischemia the contractility (RPP) fell to near zero, phosphocreatine (PCr) to 35%, ATP to 50%; creatin-phosphokinase (CPK) rose 7 fold and Pi 1.5 fold; pH was not modified. During reperfusion, all parameters recovered in part: PBN developed a marked protective activity on all tested parameters which gained a nearly normal value. The present investigation confirms that radicals play a significant role in the reperfusion injury. The protection exerted by PBN might be related to the continuous although reduced perfusion of the spin trap during the ischemic period, leading to a higher level of available drug in the time window critical for free radical production. This conclusion is supported by the comparison of the effects exerted by PBN in case of 30 min of total ischemia.

9.4 *IN VIVO* ESR STUDIES ON RADICAL REACTION DURING FEMORAL ISCHEMIA-REPERFUSION INJURY OF MICE

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Previously, we measured nitroxide radicals after administered into femoral muscle of mice using *in vivo* ESR, and demonstrated the diffusion and reduction of radicals in muscle. These phenomena also depended on ischemia-reperfusion injury of femoral muscle in mice. Spin labeled compound, amino-TEMPO, was injected into femoral muscle of mice after occlusion and before reperfusion and found that the spin clearance increased by ischemia-reperfusion injury. In the present paper, we investigated the effect of SOD on the radical reaction during ischemia-reperfusion injury.

Female ddY mice (18-25g) were anesthetized with the injection of pentobarbital (130mg/kg). Ischemia was prepared by tieing thigh with a thread for 20min, and then followed with reperfusion. Amino-TEMPO was dissolved in isotonic buffer (10mM sodium phosphate, pH 7.4) at 10mM concentration. SOD was dissolved in the solution containing spin labeled compound. 50μl of amino-TEMPO solution with or without SOD were administered to the left femoral muscle of mice and ESR spectrum was successively observed with *in vivo* ESR spectroscopy. After that, the right femoral muscle of the same mice was occluded. One minute before reperfusion, amino-TEMPO solution with and without SOD was administered to right muscle, and ESR spectrum was observed after reperfusion.

ESR spectrum of 10mM amino-TEMPO in muscle consisted of sharp triplet lines. The signal decreased obeying first order kinetics, and the rate constant was calculated from the decay curve. The ratio of the clearance constant at right thigh to those at left one was used to estimate the effect of SOD on the radical reaction during ischemia-reperfusion. Occlusion and reperfusion treatment of the right thigh increased the ratio, indicating that ischemia-reperfusion injury enhanced the radical reduction. The ratio of without SOD was significantly smaller the mouse than that with SOD, indicating the contribution of O_2^- to nitroxide reduction in muscle.

9.5 KINETICS OF ACTIVATED OXYGEN PRODUCTION AND LIPID PEROXIDATION IN ISCHEMIA AND REPERFUSION

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Reactive oxygen formation and lipid peroxidation has been suggested to contribute to brain injury associated with ischemia and reperfusion. In this study lucigenin-enhanced chemiluminescence was used to evaluate oxygen free radical production. Conjugated dienes, lipid hydroperoxides, thiobarbituric acid-reactive material and fluorescence products were measured to estimate lipid peroxidation during ischemia and reperfusion of rat brain tissue in vitro. Chemiluminescence markedly increased to a peak during the first several minutes of reperfusion followed by a slow decline to a steady state level. Light emission was inhibited by SOD, thiols (GSH, D-penicillamine), flavonoids (quercetin, rutin, catechin), but not by iron chelators. Statistically significant increase of lipid peroxidation products was observed not earlier than 20 min after the onset of reperfusion. During this period, decrease of GSH content and of glutathione peroxidase and glutathione reductase activities was observed. The lag period was shortened by $FeCl_3$ or by additional source of superoxide. Reperfusion-induced lipid peroxidation was inhibited by chain breakers (butylated hydroxytoluene, alpha tocopherol), iron chelators, thiols, flavonoids but not by catalase, $HO^{\cdot}$ and $^1O_2$ scavengers. SOD increased the lag phase duration by a concentration-dependent manner. Based on these results the following sequence of events has been proposed to take place in reperfused tissue: almost immediately after the onset of reperfusion drastically increases the production of oxygen-derived free radicals. These species damage some of the cell protecting systems and decrease GSH level. They seem to cause liberation of iron from its stores which plays the key role in the initiation of peroxidation.

PLASMA Cu-Zn SUPEROXIDE DISMUTASE CONCENTRATIONS AFTER ACUTE MYOCARDIAL INFARCTION 9.6

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The level of Cu-Zn superoxide dismutase (SOD) was measured in patients with acute myocardial infarction (AMI;n=5) or with AMI who underwent thrombolysis treatment (AMIT;n=9). The Cu-Zn SOD was quantified by ELISA using 2 different monoclonal antibodies against SOD.

Cu-Zn SOD (μg/l)			
	4-6 hrs	24hrs	7 days
AMI	64±9	70±20	47±27
AMIT	113±119	69±31	40±19

In comparison to control subjects (Cu-Zn SOD = 15.7 μg/l n=9), the patients with AMI showed high levels of plasma Cu-Zn SOD which remained elevated in the following 7 days. The thrombolysis treatment enhanced the plasma Cu-Zn SOD level in the first 4-6 hrs after myocardial infarction, but no differences were found between the group up to 7 days after infarction.
These results suggest that release of Cu-Zn SOD from injured cardiac muscle decreases the $O_2^{\cdot}$ - scavenger properties of the heart.
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9.7 SOURCES OF OXYGEN RADICAL GENERATION AFTER INTESTINAL REPERFUSION

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Haemorrhagic mucosal injury after intestinal ischemia is mediated by oxygen radicals (OR). The enzyme xanthine oxidase is believed to be the primary source of these reactive oxygen metabolites. The second potential source of oxygen radicals are activated neutrophils. Aim of these experiments was to quantitate the amount of OR after reperfusion and the source which is responsible for the enhanced OR formation. Cats were subjected to 60' intestinal ischemia and 60' reperfusion. We measured conjugated dienes (CD) and myeloperoxidase (MPO). Radical formation was measured directly with the reduced spin label OXANOH, which is capable to react with OR. 5 cats were treated before ischemia with a monoclonal antibody (IB4). This antibody prevents the adhesion and subsequent activation of neutrophils. 5 cats were injected allopurinol to inhibit the xanthine oxidase. Untreated cats showed a significant increase of CD, MPO and enhanced generation of OR. IB4 and allopurinol treated cats showed no significant elevation of MPO or CD after reperfusion. The formation of OR in IB4 treated cats after reperfusion is diminished to 54%. Allopurinol effects a further (33%), but not a complete reduction of OR formation. So a third unknown source of OR seems to exist. This source doesn't lead to lipid peroxidation.

EFFECTS OF VARIOUS ARTERIAL CLAMPING PERIOD ON O_2 PRODUCTION AND ARTERIAL SMOOTH MUSCLE CONTRACTION 9.8

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It is well known that during the ischemia reperfusion phenomenon free radicals are produced and they damage the arterial wall.
Our experiments were performed on dogs for elucidate the effect of various arterial clamping period on adrenalin induced arterial smooth muscle (arteria femoralis) contraction. After various clamping period (from 15 min to 120 min) we measured the O_2 production by Cytochrom C reduction (from 1 min to 4 days). Following we sacrifized the dogs and we performed on parallel arterial samples (clamped and non clamped) the contraction studies by using various concentrations of adrenalin (10^{-5}M -10^{-8}M). During reperfusion the O_2 production significantly increased during the first 30 min, later normalized and we observed a second peak during the 2nd and 3rd days of measurement. The adrenalin induced contraction differed markedly between the two arterial samples. The difference in the contractile activity was demonstrable after the 60th min of clamping and increased linearly with the time. The concentration of 10^{-8}M adrenalin was ineffective. After our results we can say that the period of arterial clamping significantly influence the arterial contractility. Moreover the developement of thrombosis in the clamped arterial segment is largely increased. The period of clamping should be as short as possible and the harmful effects of free radicals reduced.

9.9 HISTOCHEMICAL STUDY ON THE EFFECTS OF ISCHEMIA ON XANTHINE OXIDASE ACTIVITY IN RAT INTESTINE AND LIVER

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A histochemical method was applied to demonstrate the O-form of xanthine oxidoreductase activity in rat intestine and liver using unfixed cryostat sections and a semipermeable membrane. The gelled incubation medium contained hypoxanthine as substrate, cerium ions which capture the enzyme product, hydrogen peroxide, and sodium azide to inhibit catalase and peroxidase activities. In a second step reaction diaminobenzidine was polymerized in the presence of cobalt ions by decomposition of cerium perhydroxide. Large amounts of final reaction product were found in the cytoplasm of enterocytes and goblet cells of control small intestine. A very low xanthine oxidase activity was found in rat liver. Highest activity was present in endothelial cells, whereas in liver parenchymal cells, a more pronounced activity was found in pericentral than in periportal hepatocytes. No reaction product was generated when sections were incubated in the absence of substrate or in the presence of substrate and allopurinol, a specific inhibitor of xanthine oxidase activity. After storage of tissue blocks for 2 h at 37°C enzyme activity was significantly reduced in the apical region of epithelial cells in small intestine, whereas a high activity was present in the basal region of these cells. This period of ischemia did not affect the enzyme activity in liver. It was concluded that intracellular conversion of xanthine dehydrogenase into xanthine oxidase during ischemia may not be responsible for cell damage during reperfusion.

9.10 SUPPLEMENTATION WITH α-TOCOPHEROL AND ß-CAROTENE IMPROVED THE ELEVATED SENSITITIVITY OF THE MYOCARDIUM TO ISCHEMIA/REPERFUSION INJURY IN SMOKED-EXPOSED RATS

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Previous results from our laboratory showed that rats subjected to a small concentration of cigarette smoke had an elevated sensitivity of the myocardium to ischaemia/reperfusion. The aim of the study was to determine whether supplementation of anti-oxidant vitamins (α-tocopherol and ß-carotene) inhibited at least some of the damage induced during ischaemia/reperfusion in smoke-exposed rats. Mitochondrial function, LMWI and α-tocopherol were measured. Rats were subjected to low concentrations of cigarette smoke twice a day. The one group received drinking water supplemented with α-tocopherol and ß-carotene during the 2 months of smoke-exposure. The supplemented group showed significant less mitochondrial function impairment compared to controls. The LMWI content was not significantly lower in the supplemented group but the α-tocopherol content was much higher. Supplementation with antioxidant vitamins to smoke exposed rats partly protected against ischaemia/reperfusion injury.

9.11 PROTECTIVE EFFECT OF PYRUVATE ON RAT HEART MITOCHONDRIAL GLUTATHIONE DURING ISCHEMIA AND REPERFUSION.

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Hypoxia or ischemia followed by reperfusion determines a large release of glutathione from isolated/perfused rat heart. The effects of ischemia/reperfusion or hypoxia/reperfusion on the release of cytosolic and mitochondrial glutathione were compared. During ischemia, mitochondrial glutathione is released from the mitochondrion into the cytosol and, in turn, into the extracellular environment. Reperfusion causes a large release of total glutathione, while the mitochondrial pool is only slightly decreased. After hypoxia (without glucose) glutathione is mainly lost from the cytosol, while the mitochondrial pool is preserved and is not modified by the subsequent reperfusion. Pyruvate, which protects the heart by inducing a complete recovery of the contractile force after ischemia, largely prevents the loss of glutathione, particularly from the mitochondrial compartment. The effects of pyruvate are discussed and some of its actions such as hydrogen peroxide breakdown, production of ATP during ischemia and diminution of the intracellular concentration of inorganic phosphate, are examined.

9.12 FRUCTOSE-1, 6-BISPHOSPHATE PREVENTS OXIDATIVE STRESS IN ISOLATED AND PERFUSED RAT HEART.

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Fructose-1,6-bisphosphate (FDP) is an intermediate of the glycolytic process also utilized as a cardioprotective drug. In isolated rat heart, perfused with the Langendorff technique at constant flow, FDP (0.05 to 0.5 mM) markedly increases the contractile force. The infusion of hydrogen peroxide (0.1 mM) during normoxic perfusion causes serious alterations in the contractile capacity reflected by an increase in the resting tension, leading, in about one hour, to a complete arrest of the functions in hypercontraction (stone heart). The inclusion of 0.2 mM FDP in the perfusion medium relieves the toxic effect of hydrogen peroxide observed as duration of the contractile activity which is increased of about fifty per cent. During ischemia/reperfusion and treatment with oxidizing agents such as diamide or hydrogen peroxide there is a large loss of glutathione from the heart; the presence of FDP in the perfusion medium prevents the loss of glutathione from the mitochondrial compartment. The effect of FDP in terms of a direct or indirect (through its transformation into other metabolites) intervention in counteracting the oxidative stress is discussed.

9.13 INHIBITION OF PROTEIN SYNTHESIS IN HEPATOCYTES DURING HYPOXIA: AND EARLY CYTOTOXIC EVENT OR AN ADAPTATIVE CELLULAR RESPONSE?

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Many energy-dependent functions, such as biosynthetic activity or ionic homeostasis are modified when cells or tissues become hypoxic, thus leading in some cases to cellular death. However, it remains to be clarified which of the changes induced by hypoxia are not related to cell death. By using isolated rat hepatocytes as experimental model, the biochemical effects of hypoxia were studied in cells incubated either in normoxia or in hypoxia. Our main findings are: 1) protein synthesis is totally and immediately stopped, while intracellular ATP content decreases progressively when cells are incubated in hypoxia; 2) hypoxic cells are more sensitive to H_2O_2-mediated injury; and 3) addition of fructose protects against hypoxic cell injury. Due to the rapidity of the changes in protein synthesis, it appears that cells respond to oxygen limitation rather than ATP depletion. The rationale of such a mechanism is to lead cells in a state of 'metabolic arrest', thus keeping ATP for more critical cellular functions. Such a hypothesis implies the existence of an 'oxygen sensor' indicating cells whether to stop or to reinitiate their biosynthetic activities.

(*) V. Lefebvre is Research Assistant of the National Fund for Scientific Research (Belgium).

9.14 ARGIMESNA ANTIOXIDANT PROPERTIES IN RABBIT MYOCARDIUM

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Free oxigen radicals are considered playing an important role on myocardial ischaemia and reperfusion damage.
Argimesna (AR), as thiol cofactor, was evaluated as protective agent against ischaemia and reperfusion damage in isolated and perfused Langendorff rabbit hearts. The damage was evaluated as mechanical function, CPK release, isolated mitochondrial function and tissue glutathione and protein-SH contents.
AR 10^{-6} M was delivered before (60 min, 22 ml/min) and during (60 min, 1 ml/min) ischaemia and reperfusion (30 min, 22 ml/min).
Results : in aereobic perfusion, AR did not produce any significant alteration of systolic and diastolic pressure. Versus control hearts, before and during ischaemia and reperfusion, AR delaied and reduced the rate of diastolic pressure on reperfusion (35.1 vs 68.15 mmHg; $p < 0.01$) and increased the recovery of systolic pressure (48.0 vs 23.7 mmHg), reduced CPK release ($p < 0.05$) and tissue ATP and CP ($p<0.01$) as also tissue SH content (glutathione and SH-proteins) depauperation..
Our data indicate that AR 10^{-6} M has a protective effect against ischaemia and reperfusion damage : in our opinion, AR could work as reduced equivalent donor able to reduce the oxygen toxicity.

9.15 Active oxygen species production and vitamin E levels in human articular chondrocytes.

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We investigated the ability of human chondrocytes to produce active oxygen species, hydrogen peroxide (H_2O_2) and hydroxyl radical (°OH), and the effects of cytokines (Il1α and Il1β at 50 U/ml) on this production. We were also interested in the intracellular levels of vitamin E (vit E) as a major liposoluble antioxidant. Human chondrocytes were isolated from knee articular chondrocytes by collagenase digestion and cells were maintained in a serum free culture medium for variable periods. H_2O_2 production was assayed by a direct fluorimetric technique (oxidation of diacetyldichlorofluorescin in the presence of a peroxidase). Unstimulated cells (1.10^6) produced around 10^{-8}moles H_2O_2 and this production was increased until 3 to 4 times (until 2.10^{-7} moles) by Il1α . °OH were quantified by an indirect method: gas chromatography measurement of the ethylene generated from γ-methiol-keto-butyric acid by °OH attack. After preincubation with Il1β, chondrocytes produced 14 times more °OH than the control cells, and this production °OH reached until 40 times the control value after preincubation with Il1α. Intracellular significant amounts of Vit E were measured by HPLC with a progessive decrease in the intracellular level : for 1.10^6 cells, we measured 20 to 30 ng Vit E after collagenase digestion, 9 to 11 ng after one day of culture and 4 to 6 ng after 2 days of culture. Human chondrocytes in culture are thus able to produce active oxygen species and cytokines treatment enhances this production. Cultured chondrocytes possess vit E, but they slowly lose this natural antioxidant defence.

9.16 ALLOPURINOL AND REPERFUSION STRESS DURING OPEN HEART SURGERY

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Allopurinol, an inhibitor of the enzyme xanthine oxidase which helps to generate free radicals, has been tested during open heart surgery. Patients were divided in two groups, control (n=8) and allopurinol (n=10). Allopurinol group received 600 mg of allopurinol before operation. Blood samples were taken from coronary sinus an the radial artery before the cardiopulmonary bypass, 2, 10 and 20 minutes after the aortic cross clamp was removed and 5 minutes after the cardiopulmonary bypass. In blood sample were determined erythrocyte oxidized (GSSG) and reduced (GSH) glutathione, plasma malondialdehide (MDA), creatine kinase- MB isoenzyme (CK-MB) and lactic acid. A significant difference between two groups was found in the transcardiac gradient of erythrocyte GSSG/GSH ratio immediately after the crossclamp release. Although there was a significant rise in gradient of MDA, CK-MB and lactic acid, there was no difference between the two groups. Obtined data suggest that the allopurinol diminishes oxygen free radicals production during reperfusion of patients with coronary artery disease undergoing open heart surgery.

9.17 ISCHEMIA-REPERFUSION INJURY: BIOCHEMICAL ALTERATIONS IN PEROXISOMES OF RAT KIDNEY.

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We studied the effect of ischemia-reperfusion on the structure and function of kidney peroxisomes. Ischemic injury changed the density of peroxisomes (from 1.21 gm/cm^3 to 1.14 gm/cm^3). The number of peroxisomes moving from the normal density to a lower density increased with ischemic injury. Latency experiments indicated both populations of peroxisomes to be of intact peroxisomes. Immunoblot analysis with antibodies against peroxisomal matrix and membrane proteins demonstrated that after 90 min of ischemia a significant number of matrix proteins were lost in lighter population suggesting that functions of these peroxisomes may be severally affected. Reperfusion following ischemic injury resulted in loss of peroxisomal matrix proteins in both peaks suggesting that peroxisomal functions may be drastically compromised. The decrease of catalase activity during ischemia was due to its inactivation and proteolysis following reperfusion. Since peroxisomes participate in various cellular functions, therefore, the observed changes in the structure and function of peroxisomes as a result of ischemic-reperfusion injury underlines the importance of this organelle in the pathophysiology of vascular injury. Funded by DCI and VA Merit Review.

9.18 ALTERATIONS OF PEROXISOMAL FUNCTIONS IN ISCHEMIA-REPERFUSION INJURY

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To understand the molecular mechanism leading to the loss of peroxisomal ß-oxidation in ischemia, we examined the individual enzyme activities of ß-oxidation system and the overall fatty acid oxidation in peroxisomes isolated from kidney exposed to different periods of ischemia-reperfusion injury. The ß-oxidation decreased with an increase in ischemic injury (53 and 43% of the control after 60 and 90 min ischemia, respectively). Reperfusion for 24 hr restored the peroxisomal ß-oxidation in kidney exposed upto 60 min of ischemia,however, 90 min of ischemic injury was irreversible. The individual enzyme activities of lignoceroyl-CoA ligase, acyl-CoA oxidase, bifunctional enzyme and thiolase were decreased after 90 min of ischemia. This decrease in enzyme activities was more pronounced following reperfusion. Immunoblot analysis indicated that the major loss of these enzyme activities was due to their inactivation. These results demonstrate that the peroxisomes experience free radical injury following ischemia-reperfusion as evidenced by loss of function without loss of peroxisomal enzyme proteins. Supported by DCI, VA Merit Review and NS-22576.

9.19 PENTOXIFYLLINE IMPROVES LIPID PEROXIDATION IN HEPATIC ISCHEMIA-REPERFUSION INJURY IN THE RAT

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Pentoxifylline (PTF) is a methylxanthine which improves microcirculation and inhibits TNF, partially preventing its action in leukocyte adherence. Partial warm ischemia (70%) was induced in male Wistar rats (250-300 g). 3 groups were studied: I (n=8), sham-operated; II (n=15), non-treated ischemia/reperfusion (I/R); III (n=15) PTF-treated I/R (50 mg/kg im 30 min before reperfusion). Blood samples and liver biopsies were obtained after 1, 6, 24 h of reperfusion. Serum AST, ALT, LDH were measured. Lipoperoxidation was determined after 15 min of reperfusion in liver homogenates, incubated in prooxidant system using the TBA test. Extent of necrosis was assessed by light microscopy. Mean serum AST values after 1, 6, 24 h reperfusion rose significantly less in group III than in group II: 4118, 5123, 750 vs 8200, 13193, 7146, respectively ($p<0.05$). The rise in AST and LDH was significantly lower in group III. Mean liver MDA values (nmol/g wt) rose less in group III than in group II after 0, 30, 60 min incubation: 499, 451, 633 vs 556, 640, 848, respectively. The extent of necrosis at 24 h was over 80% in group II, and under 35% in group III. Conclusion: PTF protects the liver from I/R injury and improves lipid peroxidation, possibly by diminishing the O_2 chain reaction begun in the hypoxanthine-to-xanthine reaction by product saturation.

9.20 LIPID PEROXIDATION IS NOT THE MAIN MECHANISM OF LIVER DAMAGE IN PRIMARY NON FUNCTION AFTER TRANSPLANTATION.

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Free radicals production and lipid peroxidation are two possible mechanisms involved in the ischemic damage of transplanted organs. In order to elucidate this hypothesis we studied the behaviour of plasma malondialdehyde (MDA), a secondary product of lipid peroxidation, in 4 subjects (2 males, 2 females, mean age 37 yrs) who underwent to liver transplantation, 2 of whom died within 10 days for primary non function of the liver. Venous blood samples for MDA, transaminases (AST, ALT), prothrombin time (PT), bilirubin (BIL) and lactic acid (LA) were drawn every six hours, in the first week following the operation. Peripheral oxygen consumption (VO2I) was also measured at the same time using the Fick calculation. Plasma MDA was assessed by thiobarbituric acid reaction and fluorimetric measurement. Mean values of MDA concentrations (±SE) were significantly higher in the patients who promptly recovered after liver transplantation (3.06±.05 vs 2.89±.05nmol/ml)($p<.005$). The same patients showed significantly higher VO2I (243+4.9 vs 112+1.2 ml/min-1/m-2)($p<.0001$). As expected AST, ALT, BIL, LA were significantly higher and PT significantly prolonged ($p<.0001$) in 2 subjects who died for primary non function of the liver. The plasma levels of MDA significantly correlated with PT ($p<.0001$) and VO2I ($p<.0001$), whereas no significant correlation was found between MDA and AST or ALT. In conclusion lipid peroxidation does not seem to be related to the pathogenesis of hepatocellular damage in the primary disfunction of transplanted liver. On the contrary MDA seems mostly influenced by hepatic synthetic and oxidative function needed for its production.

9.21 DISTRIBUTION AND ACTIVITY OF XANTHINE OXIDOREDUCTASE IN HUMAN TISSUES.

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Localization and activity of xanthine oxidoreductase (dehydrogenase and oxidase forms) were studied in post mortem material and biopsies of various human tissues with a recently developed histochemical method using unfixed cryostat sections, polyvinyl alchol as tissue stabilisator, 1-methoxyphenazine methosulphate as intermediate electron acceptor and Tetranitro BT as final electron acceptor. High enzyme activity was only found in liver and jejunum, whereas all other organs studied showed no activity. In the liver, enzyme activity was found in sinusoidal cells and both in periportal and perivenous hepatocytes. In jejunum, enterocytes and goblet cells as well as the lamina propria underneath the epithelial lining showed activity. Oxidase activity and total dehydrogenase and oxidase activity of xanthine oxidoreductase as studied biochemically were found in human liver and jejunum, but not in kidney and spleen. This confirmed the histochemically obtained results for these organs. Autolytic rat livers several hours after death were studied histochemically to exclude artefacts due to postmortal changes in the human postmortal material. Loss of activity could not be observed in this autolytic material. In addition, the percentage xanthine oxidase did not change significantly in autolytic rat livers compared with control livers as measured biochemically. The localization found in human tissues is discussed with respect to the localization found before in rat tissues. Furthermore, the low conversion rate of xanthine dehydrogenase during autolysis is discussed with respect to ischemia-reperfusion injury.

9.22 PROTECTION BY RECOMBINANT ATL-DERIVED FACTOR/HUMAN THIOREDOXIN (ADF/HTX) AGAINST ISCHEMIA-REPERFUSION TISSUE INJURY

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ATL-derived factor/human thioredoxin (ADF/hTx) is a polypeptide consisting of 104 amino acids with two redox-active half cystine residues in an exposed active center, having the amino acid sequence: -Cys-Gly-Pro-Cys-. ADF/hTx is ubiquitously distributed in a variety of organs and induced by physical and biological stresses, such as ultraviolet or X-ray irradiation and viral infection. ADF/hTx was shown to reduce reactive oxygen species such as hydrogen peroxide and alkyl peroxides. ADF/hTx was also revealed to have protein disulfide-isomerase (PDI)-like protein refolding activity for denatured enzymes.

To determine pharmacokinetics and *in vivo* effects of ADF/hTx, we produced highly purified ADF/hTx using recombinant DNA technics in *E. coli*. After a single intravenous administration, recombinant ADF/hTx (rADF/hTx) was eliminated bi-exponentially from the serum in mice. The distribution half-life was 3.3 min and the elimination half-life was 66 min. The administrated rADF/hTx was distributed especially in skin and kidney. The maximum uptakes by these organs were observed about 30 min after administration. Next we determined the effects of administrated rADF/hTx on animal ischemia-reperfusion injury model. The rADF/hTx administrated intravenously suppressed ischemic paw edema of mice. These data suggest that administrated rADF/hTx distributed to skin and probably around endothrial cells protected tissue damage caused by ischemia-reperfusion.

9.23 THE EFFECT OF NON-ENZYMATIC ANTIOXIDANTS ON THE POST-ISCHEMIC REPERFUSION INJURY OF THE HEART

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The majority of reports on ischemia-reperfusion injuries suggest the involvement of an imbalanced relationship between reactive prooxidants and antioxidants. However, direct evidences of the pathogenetic role of uncontrolled prooxidant formation is still lacking. The main reason for that is the lack of suitable methods to demonstrate the site(s) of prooxidant generation in the intact organ. Many biochemical and structural alterations at the molecular level occurring during reperfusion can be explained without the involvement of reactive oxygen species. The influence of antioxidants on the recovery of functional and electrophysiological parameters was studied to evaluate the role of "oxidative stress" in the development of reperfusion injury. Langendorff perfused rat hearts were subjected to normothermic global ischemia (30 min) and subsequent reperfusion (30 min). Trolox C, a water-soluble analog of alpha-tocopherol, was infused directly into the aorta cannula 5 min prior to the onset of ischemia and was continued throughout reperfusion period. Left ventricular developed pressure (LVDP), end-diastolic pressure (LVEDP), contractility, coronary flow and ECG were recorded. Nontreated hearts showed a low rate of recovery (contractility 27%, LVDP 36%), and an increase of LVEDP. The recovery of LVDP and contractility was significantly improved ($p < 0.05$) in hearts preloaded with Trolox (75% and 67%, respectively). Trolox decreased LVEDP in comparison to the untreated group. Trolox was also effective in reducing severe rhythm disturbances. These findings suggest the involvement of prooxidants in the ontogenesis of functional reperfusion injuries of the heart.

9.24 ALTERATIONS OF GLUTATHIONE STATUS IN BLOOD OF PATIENTS UNDERGOING CARDIOPLEGIC ARREST AND REPERFUSION

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The alterations of erythrocyte and plasma glutathione redox status (taken as indirect index of oxidative stress) were measured in 13 patients undergoing cardioplegic arrest and reperfusion using blood cardioplegia. A marked increase of glutathione disulfide (GSSG) and glutathione-protein mixed disulfide (Cys-SG) concentration and a shift of the glutathione redox status toward oxidation were detected during reperfusion in the plasma effluent from the coronary sinus. Comparable results were obtained in the whole blood (erythrocytes). Furthermore, a moderate increase of aldehyde-protein fluorescent adducts (taken as chemical signature of oxygen free radical-induced injury) has been measured in plasma during heart reperfusion. No correlation was found between the duration of the ischemic period and the extent of alterations of any of the above-mentioned parameters.

Conclusion: taken together these results support the occurrence of oxidative stress in human hearts subjected to reperfusion after cardioplegic arrest. Moreover, the demonstration that glutathione oxidation takes place even in erythrocytes suggests that erythrocyte glutathione is efficiently employed as reductant in protection against oxidative stress.

9.25 Ca^{2+}-CHANNEL BLOCKERS DO NOT PROTECT PERFUSED HEPATOCYTES FROM ANOXIC INJURY

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The effects of verapamil, nifedipine and diltiazem were studied during anoxia for 24 in isolated rat hepatocytes. Under control conditions, cytosolic free calcium (Ca_i^{2+}) was 126 ± 10 nM. In the control group, anoxia increased Ca_i^{2+} with a first rise within 15 minutes and a maximum value of 515±78 nM after 1 hr ($p<0.001$). LDH increased 6-fold during the second hour ($p<0.001$). During reoxygenation, Ca_i^{2+} and LDH returned to control levels within 45 minutes. Ca^{2+} channel blockers (10^{-7} to 10^{-5} M) added to the perfusate during the anoxic period did not inhibit the rise in Ca_i^{2+} and in LDH release. On the contrary, high concentrations (10^{-6} to 10^{-5} M) of the blockers nifedipine and diltiazem significantly increased LDH release. The increase in Ca_i^{2+} during anoxia was caused by an increased Ca^{2+} influx from the extracellular fluids because it was totally abolished in Ca^{2+}-free media. Since the rise in Ca_i^{2+} was not suppressed by Ca^{2+} channel blockers, Ca^{2+} influx must have occurred not through Ca^{2+} channels but on the Na^+-Ca^{2+} antiporter which is the only other recognized Ca^{2+} influx pathway. These studies also suggest that cell injury and LDH release induced by anoxia may be related directly to the increase in Ca_i^{2+}.

9.26 GLUTATHIONE AND PROTEIN THIOL HOMEOSTASIS IN CEREBRAL ISCHEMIA AND REPERFUSION

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Glutathione (GSH) and protein thiol (PrSH) homeostasis was examined in rat brain regions during reperfusion (R) following cerebral ischemia (I). I was induced by (i) bilateral carotid artery occlusion (BCO) alone (reducing cerebral blood flow (CBF) by 50%) and (ii) BCO with simultaneously induced hypotension (reducing CBF by 90%). After 1 hr of R following I induced by BCO alone, the GSH levels in striatum (ST) and hippocampus (HP) were depleted by 68 and 56% respectively and lipid peroxidation products increased. Less than 5% of the depleted GSH was recovered as GSSG, and the rest as PrSSG, with concomitant loss of PrSH. The total GSH recovered as GSH+PrSSG in ST and HP was 115 and 159% of Sham operated controls. After 24 hr of R the PrSSG and PrSH levels were comparable to controls and the GSH/PrSH homeostasis was restored. In animals subject to hypotension and BCO, GSH depletion was observed after 1 hr with loss of PrSH and increase in PrSSG levels. Increased recovery of GSH (GSH+PrSSG) was not observed and the GSH/PrSH homeostasis was not restored after 24 hr. Thus, a major consequence of oxidative stress induced by I/R is loss of GSH with concomitant gain in PrSSG and disturbance of Protein thiol homeostasis.

9.27 TIME COURSE AND MECHANISM OF OXIDATIVE STRESS AND TISSUE DAMAGE IN THE RAT LIVER SUBJECTED TO ISCHEMIA-REPERFUSION.

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The time course of the biochemical changes in the main cellular sources of oxygen free radicals as well as the related oxidative stress and tissue damage were evaluated in an in vivo model of zonal ischemia-reperfusion in rat liver. Rats were subjected to ligature of the right branches of the hepatic artery and portal vein for 60, 120 and 180 min, and the liver was then reperfused for 30 min. After 180 min of ischemia oxyradical production (assayed by the spontaneous chemiluminescence) decreased to zero, mitochondrial function decrease by 80% and xanthine oxidase activity increased by 26% without changes either in the markers of cell damage (LDH, AST, ALT and water content) or in the activities of superoxide dismutase, catalase and glutathione peroxidase. After reperfusion, marked increases were detected both in oxyradical production and in the serum activities of tissue damage markers. Mitochondrial function as well as the activities of antioxidant enzymes and the level of non-enzymatic antioxidants were decreased. The severity of the post-reperfusion changes correlates with the length of the ischemic period. Hydrogen peroxide concentration and production rates were 5 times higher after ischemia-reperfusion than in control conditions. The use of mitochondrial inhibitors and uncouplers strongly suggest mitochondria is responsible for the increased H_2O_2 concentration and production rate in the reperfused liver. These results support a role for mitochondria as primary oxyradical source in liver ischemia-reperfusion in a quantitative and kinetic fashion.

9.28 INTRACELLULAR CATALASE INHIBITION DOES NOT IMPAIR POST-ISCHEMIC RECOVERY IN RAT HEART.

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The effect of inhibition of myocardial catalase upon post-ischemic recovery was studied. Myocardial catalase activity was inhibited by 90% (1792±145 vs 184±23 U/g) with 3-amino-1,2,4-triazole (ATZ, 1.5 g/kg i.p.). Isolated rat hearts (n=7/group) were subjected to 35 min global ischemia (37° C) and 30 min reperfusion. Results (mean ± SEM) were expressed as percent recovery of coronary flow rate (CFR), developed pressure (DP), rate-pressure product (RPP) and leakage of lactate dehydrogenase (LDH, IU/15 min/g) during reperfusion. Catalase (CAT, U/g) and glutathione peroxidase (GPx, U/g) activity was determined after 30 min reperfusion.

a=$p<0.05$, control vs ATZ

	Control	ATZ-treated
CAT	1756±86	244±14[a]
GPx	55.3±1.6	61.0±1.4[a]
CFR	74±2	93±9
DP	57±2	79±7
RPP	57±3	69±6
LDH	4.02±0.25	4.45±0.39

We concluded that intracellular inhibition of myocardial catalase did not impair the extent of contractile recovery and LDH release during reperfusion of ischemic rat heart.

9.29 ROLE OF OXIDATIVE STRESS IN CIRCULATORY SHOCK ASSOCIATED WITH HEPATIC DAMAGE

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An increasing number of studies suggests an involvement of free-radical mediated oxidative reactions in the hepatic damage which could occur during circulatory shock. Especially after acute cardiovascular failure or hypovolemic shock there is an hepatic damage due to ischemia followed by reperfusion when circulation is reequilibrated. This condition is accompanied by the appearance of oxidative damage due to oxygen free radical production. In the shock patients that exhibited marked elevation of serum levels of AST, ALT and hepatic isoenzyme LDH5 (ischemic hepatitis), a marked increase of intermediate and final products of lipid peroxidation was observed; in particular malonaldehyde (MDA) content in erythrocytes, and the fluorescent adducts between protein and MDA or 4-hydroxynonenal, and the levels of hydroperoxides in the plasma were enhanced. In parallel there was a decrease of antioxidant defenses in terms of erythrocyte glutathione content.

These data suggest that the free radical-mediated oxidative damage could play an important role in the pathogenesis of tissue injury following reperfusion after hepatic ischemia.

Session 10

Free Radicals in Medicine II (Nervous System, Muscle)

10.1 OXIDATIVE STRESS AND DAMAGE INDICATORS IN ANIMAL AND HUMAN EXERCISE.

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Several parameters and criteria were used to assess exercise-induced oxidative damage in various tissues of animals and humans. Animal studies showed an increased production of free radicals and elevated levels of lipid peroxidation. However, studies on oxidative changes in human plasma due to exercise were not as consistent, as some studies did not show a significant effect of damage to blood components. Recent studies on protein oxidation as evaluated by protein carbonyl accumulation in muscles of rats, demonstrated exercise-induced oxidative damage to proteins. Thus, levels of protein carbonyls in muscles were increased by +107%, $p<0.001$, in endurance trained rats for 12 weeks. Similar studies on rats subjected to a single bout of endurance exercise to exhaustion revealed an increase of 17,3% in carbonyl levels compared to sedentary controls.
However, animals fed with a-tocopherol or tocotrienols had a much lower level of carbonyls in muscle protein of sedentary animals (-32.2%, $p<0.01$) which was also reduced in exercised animals. The studies show that α-tocopherol and tocotrienols can protect proteins from exercise-induced oxidative damage.

10.2 FREE RADICALS, ANTIOXIDANTS AND CALCIUM HOMEOSTASIS WITH REFERENCE TO MALIGNANT HYPERTHERMIA

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The regulation of intracellular [Ca^{2+}] in skeletal muscle at rest and during contraction depends on mechanisms such as Ca^{2+}-ATPases, Na^{+}-Ca^{2+} exchangers and the voltage-sensitive ryanodine receptor. The location of these mechanisms within the membranes of sarcolemma, sarcoplasmic reticulum and mitochondrion may enhance their susceptibilty to free radical-mediated damage and result in uncontrolled increases in cytosolic [Ca^{2+}] and cell death.

The pharmacogenetic disorder, malignant hyperthermia (MH), is triggered by volatile anaesthetics and is characterised by muscle rigidity and a rapid, invariably fatal rise in body temperature. The biochemical lesion responsible for MH remains uncertain, but it has been variously ascribed to faults in many of the Ca^{2+}-regulatory mechanisms. Peroxidation results in rapid efflux of Ca^{2+} from organelles and compared with controls, tissue incubations from MH susceptible individuals produce more pentane, thiobarbituric acid reactive substances and electron spin resonance-detected adducts of linoleic acid; these can be decreased by vitamin E supplementation. A proposed mutation which results in excess free fatty acid release by hormone sensitive lipase provides additional evidence for a pivotal role of free radical-mediated peroxidation in MH.

10.3 FREE RADICALS, DOPAMINE, LEVODOPA AND PARKINSON'S DISEASE.

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The mechanism for the accelerated loss of dopamine neurons in Parkinson's Disease is unknown. One hypotheses focuses on the potential for oxidative stress associated with the natural metabolism of the neurotransmitter dopamine (DA). An ESR, spin trapping technique has been used to study the kinetics of free radical reactions from DA and its precursor levodopa. DA catabolism by monoamine oxidase resulted in the formation of the hydroxyl (OH) adduct of the spin trap 5,5-dimethyl-1-pyrroline N-oxide. Quantitative analysis indicates that the number of OH released to the solution is comparable with the number of DA oxidized. OH adduct formation is eliminated by denaturing the enzyme and decreased by the addition of various enzyme inhibitors and radical scavengers. Results with added metal ions and chelators implicate redox-cycled copper ion in the system. Under these conditions, the OH from DA was greater than that from serotonin or norepinephrine. Free radicals have also been detected by the autoxidation of DA or levodopa in the presence of metal ions. The role of protective agents and transition metal ions in these systems are under study. These results support the oxidative stress hypothesis associated with DA which plays a role in the tissue injury and cell dysfunction seen in Parkinsonism. (Supported in part by a grant to F.F.Oldfield from the Parkinson's Disease Foundation.)

10.4 INDUCTION OF POLY(ADP-RIBOSE)POLYMERASE IN NEUROBLASTOMA CELLS BY ANTIBODY-GLUCOSE OXIDASE CONJUGATES AND ITS ENHANCEMENT BY ASCORBIC ACID

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Most neuroblastoma cells express the ganglioside GD_2. Using the anti GD_2 antibody 14.18 conjugated to glucose-oxidase enough H_2O_2 on neuroblastoma cell surface was generated to result in killing the cell line SK-N-LO. Specific binding was examined by fluorescence microscopy in parallel. Poly(ADP-ribose) polymerase (PADPRP) was used as an indirect parameter for DNA-strand break formation by the generated H_2O_2. The amount of H_2O_2 produced in the presence of different glucose concentrations was determined and correlated with PADPRP activity. Killing of neuroblastoma cells by the antibody-glucose-oxidase conjugate is facilitated since these cells cannot effectively scavange H_2O_2. Induction of PADPRP activity was significantly enhanced in the presence of ascorbic acid. Since H_2O_2 can be delivered by this method to the close proximity of neuroblastoma tissue, the use of glucose oxidase antibody-conjugates may be of therapeutical benefit in neuroblastoma treatment, especially in combination with ascorbate. Advantages and side effects of this possible therapy will be discussed.

10.5 ACTIVE OXYGEN RADICAL SPECIES AS A FIRST SIGNAL STRESS

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It is well known that a stress stimulates hypothalamus, hypophysis and adrenal cortex system and also sympathetic nerve system which stimulates the release of catecholamines such as adrenaline (AD), noradrenaline (NA) and dopamine (DA). But it is still not known what is a first signal of stress, which stimulates this hypothalamus system. Selye pointed out that even if all of the input nerve tract to the hypothalamus are cut off in the deafferentation animal, the stress reactions are still occurred and he suggested that an unknown hormonal factor stimulates hypothalamus system.

Here we show that the active oxygen radical species may be the first signal of stress, and this active oxygen radical species are the hormonal factors which stimulate the hypothalamus system.

The effect of ß-carotene on the stress reaction of vigil was studied. Urinary levels of AD, NA and 11-oxy-17 KS and plasma ACTH levels were altogether increased and systolic blood pressure also increased by vigil stress. ß-carotene inhibited all stress reactions, suggesting that the effect site of ß-carotene may be hypothalamus. In other words, the active oxygen radical species stimulate the hypothalamus functions.
The effect of ß-carotene is attributed as the scavenging of radicals which was observed the inhibitory effect on urinary MDA levels.

10.6 THE EFFECTS OF THE FREE RADICAL MODULATOR, TUMOR NECROSIS FACTOR-α, ON ACHILLES TENDON HEALING

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Tumor necrosis factor-α (TNF-α) is a cytokine that amplifies superoxide release by phagocytic cells. TNF-α also augments the ability of healthy cells to protect themselves from oxidant injury by inducing intracellular manganous superoxide dismutase. The aim of this study was to determine if TNF-α modified the functional and mechanical recovery of injured rat Achilles tendons.

Twenty one male Sprague-Dawley rats (weight 250-300g) were randomly allocated into two groups. The TNF-α group received an intraperitoneal injection of 5 μg TNF-α in phosphate buffered saline (PBS) 24 hrs and 1 hr prior to surgical transection of the right Achilles tendon. The control group received PBS alone. All evaluations were performed in a blinded fashion. The functional recovery was objectively determined using the measurements of hind paw prints (Achilles Functional Index; AFI) pre-operatively and on post-operative day 1,3,5,7,9,11,13 and 15. On day 15 the animals were sacrificed and biomechanical evaluations were performed on both the injured and uninjured Achilles tendon constructs.

All animals had a significant initial functional impairment that gradually improved over 15 days. The functional recovery was greater in the TNF-α pre-treated group, a difference that became most obvious on day 15 (TNF-α = -9.6 ± 3.0, control = -23.4 ± 4.2; mean AFI ± SEM; $p < 0.02$). There were no significant differences in failure load, ultimate deformation or mean stiffness.

In summary, pre-treatment with TNF-α significantly improved the functional recovery of rats subject to Achilles tendon division, but did not alter the mechanical properties of the Achilles tendon constructs harvested 15 days following Achilles tendon division.

10.7 THE EFFECTS OF ALLOPURINOL ON THE FUNCTIONAL RECOVERY OF SCIATIC NERVE CONTUSION

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The object of this study was to consider the effects of allopurinol (a competitive inhibitor of xanthine oxidase) on the functional recovery of rats subject to sciatic nerve contusion. Two experiments were carried out. In each, twenty-four outbred male Sprague-Dawley rats, were randomly allocated to three groups: (1) sham operated group - sciatic nerve exposure only, (2) sciatic nerve contusion without active pre-treatment, (3) sciatic nerve contusion with intra-peritoneal injections of allopurinol (20 mg/kg), 24 hours and 1 hour pre-operatively. The two experiments differed in the force used to contuse the nerve. In the first experiment, a 0.98 N crush was applied for 10 minutes, while in the second experiment 147 N crush was applied for 10 minutes. The functional recovery was objectively determined using the measurements of hind paw prints (Sciatic Functional Index; SFI).

Rats subjected to sham operations exhibited no functional deficit, while rats subject to sciatic nerve contusion had obvious functional deficits with longer, narrower hind paw prints on the affected side. The time for recovery from 0.98 N crush (11 days) was shorter than that for 147 N of crush (60 days). In the 0.98 N crush experiment there were no significant differences between the time courses of recovery for the allopurinol treated and untreated animals. In the 147 N crush experiment there was little difference between the two groups until 60 days post-operatively, when the allopurinol pre-treated group were slightly less disabled than the control group.

These results indicate that pre-operative intra-peritoneal allopurinol offered some small functional benefit to rats with severely contused sciatic nerves. Xanthine oxidase-mediated free radical damage may play a small role in severely contused peripheral nerves.

10.8 EFFECTS OF ANTIOXIDANT BUTYLATED HYDROXYTOLUENE (BHT) ON HORMONAL REGULATION AND ESR SIGNALS IN ADULT AND OLD RATS

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The effects of a single administration of BHT on the intensity of ESR signals in blood plasma, as well as on the plasma concentrations of ACTH, 11-OHCS, TSH and T_3 hormones within 48 hours following the drug administration were studied in the experiments on adult (4-6 months) and old (24-26 months) male Wistar rats. Changes due to BHT took place in the intensity of ESR transferrin and ceruloplasmin signals in the blood. There were also significant BHT-induced changes in the plasma concentrations of ACTH, 11-OHCS, TSH and T_3 hormones. The amplitude and the direction of the changes depended upon the type of hormones, the time period that had elapsed after BHT injection, and the animal's age. The physiological effects of BHT in vivo seem to be mediated via the system of the neurohumoral regulation.

10.9 NERVE GROWTH FACTOR AND ANTIOXIDANT ENZYMES IN REAGGREGATION CULTURES OF FETAL RAT BRAIN CELLS

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Since we have shown that X-irradiation causes a doubling of the amount of NGF in reaggregation cultures of fetal brain cells[1], we treated cultures with NGF to see if it affected the antioxidant enzymes.

The experiments were performed using reaggregation cultures of rat brain cells mechanically dissociated on embryonic day 15, and grown as spheroids in serum-free medium. We have earlier shown that the development of the studied antioxidant enzymes in this system and *in vivo* is similar[2]. In a chronic experiment 40 ng/ml ß-NGF was added to the medium day 2 in culture and thereafter. In an acute experiment NGF was added from day 35 to day 42. SOD-activity was measured as the inhibition of spontaneous oxidation of 6-hydroxydopamin, using 5 mM KCN to discriminate between Mn and CuZn forms. Catalase was assayed with a Clark-type electrode. GSH-Px activity was measured fluorimetrically with H_2O_2 as substrate.

The chronic NGF-treatment resulted in a significant increase in catalase activity at both day 19 and day 30 in culture. Total SOD activity was decreased at d19 but it was not different from control activity at d30. CuZnSOD activity was decreased at d19, and MnSOD activity was increased at d30. GSH-Px activity was increased at d30. When NGF was administered acutely, after the maturation of the antioxidant enzymes in this cultures, no effects were seen on the antioxidant enzyme activities.

The NGF-induced catalase and GSH-Px increases observed in the present study and by others[3,4], in conjunction with previous findings[1] suggests that one of the mechanisms behind the trophic effects of NGF might be modulation of the antioxidant defense systems.

1) Dimberg,Y *et al.* Accepted in Int.J.Rad.Biol. (1992).
2) Aspberg,A & Tottmar,O. Accepted in Dev.Brain Res. (1992).
3) Nistico,G *et al.* Neurosci.Lett. 130:117-119 (1991).
4) Jackson,GR *et al.* J.Neurosci.Res. 25:360-368 (1990).

10.10 DEPRENYL INCREASES LIFESPAN OF NMRI-MICE

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The purpose of this study was to investigate the influence of selegilin HCL on the longevity of immunosuppressed nude mice. Athymic NMRI-mice lack mature T-cells and specific IgG antibodies. Cross-reacting IgM antibodies accumulate and cause premature aging. NMRI-mice are raised and kept in the Tierversuchs-anlage of the University Clinics Frankfurt (Head Prof. Dr. med. vet. H.P. Fortmeyer) under germreduced conditions. At the beginning of the study, the mice were 10 weeks old. In the deprenyl group 4mg of selegilin HCl was admixed to 10kg of normal diet. An average consumption of 5.7g per mouse and day correlates to 2.3µg selegilin HCl per mouse and day or 70µg per kg body weight. 11 weeks after selegilin feeding was started, 1 mouse out of 14 was still alive in the control group versus 9 out of 14 animals still living in the selegilin-treated group. The study is continued. Selegilin is used as a selective MAO-B-inhibitor in therapy of Parkinson's disease, in which free radical involvement has been discussed. Increased life expectancy of selegilin-treated patients was reported; extension of lifespan was proven in experiments with aged male rats.

10.11 OXIDATIVE DAMAGE: A POTENTIAL INDUCER OF HEAT SHOCK PROTEINS IN DUCHENNE MUSCULAR DYSTROPHY

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Duchenne muscular dystrophy (DMD) is a severe, progressive X-linked muscular dystrophy associated with a dystrophin mutation which in turn leads to muscle fibre membrane damage, calcium influx and intracellular proteotoxicity. We investigated the expression in this condition of heat shock genes which are known to be transcriptionally activated in response to proteotoxic agents. Cryosections were stained immunocytochemically with HSP 90,73,72,65 and ubiquitin employing an immunoperoxidase biotin-streptavidin system. An elevated expression of HSP 73,72,65 and ubiquitin was revealed in hypercontracted fibres while HSP 90 antibodies localised to regenerating and necrotic fibres invaded by macrophages. A preferential localisation of HSP 65 and ubiquitin was evident in type 2 fibres of normal muscle. Distinct induction patterns of these HSPs were seen in normal muscle following an *in vitro* heat shock treatment. The increased expression of HSPs in certain fibres of DMD may represent a protective mechanism against the metabolic stress characteristic of the disease.

10.12 RELEASE OF MDA - TBARS AFTER REVASCULARISATION OPERATIONS IN HUMANS

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Reperfusion injury is thought to be in part due to lipid peroxidation and play an important role for clinical outcome. According to the method of Wong we have measured the MDA-TBARS by HPLC peroperatively (30 minutes before and occassionally 1, 2, 3 hours after reperfusion start) during revascularisation operations concerning different organ systems in humans. All together MDA-TBARS show a strong increase within the 1st hour after revascularisation onset and decrease to its baseline value during the next 2 to 3 hours. The different groups are as follows (mean MDA nmol / ml) :

Leg revascularisations, (n = 31): 0.68, 1.14, 0.94, 0.69

kidney transplantation, (n = 9): 0.67, 1.40, 0.81, 0.77

cerebral revascularisation, (n = 27): 0.91, 1.1, 1.14, 0.93

arthroscopy, (n = 17): 0.61, 1.1, 0.88

Summ it up we can conclude that lipid peroxidation is a short lasting event which takes place immediately after reperfusion onset. Thus, implies that successful therapy is limited immediately prior or simultaneously with reperfusion start.

10.13 MITOCHONDRIAL DNA TRANSCRIPTION IN AGING RAT.

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Miquel and Fleming in their hypothesis of cell aging proposed a pathway of senescent disorganization in which mitochondria and mitochondrial (mt)DNA are the primary target of free radical injury (1). Age-dependent modifications of mtDNA have been recently reported . We were able to show that senescence induces a reduction of mt transcripts in brain and heart (2). This reduction depends on reduced RNA synthesis (3)and on a reduced percentage of mtDNA molecules harboring the third DNA strand . However reduced RNA synthesis is reversed by a pre-treatment of senescent rats with acetyl-L-carnitine (1h,300 mg/Kg b.w., i.p.). This strongly suggestes that mt transcription apparatus is not irreversibely damaged in aging and that mt gene activity is metabolically mediated.

1) Miquel,J. and Fleming, J.(1986)in:Free Radicals,Aging, and Degenerative Diseases (Johnson, J.E. et al eds) Modern Ageing Research Vol.8 pp. 51-74.
2) Gadaleta, M.N. et al. (1990)E.J.B.187,501-506
3) Fernandez-Silva, P. et al. (1990) Biochem. Biophys.Res.Commun.176,645-653.

10.14 OXYGEN FREE RADICALS IN DEMYELINATING AND INFLAMMATORY DISORDERS OF THE NERVOUS SYSTEM

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A role for oxidative damage caused by free radicals was investigated in sera and CSF from patients with clinically definite multiple sclerosis (a centrally demyelinating disorder), aseptic meningitis and Guillian-Barre (GB) syndrome (an acute peripheral demyelinating disorder). A control group consisted only of patients with either chronic headache/migraine, benign intercranial hypertension or psychological disorders. Blood and CSF from all patients were subjected to routine haematological, biochemical and bacteriological analysis.

The demonstration of increased end products of lipid peroxidation is the evidence most frequently quoted for the involvement of free radicals in human disease. Hence, lipid peroxidation was assessed by measuring the levels of TBARS using an HPLC-based assay. In addition, ascorbate, dehydroascorbate, protein carbonyls and bleomycin-detectable iron were measured. Serum lipid peroxidation was significantly elevated in GB patients prior to treatment (plasma exchange or steroids) when compared with post treatment levels, controls or the other groups. Serum ascorbate levels appeared depressed in both GB and meningitis patients. Levels of serum protein carbonyls were found to be increased only in the meningitis group.

10.15 TURNOVER OF VITAMIN E IN NEURAL AND OTHER TISSUES

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Vitamin E (α-tocopherol) is necessary for the maintenance of normal neural structure and function in both man and experimental animals. To gain an understanding of the neurobiology of α-tocopherol we have investigated the turnover of natural RRR-α-tocopherol in various neural and non-neural tissues in vitamin E deficient and sufficient animals. The study design was as follows: one group of rats received a vitamin E deficient diet for 16 weeks and a 2nd group the same diet to which unlabelled (d0) tocopherol (36mg/kg) was added (phase 1). Both groups then received deuterated (d3) tocopherol for 12 weeks (phase 2), before returning to their original diets for a further 16 weeks (phase 3). The results can be summarised as follows:
1) We have confirmed that the turnover of tocopherol is slower in neural compared to non-neural tissues. 2) The loss of tocopherol from neural tissues can be characterised by a 1st order rate constant. 3) The ratio of d3 / d0 tocopherol during phase 3 in the tissues of rats receiving a vitamin E deficient diet showed a consistent but opposite pattern in the neural compared to the non-neural tissues. This could be explained by a redistribution of the most recently acquired (i.e. the d3) tocopherol from the non-neural to the neural tissues. 4) The turnover of tocopherol in neural tissues during phase 3 appeared to be slower in the deficient than the sufficient animals. 5) Animals that had been vitamin E deficient showed an increased uptake of d3 tocopherol compared to controls during phase 2. These results suggest that there is a mechanism whereby neural tissues conserve vitamin E at the expense of the other non-neural tissues.

10.16 THE PROTECTIVE EFFECTS OF MATERNAL VITAMIN E ON THE FETAL CEREBRAL DYSFUNCTION

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The vulnerability of the fetal brain to free radical attacks is well known. Vitamin E is a radical scavenger and an effective protective agent against the formation of lipid peroxide. The present study was on the possible prevention of brain damage in several animal models by using supplementary vitamin E in utero.
Results: 1) Fetuses from rats given 0.03% dl-α-tocopherol acetate(vitamin E) as a drinking fluid and X-irradiated with 100 rad on gestational day (gd) 13 were examined on gd 21. Administration of vitamin E in irradiated cerebrum resulted in increased weight, DNA concentration and the dendritic branching development, and a decreased lipid peroxide formation. 2) The administration of 0.02% vitamin E with 10% ethanol during pregnancy resulted in an increased weight and a decreased lipid peroxide formation in the fetal rat cerebrum on gd 21. 3) As a possible preventive measure for brain dysfunction in Menkes disease, vitamin E was given prenatally in brindled mutant mice. The maternal administration of vitamin E resulted in decreased fetal and neonatal death of offspring, especially those of hemizygous males. In conclusion, this study provides evidence of the protection by vitamin E of neuronal development in fetus, through its antioxidant properties, against attacks by free radicals and/or lipid peroxide. Ref. Int J Devl Neurosci, 9:509-17, 1991.

10.17 SUPPRESSION OF EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS BY TREATMENT WITH CATALASE AND SUPEROXIDE DISMUTASE

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Experimental allergic encephalomyelitis (EAE) is a well known model for the demyelinating disease multiple sclerosis. EAE can by induced by immunization of Lewis rats with a homogenate of guinea pig spinal cord and Freund's complete adjuvants. After 10 days the immunized rats become paralyzed. Within the central nervous system of these animals large perivascular infiltrates of mononuclear cells are present, which consist for 50% of macrophages (mφ). These mφ have been shown to play an important role in the pathogenesis of EAE, since elimination of mφ causes a marked suppression of the clinical signs (1). In the present study, we have examined the possible pathogenic role of oxygen radicals secreted by the infiltrating mφ, by *in vivo* treatment with oxygen radical scavengers. Treatment of EAE rats with either catalase or superoxide dismutase (10,000 units/day/kg) from day 7 on after immunization, resulted in a marked suppression of the clinical signs. These results indicate that reactive oxygen species, probably secreted by the infiltrating macrophages, do indeed contribute to the demyelination during EAE. Similar findings have been reported in experimental allergic neuritis, the peripheral counterpart of EAE (2). Our results underline the importance of macrophages and the products secreted by these cells in the pathogenesis of demyelinating diseases of the (central) nervous system.

1) Huitinga et al., J. Exp. Med. 1990, 172: 1025 ; 2) Hartung et al., Ann. Neurol. 1988, 23: 453

10.18 OXIDATIVE STRESS DURING SEPSIS IN LIVER AND MUSCLE: A COMPARATIVE STUDY.

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Sepsis is generally defined as an infection associated with systemyc manifestations. It has been suggested that lipid peroxidation may play a role in the pathogenesis of this process. Female Sprague Dowley rats were divided in two groups: a) septic group, cecal ligation and double cecal perforation and b) sham operated. Spontaneous chemiluminescence of adductor muscle and liver were measured at 6, 12, 24, and 30 hours after the surgical procedure. Enzymatic activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were also determined at the same intervals. The results showed that ligth emission by the muscle doubled the control values (8 ± 2 cps/cm^2) at 12 hs of sepsis while in liver there was a 80% increase at 24 hs of sepsis (control: 11 ± 3 cps/cm^2). The activities of antioxidants enzymes in muscle were found to be diminished by 46% for MnSOD, 83% for CAT and 55% for GPx at 12 hs. In liver, only CAT showed a decrease (52%) at 24 hs. This data show the ocurrence of oxidative stress in muscle, partially due to the inactivation of antioxidant enzymes. On the other hand, in liver there was only inactivation of CAT without oxidative stress.

Plenary Session on Reactive Species in Metabolic Disorders

C.1 ATHEROSCLEROSIS AS AN INFLAMMATORY REACTION: Artery wall cell and lipoprotein interactions

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In a multilayer coculture, human aortic endothelial cells grown on the extracellular matrix of autologous aortic smooth muscle cells on a membrane, produced higher levels of fibronectin, collagen, TGF-ß, M-CSF, and GM-CSF, compared to the separate cultures of these artery wall cells. Inclusion of monocytes in the cocultures increased the level of matrix proteins by 3- to 22-fold. This increase appeared to be mediated by interleukins 1 and 6 and was accompanied by a marked elevation of mRNA for connexin 43. The interaction of endothelial cells and smooth muscle cells in this artery wall model resulted in a mild modification of LDL in the presence of 5-10% serum, followed by the induction of monocyte chemoattractant protein 1. Monocytes added to the endothelial side of these cocultures transmigrated into the subendothelial space at 5 to 7-fold higher numbers compared to cocultures that were not preincubated with LDL. HDL, antioxidants, antiinflammatory agents and inhibitors of lipoxygenase pathway inhibited LDL modification and the induced monocyte trasmigration in this system. The interaction of artery-wall cells, therefore, invokes an environment for mild LDL modification, monocyte attraction, potentially followed by formation of highly oxidized LDL, and eventual production of macrophage-foam cells.

LIPOPROTEIN OXIDATION AND LIPOPROTEIN-INDUCED CELL INJURY IN EXPERIMENTAL DIABETES C.2

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Recent studies show that plasma lipoproteins, because of ample lipid constituents, e.g., triglycerides, cholesteryl esters, cholesterol and phospholipids, are particularly vulnerable to free radical attack. Initiation by reactive oxygen and probable propagation by lipid radicals alters not only the physicochemical properties of lipoproteins but also the lipoprotein interactions with cells. Similarities between properties of vascular lesions and the attributes of vascular cells in culture exposed to oxidized lipoproteins have led to theories that free radical oxidation of lipoproteins may be involved in vascular lesion development. Recent evidence suggests that oxidized lipoproteins do, in fact, exist *in vivo*, in vascular lesions and, in particular instances, in plasma and tissues of diabetic animals and humans. We have reported the formation of oxidized lipoproteins in diabetic rats and the capacity of these lipoproteins to injure cells *in vitro*. Recently we have pursued the identities of the injurious lipid oxidation products on oxidized LDL and the mechanism by which these agents injure cells. The cholesterol hydroperoxide formed during *in vitro* LDL oxidation appears to be responsible for the predominant cell injuring capacity. The mechanism of cell injury is consistent with that seen by others for lipid hydroperoxide-induced cell death. Cell-associated metal ion chelators appear to retard cell death, as do general chain breaking antioxidants. Lipid peroxide inhibitors also inhibit the cell death rate. These findings offer tools to examine whether *in vivo* counterparts exist in the tissue injury accompanying diabetes.

C.3 Ethanol-inducible cytochrome P450 2E1. Mechanisms of regulation, radical formation and toxicological importance.

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Ethanol-inducible cytochrome P450 2E1 (CYP2E1) represents a major hepatic enzyme, in particular after ethanol-treatment. CYP2E1 is then induced mainly in the centrilobular liver region and its concentration in these hepatocytes can then reach 0.1 mM. CYP2E1 is also distributed in the CNS. Today, more than 75 specific substrates have been identified for CYP2E1, among them ethanol, acetaldehyde, organic solvents and dimethylnitrosoamine. It is likely that the synergistic effect of alcohol on the toxicity of these compounds is explained by CYP2E1 induction. The isozyme is leaky and effectively reduces dioxgen to radical species that effectively initiate lipid peroxidation. Dioxygen treatment of rats also cause a 4-fold induction of the isozyme and it is likely that the oxygen toxicity in part is mediated through CYP2E1. We believe that induction of CYP2E1 in the centrilobular liver region can cause oxidative stress.

In liver, CYP2E1 is mainly regulated at a posttranlational level. Isozyme-specific substrates protect the enzyme from a cAMP-dependent phosphorylation on Ser-129 and CYP2E1 is then slowly degraded according to the autophagosomal/lysosomal pathway. In the absence of substrate, Ser-phosphorylation causes heme loss and the apoprotein is rapidly degraded by a Mg-ATP-stimulated proteolytic system present in the endoplasmic reticulum. In CNS, the neuronal level of CYP2E1 is resistant to influence by ethanol, acetone and/or starvation. However, a marked induction of CYP2E1 is seen in astroglia cells after ischemia induced by occlusion of the carotid arteries. Similar induction is seen after lesions in the brain and suggests CYP2E1 to have a role in regeneration of damage in the CNS.

HEPATOTOXICITY OF EXPERIMENTAL HEMOGHROMATOSIS C.4

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In genetic hemochromatosis, the liver is the major recipient of the excess absorbed iron, and after several years of high tissue iron concentrations, fibrosis and, eventually, cirrhosis develop. Complications of cirrohsis are the most common causes of death in patients with genetic hemochromatosis. Despite the convincing clinical evidence for the hepatotoxicity of excess iron, the specific pathophysiologic mechanisms responsible for hepatocyte injury and hepatic fibrogenesis in chronic iron overload are poorly understood. In our laboratory, experimental hemochromatosis is achieved by feeding rats a diet supplemented with carbonyl (elemental) iron. The pattern of distribution and the degree of hepatic iron overload are qualitatively and quantitatively comparable to that seen in human genetic hemochromatosis. We have demonstrated evidence of iron-induced mitochondrial and microsomal lipid peroxidation and variety of associated organelle functional abnormalities, followed by portal fibrosis, in rats with chronic iron overload. Recent work from our laboratory has shown an increase in the hepatic levels of malondialdehyde (MDA), as well as significantly impaired mitochondrial metabolism of this product of peroxidized lipids, in iron-loaded rats. Additionally, Chojkier and coworkers have reported that MDA causes an increase in both collagen production and in procollagen I gene expression in cultured human fibroblasts. Taken together, these data suggest that aldehydic by products resulting from iron-induced lipid peroxidation could be an important initiating factor leading to increased hepatic fibrogenesis in iron overload. In the liver, lipocytes (Ito cells) represent the most likely source of the increased in hepatic MDA. Therefore, aldehydic peroxidation products may serve as link between hepatocellular lipid peroxidation and subsequent hepatic fibrosis in iron overload, either by directly stimulating lipocyte collagen production or by activating Kupffer cells to release profibrogenic cytokines.

C.5 GLUTATHIONE (GSH) SUPPLEMENTATION PREVENTS RAT'S LIVER DAMAGE FROM THE ACUTE CCL4 INTOXICATION

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In the present study we evaluated the protective activity of GSH against acute CCL4 intoxication in rats, especially in relation to liver fibrosis. 24 Sprague Dawley rats were treated with a single dose of CCL4 i.p. (0.25 mg/hg); 12 out the 24 rats were also pretreated with GSH i.p. (500 mg/kg) 5' before the intoxication. 6 untreated rats were the controls.
Total hepatic RNA were subjected to Northern Blot hybridization with the following cDNA clones:albumin, procollagen α1(I),TGF-β1 and GAPDH.
The densitometry scanning of the radiolabelled bands showed in the CCL4 treated animals a significant reduction of the albumin gene expression whilst in the GSH protected animals sacrificed 24 and 48 hrs after the intoxication, the mRNA levels were very close to those of the controls. A similar behaviour was observed for the procollagen α1 (I) and TGF-β 1 genes.
On the basis of these results we conclude that GSH supplementation protects the liver from the free radical injury.

C.6 Utilization of Oral Glutathione (GSH).

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Glutathione is thought to be one of the most important anticarcinogens in mammalian tissues, and significant health benefit may be obtained by increasing GSH concentrations in cells susceptible to chemical-induced injury and carcinogenesis. In recent years, we have investigated the bioavailability and utilization of dietary GSH in rodents, rabbits and humans to determine the feasibility of using GSH supplements to enhance tissue detoxication systems and to provide a data base to determine whether dietary GSH is associated with reduced risk of specific forms of cancer and other disease processes. The results show that the amount of GSH available in the diet is highly dependent upon food storage and preparation methods. Dietary supplementation with GSH increases plasma GSH, but this is under tight regulation with an apparent feed-back control from the intestine to regulate hepatic release of GSH into the blood. In the intestine, uptake of luminal GSH by the small intestinal enterocytes supports up to 80% of the detoxication of dietary electrophiles and lipid hydroperoxides. Thus, available data show that dietary GSH provides an important protection against toxic and potentially carcinogenic dietary chemicals in the intestine. Experimental and epidemiologic studies are currently underway to better define the physiological regulation of GSH utilization and the potential association of dietary GSH consumption with reduced disease risk.

C.7 ROLE OF GLUTATHIONE AS A THERAPEUTIC AGENT

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Increased free radical production and lipid peroxidation have been associated, during various pathological conditions, with an impairment of the hepatic antioxidant defence systems. In addition, the critical role played by glutathione (GSH) in the defence against oxidant stress as been well elucidated. Consequently, strategies involving antioxidant compounds or agents capable of restoring the normal hepatic GSH content, seem to be of great interest. Several studies have been carried out in our laboratory in order to evaluate the effectiveness of GSH as a therapeutic agent. In some experiments, the changes in the hepatobiliary homeostasis of GSH after i.p. administration of GSH were studied in rats depleted of the tripeptide by a prolonged fasting. Other studies include the evaluation of liver GSH and malondialdehyde content after oral GSH administration in rats acutely treated with ethanol. The effect of oral GSH administration on hepatic GSH content in animals treated with gamma-glutamiltranspeptidase (GGT) inhibitors was also evaluated. The data collected from all these studies show that oral (better than i.p.) GSH administration increases hepatic GSH levels after depletion by ethanol or prolonged fasting. The low hepatic GSH levels in animals pretreated with GGT inhibitors suggest a role of this enzyme in the extracellular degradation of GSH followed by an increase in cysteine availability and in GSH resynthesis.

Session 11

Food Antioxidants

11.1 MECHANISMS OF FOOD ANTIOXIDANTS

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Food antioxidants play a dual role: protecting the redox integrity of both foods and organisms, i.e., preventing food spoilage and the onset of degenerative diseases. Oxidative processes are inhibited by chelation of metals and breaking of chain peroxidation. Most chain-breaking antioxidants are phenolic compounds, with notable exceptions such as vitamin C. The efficacy of phenolic antioxidants under different physiological conditions (e.g., extracellular versus intracellular biochemical conditions) is governed by various parameters, such as polarity, configuration, redox properties, and kinetics. Redox potential, one of the major parameters, can be determined for phenolic antioxidants by use of the Hammett relationship and Brown substituent coefficients. These calculations are applicable to most natural antioxidants.[1] The efficacy of protection in vivo may be increased by sufficient availability of dietary antioxidants and protoantioxidants. A relationship between dietary caloric intake, antioxidant intake, and oxidative damage in vivo is based on measurements of the rate of DNA damage in humans.[2]

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11.2 CHANGES OF VITAMIN E CONTENT IN LIVER, PLASMA AND ADIPOSE TISSUE OF RATS, FOLLOWING CARBON TETRACHLORIDE INTOXICATION

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Vitamin E is probably the most efficient physiologic antioxidant in lipid environment. Its antioxidant activity is due mostly to its capacity to donate hydrogen atoms during the lipoperoxidative process. The oxidation of vitamin E has been demonstrated "in vitro", where the lack of other H-donor molecules hampers its regeneration leading to the consumption of the vitamin. "In vivo" the consumption of vitamin E during a lipoperoxidative process is difficult to evaluate because of the presence of those H-donor molecules able to regenerate the vitamin E (e.g. ascorbic acid). In this study we have measured the level of vitamin E in tissues of rats intoxicated with CCl_4, a well known lipid peroxidation inducing agent in the liver, at different times from the intoxication. Our results indicate that at 24 hours from the treatment, when lipid peroxidation is no longer detectable, a severe decrease of the vitamin E content occurs not only in the liver but, surprisingly also in plasma and adipose tissue, only indirectly involved in the toxic effects of the halogenoalkane, leading probably to a general imbalance of the cellular redox system of several organs. These results also show that there is an active transport of vitamin E stores from recipient-tissues such as plasma and adipose tissue to the liver when the latter is hit by an oxidative agent. We concluded that vitamin E supplementation would be important not only to prevent the damage caused by a free radical attack but also to avoid the consequent Vitamin E deficiency.

11.3 BHA – PRECURSOR OF A CHEMICAL MENU

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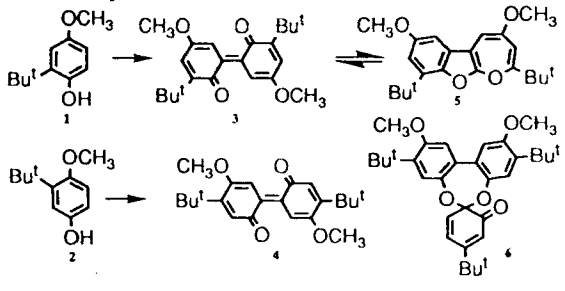
In inhibiting autoxidation the common food additive BHA is itself oxidised to a variety of products. The purpose of this paper, reviewing our work on the chemical oxidation of BHA, is to bring these oxidation products to the attention of medical scientists.

Both the hindered **1** and the unhindered **2** isomers of BHA are first oxidised to phenoxy radicals, which can dimerize to biphenyldiols, which also inhibit autoxidation, becoming further oxidised to the diphenoquinones **3** and **4**. Subsequent reactions of these quinones differ markedly. The hindered quinone **3** is in equilibrium with the oxepine **5**, and the reaction of this with singlet oxygen leads to ring cleavage. The unhindered quinone **4** gives rise both to the dioxepine **6** and to a complex molecule formed from six units of **2**. This dissociates reversibly into free radicals at ambient temperature.



11.4 RADICAL EXCHANGE REACTIONS BETWEEN VITAMIN E, VITAMIN C AND PHOSPHOLIPIDS IN AUTOXIDIZING POLYUNSATURATED LIPIDS.

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Antioxidant reactions of mixtures of Vitamin E, Vitamin C and phospholipids in autoxidizing lipids at 90°C have been studied by ESR spectroscopy. When the phospholipid contained a tertiary amine like phosphatidyl choline, the Vitamin C and the Vitamin E radicals were successively observed while these two Vitamins were sequentially oxidized. In the presence of the primary amine contained in phosphatidylserine, Vitamin C was almost immediately oxidized and the Vitamin E transformation was delayed for a few hours. Oxidation reactions led in that case neither to the observation of Vitamin C nor to Vitamin E radicals but to a nitroxide radical. The formation of this nitroxide radical can however not account for the important antioxidant properties of this mixture, as the same nitroxide can also be formed in systems with poor antioxidant activity such as Vitamin C and phospholipids mixtures.

The "in vivo" radical detoxification cascade, implying primarily Vitamin E and Vitamin C is indicative of the efficient of this mixture in biological system, but does not explain the excellent antioxidant activity of mixtures of Vitamins E, C and phospholipids in a lipid.

11.5 ANTIOXIDANT EFFECT OF CAROTENOIDS ON PHOTOSENSITIZED OXIDATION OF HUMAN BLOOD PLASMA AND LOW DENSITY LIPOPROTEIN

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Role of carotenoids as biological antioxidants is of much interest, because human accumulates a variety of carotenoids. This study was undertaken to estimate the antioxidant effect of carotenoids on photosensitized oxidation of human blood plasma. We used a water-soluble photosensitizer (methylene blue; MB) and a lipid-soluble photosensitizer (12-(1-pyrene)dodecanoic acid; P-12) as singlet oxygen generating system (Type II reaction). When human blood plasma was exposed to MB-sensitized photooxidation, phospholipid hydroperoxides (PL-OOH) and cholesterol ester hydroperoxides (CE-OOH) accumulated from the initial stage with decreasing concentration of α-tocopherol (α-Toc) and each carotenoid (lycopene, β-carotene, zeaxanthin/lutein). TBA reacting substances (TBARS) and CE-OOH increased rapidly after the depletion of α-Toc and carotenoids. Rapid increase of CE-OOH level with the depletion of α-Toc and carotenoids was also found in photosensitized oxidation of LDL. Depletion of zeaxanthin/lutein was slower in P-12 sensitized photooxidation, although these carotenoids and α-Toc consumed at the similar rate in the case of MB. These results suggest that each carotenoid acts as antioxidant on photosensitized oxidation in the plasma and LDL. It is likely that their effectiveness depends on their structural properties and the site of active oxygen to be trapped.

11.6 LOW PLASMA BETA-CAROTENE, ALPHA-TOCOPHEROL AND SELENIUM LEVELS ASSOCIATE WITH ACCELERATED CAROTID ATHEROGENESIS IN HYPERCHOLESTEROLEMIC MEN.

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We investigated the relationship of plasma β-carotene (CAR), α-tocopherol (TOC) and selenium (Se) concentrations to the progression of carotid atherosclerosis in 216 men with serum LDL cholesterol ≥4.0 mmol/l. Intima-media thickness (IMT) in common carotid arteries and carotid bulbs was measured twice in 12 months by B-mode ultrasonography. CAR and TOC were determined from fresh plasma samples using HPLC and Se with AAS. When adjusting for age, S-T_3 and LDL cholesterol concentrations, TG and protein content of VLDL and LDL, and systolic BP, there were inverse associations between CAR (regression coefficient β -0.148, p=0.012) and TOC (β -0.142, p=0.018) and IMT increase. There was a similar trend for Se (β -0.111, p=0.066). The mean adjusted IMT increase was 92% (p=0.028) greater in the lowest (≤ 0.27 μmol/l) than in the highest (≥ 0.64 μmol/l) quartile of CAR and 78% (p=0.045) greater in the lowest (≤ 22.5 μmol/l) vs. the uppest (≥ 32.7 μmol/l) quartile of TOC. These data suggest that antioxidants may have an important role in human atherogenesis.

11.7 A RAPID, SENSITIVE AND SPECIFIC METHOD FOR MEASURING MDA IN FOOD OR IN BIOLOGICAL MATERIALS

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Malondialdehyde (MDA) is one of the most studied products of lipid peroxidation. It is excreted in urine and is an indicator of lipid peroxidation in the diet and/or formed in the tissues

The classical thiobarbituric acid (TBA) test has been widely used to measure MDA. MDA-TBA chromogens were isolated and quantified by HPLC. However, in our hands with biological samples such as urine or food the resolution was not adequate. To eliminate these interfering MDA-TBA chromogens which had spectra overlapping.

We modified this classical methode. TBA reagent was replaced by diethyl TBA (DETBA). The chromogen MDA-DETBA, less polar than MDA-TBA was easily isolated by solid phase extraction using C18 cartridge and quantified by HPLC without interference at picomole levels using a fluorescent detector. The complete methodology using DETBA will be given and compared with that using TBA. Results concerning MDA in milk, in urine from traumatised patients and in thrombin activated platelets will also be presented.

11.8 FREE RADICALS IN COFFEE AND SUBSTITUTES

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Coffee, Ecco, Caro and Cocoa have been partially fractionated to isolate components showing ESR signals. Solutions were treated with XAD-2 resin, which adsorbs most organic compounds from aqueous solution or suspension. The resin was then eluted with methanol/water (80:20) and the extract evaporated to leave a solid residue which was separated into methanol soluble and methanol insoluble fractions. The methanol insoluble material in all cases showed the strongest ESR signal. This material appears, surprisingly, to be non-aromatic.
The methanol soluble fractions showed either very weak or no ESR signals. In the case of coffee, further chromatography indicated the presence of feruloyl quinides, but these were not found in the other beverages.
We are now working on further fractionation and characterisation of the components responsible for the ESR signals.

11.9 ANTIOXIDANT PROPERTIES OF ETHANOLIC EXTRACT OF PARIPAROBA (POTHOMORPHE UMBELLATA (L.) Miq.)

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Oxidative stress has been implied in many pathological process as inflammation, cancer, aging and cell death. The brazilian flora is particularly rich in medicinal plants employed for many purposes. Among them the Piperaceae family and specially the Pariparobas are widely employed for liver diseases. This study was conducted in order to investigate the "in vitro" and "in vivo" antioxidant capacity of Pariparoba - Pothomorphe umbellata. Rat brain homogenates were submitted to different concentration after 1 hour incubation at 37°C with and without ethanolic extract dissolved in phosphate buffer pH 7.0. The $Q_{1/2}$ (concentration required to decrease 50% of the oxidation in the absence of the extract) for root, stem and leaf were 4.45, 18.68 and 40,46 ug/ml) respectively. Visible chemiluminescence emission associated with the autoxidation of brain homogenate was also measured in the presence of different concentrations of root extracts (the most potent one concerning antioxidant activity). A correlation coefficient of 0.9476 was obtained with concentrations varying from 2.1 to 8.3 ug/ml. "In vivo" antioxidant activity was measured as plasma antioxidant capacity(PAC). Rats receiving roots extracts(3 doses of 24 mg/kg b.w. in 24h) showed 26% increase in PAC when compared with untreated animals.

11.10 REACTION OF β-CAROTENE WITH AN ALKYLPEROXYL RADICAL IN BENZENE

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β-Carotene is sensitive to oxidation and thought to trap lipid-peroxyl radicals because it inhibits lipid peroxidation in vitro and in vivo. Its oxidation in foods or living cells is most likely a result of its reaction with lipid-peroxyl radicals. To elucidate the antioxidative mechanism of β-carotene, we have studied the reaction products of β-carotene with an alkylperoxyl radical.

β-Carotene was reacted with an alkylperoxyl radical at 37°C in benzene. 2,2'-Azobis(2,4-dimethylvaleronitrile) was used to genarate the alkylperoxyl radicals. The reaction products were isolated by reverse-phase and normal-phase HPLC. Their structures were characterized by ultraviolet, ^{1}H and ^{13}C nuclear megnetic resonances and mass spectrometry. They were 12-formyl-11-nor-β,β-carotene (1), 19-oxomethyl-10-nor-β,β-carotene (2), 5,6-epoxy-5,6-dihydro-β,β-carotene (3), 13,15'-epoxyvinyleno-13,15'-dihydro-14,15-dinor-β,β-carotene (4a), 15',13-epoxyvinyleno-13,15'-dihydro-14,15-dinor-β,β-carotene (4b) and 11,15'-cyclo-12,15-epoxy-11,12,15,15'-tetrahydro-β,β-carotene (5). The results indicate that the peroxyl radicals can attack to every double bonds of the conjugated polyene structure.

11.11 **ANTIOXIDANT AND PRO-OXIDANT ACTIONS OF DIETARY COMPONENTS**

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Antioxidant are of interest to food scientists and food manufacturers. Control of lipid peroxidation in foods containing lipids is often achieved by addition of antioxidants. There is an increasing interest in the use of 'natural' antioxidants, such as tocopherols, flavonoids or rosemary extract, in the preservation of food material.

The antioxidant activity of food additives is frequently assessed only in lipid systems. However, phenolic antioxidants such as propyl gallate, BHT, BHA, flavonoids and gossypol are capable of stimulating free radical damage to non-lipid components such as carbohydrates and DNA. Hence they can cause damage in biological systems different from those in which they exert protection. We will discuss the pro-oxidant/antioxidant actions of food additives and components and methods for the characterization of the properties of new 'antioxidants'. Until these problems are elucidated, great care should be taken in the fortification of foods with flavonoids and other phenolic compounds.

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ARUOMA O I & HALLIWELL B (1991) Free Radicals and Food Additives. Taylor & Francis: London.
HALLIWELL B (1990) How to characterize a biological antioxidant Free Radical Res. Commun. 9: 1-32

11.12 ANTIOXIDANT AND PROOXIDANT EFFECT OF BILE-PANCREATIC JUICE ON PHOSPHOLIPID PEROXIDATION

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Mastication and digestion of ingested foods seem to promote lipid peroxidation leading to oxidative damages in gastrointestinal tracts. The purpose of this work is to know whether or not the secretion of digestive fluids in duodenum participate in the antioxidant defenses against such damages. Liposomal suspension of soybean phosphatidylcholine (PC, final conc.:6 mM) was mixed with rat bile-pancreatic juice (RBP) and peroxidized by a water-soluble radical initiator or Fe^{2+}/lipid hydroperoxides. The peroxidation products, PC-hydroperoxides (PC-OOH) and/or TBA reacting substances, were measured by using HPLC. In both reaction systems, RBP promoted lipid peroxidation at lower concentration (5 %, v/v) and inhibited at higher concentration (50 %, v/v). Addition of sodium deoxycholate to the liposomal suspension exhibited this biphasic effect depending on its concentration (1-100 mM). TLC and HPLC analyses show that fatty acid hydroperoxides (FA-OOH) is released from PC-OOH by phospholipase activity of pancreatic juice and that FA-OOH disappears rapidly without converting to hydroxy fatty acid derivative. These results suggest that bile-pancreatic juice exerts both antioxidant and prooxidant effect depending on the concentration of bile salt. It is unlikely that PC-OOH and/or FA-OOH accumulate in this extracelluar fluid as a result of phospholipid peroxidation.

Session 12

Lipid Peroxidation

12.1 EFFECTS OF PRO- AND ANTIOXIDANTS ON SUPEROXIDE PRODUCTION AND LIPID PEROXIDATION

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Contrary to previous data based on cytochrome c assay, lucigenin-dependent chemiluminescence test shows a high level of superoxide production by rat liver microsomes. Doxorubicin inhibited superoxide production and enhanced microsomal lipid peroxidation. Another "pro-oxidant" quinone menadione inhibited both processes. The flavonoid rutin and its metal complexes also inhibited both processes, and their inhibitory activity correlated well with the ability to dismute superoxide ion. We conclude that superoxide ion is a genuine initiator of lipid peroxidation in microsomes, its major role being the reduction of Fe(III) into active Fe(II) ions. Doxorubicin may enhance lipid peroxidation chelating Fe(III) to form more reducible Fe(III) complex. However, menadione, which is unable to bind metal ions, can only inhibit peroxidation reacting with superoxide ion. The ability of "pro-oxidant" quinones to enhance superoxide production in microsomal membranes seems now questionable.

12.2 METABOLISM OF THE LIPID PEROXIDATION PRODUCT 4-HYDROXYNONENAL IN LIVER AND SMALL INTESTINE

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4-Hydroxynonenal (HNE) is a major product formed by lipid peroxidation of ω-6 polyunsaturated fatty acids such as linoleic acid and arachidonic acid. It exhibits cytotoxic, mutagenic, and genotoxic properties. During reoxygenation of isolated rat hepatocytes or rat small intestine following 60 min of anoxia/ischemia an increase of the HNE concentration was observed. Nevertheless, the increase of the HNE tissue level was short-termed. It seems to reflect only a part of the drastic acceleration of the HNE formation rate under conditions of ischemia and reoxygenation. The rapid disappearance of endogeneous formed HNE is probably due to immediate reactions of the HNE with cellular compounds and the metabolism of HNE. Therefore, the fate of HNE which was exogeneously added to cells was studied. It was shown that HNE was rapidly metabolized in all cell types which were investigated. In cell suspension with 10^6 cells/ml at 37° and pH 7.4 about 95% (hepatocytes) or 75% (enterocytes of the small intestine) of 100 μM HNE added were consumed after 3 min of incubation. In both rat hepatocytes and enterocytes we investigated different pathways of the HNE metabolism. The primary metabolic products of HNE which were identified in hepatocytes and enterocytes were the glutathione-HNE-1:1-conjugate, 4-hydroxynonenoic acid, and 1,4-dihydroxynonene as corresponding alcohol of HNE. With increasing duration of the incubation the fraction of secondary HNE metabolites steadily increased. As secondary products until now the glutathione-dihydroxynonene conjugate and water, which is formed in the β-oxidation of the hydroxynonenoic acid, were identified. Additionally, the protein binding of HNE was quantified. The rapid metabolism of HNE and other aldehydic products of lipid peroxidation could be an important part of the intracellular antioxidative defense system.

12.3 HYDROXYSTEARIC ACID: AN UNUSUAL LIPID PEROXIDATION PRODUCT AFFECTING IN VITRO CELL PROLIFERATION.

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Lipid peroxidation products may affect cell proliferation. Recently we have identified in whole cells lipid extracts from murine C108 Lewis lung carcinoma two unusual derivatives of stearic acid, characterized by an hydroxyl group position either 9 or 10 (HSA). The content of these products was inversely correlated to the percent of cell density; furthermore, the HSA in vitro administration to C108 cells was affecting cell proliferation and cell viability (1,2).

We report here further results obtained by studying human cell lines, i.e. HT29 colon carcinoma cells and I407 embryonal intestine used as a control. Both lines have the same embryonal origin as murine C108. In the human cells HSA still inhibits proliferation in a dose and a time dependent manner; moreover, the inhibitory effect is completely reversible. Flow cytometric analysis of their cell cycle showed that human HT-29 are specifically blocked in G1, while murine C108 are specifically blocked in G2-M.

In order to study HSA uptake at the different exogenous concentrations and its subcellular localization, we synthesized radioactive labeled ^{3}H-HSA by hydration of methyl oleate. In C108 cells HSA incorporation increases from 1 μg/10^6 cells (exogenous 10 μM HSA, 5 days administration) to 3 μg/10^6 cells (exogenous 50 μM HSA, 5 days administration). At these concentrations HSA is cytostatic. 100 μM exogenous HSA is, in contrast, strongly cytotoxic and its uptake increases to 6.5 μg /10^6 cells after 3 days administration.

From these studies we hypotize that HSA shows to be an additional useful reagent for investigating cell cycle kinetics and a new agent with antiproliferative activity. Studies are in progress to clarify which mechanism underlies the observed blocks in specific phases of the cell cycle.

1)*Cavalli et al.*, Biochem.Biophys.Res.Commun., 178, 1260-1265,1991.
2) *Casali et al.*, Cellular and Molecular Biology, 1992, in press.

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12.4 POLYUNSATURATED FATTY ACID PEROXIDATION MECHANISM SPECIFIC TO HUMAN BREAST CANCER CELLS (ZR-75-1) PRODUCING SELECTIVE CANCER CELL - KILLING IN RESPONSE TO GAMMA- LINOLENIC ACID + Fe (II)

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Gamma-linolenic acid: 6, 9, 12 cis-octadecatrienoic acid is an essential fatty acid which selectively kills human cultured cancer cells in a dose dependent manner. Polynsaturated fatty acid peroxidation in cancer cells was closely associated with this selective killing effect. Therefore we investigated by Gas chromatography-mass spectrometry and high performance liquid chromatography the mechanism of polynsaturated fatty acid peroxidation in human breast cancer (ZR-75-1) cells in comparison to human normal skin fibroblast (CDD-41Sk) exposed to gamma-linolenic acid with ferrous iron. Peroxidation of polyunsaturated fatty acid was indicated by UV absorption in the 235 nm region in lipid extracts from ZR cells but not from 41Sk cells. The peroxyl group was identified by gas chromatography-mass spectrometry analysis both of triphenylphosphine oxide formed from the peroxide oxidation of triphenylphosphine and of the derivatized polyunsaturated fatty acids as fatty alcohols. The sample was methyl esterified, hydrogenated and silylated (tert- butyldimethylsilyl ether). These derivatives gave mass spectra-indicating 20 carbon hydroxy saturated fatty acids (15-OH, 12-OH and 8-OH regional isomers) and an 18 carbon hydroxy saturated fatty acid (13-OH). These results indicate that peroxidations are occurring at the 15, 12- and 8- carbon positions of 20 carbon and at the 13-carbon position of 18 carbon polyunsaturated fatty acids respectively in ZR-75-1 cells. These reactions are not detectable in normal cells. Therefore a peroxidation mechanism specific to cancer cells was identified. This suggests the possibility of use of gamma-linolenic acid as a highly selective anticancer agent.

12.5 EFFECTS OF OXYSTEROLS ON ARACHIDONATE METABOLISM AND CALCIUM FLUX IN PLATELET.

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Oxysterols (OS) produced from cholesterol (CHOL) have numerous biologicals activities mostly related to atherogenicity. We have previously indicated that 25-hydroxy-CHOL (25OH) inhibited platelet-induced aggregation and secretion whereas 22-hydroxy-CHOL (22OH), CHOL-5,6α-epoxide (EPOX) and cholestane-3β,5α,6β-triol (TRIOL) potentiated these parameters (Thromb Res (1988) 50 221). We now investigated the effects of these compounds on arachidonate (AA) release and metabolism and on Ca^{2+} permeability. OS have been incorporated as albumin-bound complexes into washed rat platelets prelabeled with [^{14}C]-AA. Results indicate that upon thrombin-stimulation, the 25OH-loaded platelets produced less thromboxane B_2 than control platelets. By contrast, the platelets loaded with TRIOL, EPOX and 22OH released more AA and metabolites. Also, the kinetic study of the passive calcium diffusion by either 25OH- or 22OH-loaded platelets showed a slight decrease or a drastic increase of calcium binding, respectively. Moreover, results of the thrombin-induced Ca^{2+} uptake by OS-loaded platelets have shown an increase uptake with 22OH and a decrease with 25OH. From these data, we conclude that variations of Ca^{2+} movements might explain the reported modulation of platelet activity after *in vitro* incorporation. Since OS can be produced endogenously or ingested from diet, these data might be relevant in vascular diseases.

12.6 SPONTANEOUS VISIBLE URINARY CHEMILUMINESCENCE AND OXIDATIVE STRESS.

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The spontaneous visible urinary chemiluminescence is considered to be due to the decomposition of oxidized biomolecules. As such it can be expected to be a marker of in vivo oxidative stress. Urinary chemiluminescence levels have been measured at pH = 7, in hyperthyroid subjects, smokers and children with Duchene molecular distrophy. For each of these groups, the emitted chemiluminescence levels are greater than those measured for the corresponding controls.

12.7 ARE DIOXETANES CHEMILUMINESCENT INTERMEDIATES IN LIPOPEROXIDATION?

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Lipid peroxidation has been pointed out as a key chemical event of the oxidative stress associated with several inborn and acquired disorders. Disruption of organelle and cell membranes together with calcium homeostasis alterations are main supramolecular events linked to lipoperoxidation. Triplet carbonyls and $^1O_2$ are the putative emitters of the chemiluminescence that accompanies the lipoperoxidation (Russell mechanism). However, it is long known that $^1O_2$ reacts with α-hydrogen-containing olefins to yield the corresponding ene-hydroperoxide (1,3-ene reaction). We re-study here the reaction between linoleic acid and $^1O_2$ generated both photochemically and by thermolysis of 1,4-dimethylnaphthalene endoperoxide (1,4-DMNO$_2$). We have found by ^{1}H NMR spectroscopy that the main products are indeed ene-hydroperoxides, after equimolar addition of $^1O_2$. A minor chemiluminescent product is indeed formed along with the products under heating at 37°C, although with a very low quantum yield ($\phi = 1x10^{-7}$). No energy transfer to 9,10-dibromoanthracene is observed. Hexanal is the expected major product, probably governed by Hock cleavage. Photooxygenation of tetraethylethylene is shown to form 0.2% of the corresponding dioxetane. This is in line with previous data demontrating that formation of dioxetane during 2 + 2 cycloaddition is also formed by steric hindrance. Altogether these results reinforce the fact that light emission arising from lipoperoxidation can not be attributed to a dioxetane as a significative abundant intermediate.

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12.8 CRITICAL ASPECTS OF ONLINE DIENE MONITORING

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Conjugated Dienes are the first products after initiating lipid peroxidation in lipoproteins and thus the assay of continuous monitoring their 234 nm absorbance in vitro was improved to analyze six LDL samples in parallel. Fast preparation of all lipoproteins together with minimizing impurities is achieved by step gradient ultracentrifugation in swingout rotor. To circumvent uncontrolled oxidation, gel permeation chromatography replaces dialysis to remove additives, used for preparation of lipoproteins. Prepacked desalting columns (Econo-Pac 10DG, BioRad) proved especially useful to remove small molecules like salts, antibiotics or EDTA, within 5 min, compared to 15 to 20 hrs, by dialysis. A photometer with six fold automatical cell positioner together with computerized data acquisition combines high time resolution (20 sec. minimum) with several hours monitoring (typical 6 hrs.). Only small amounts of sample (0.25 mg LDL mass, equals 50 µg LDL protein, 1 ml) are required to obtain full information of the time-course of lipid peroxidation. Substances absorbing in the 234 nm region will intefere considerably with this method. A wide variety of oxidation initiators (e.g. metal ions, organic radical initiators or enzymes) watersoluble antioxidants (abscorbate, urate, glutathione etc.) as well as lipid soluble like tocopherols, carotenoides or probucol were studied. It is known that reasonable alterations in the lipoprotein particles take place after the dienes reached their maximal concentration (decomposition phase). Especially immunological methods proved useful to indicate protein-aldehyde adducts involved in these changes. Nevertheless all these alterations are triggered at the very beginning of oxidative stress (lag phase).

12.9 STAGES OF DEVELOPMENT OF LIPID PEROXIDATION IN ORGANISM

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Activation of the lipid peroxidation is an important part of structural and functional alterations of the biological membranes in stress or in cases of any other pathology. Still, results, obtained in the same study sometimes vary significantly. Different levels of lipid peroxidation can be demonstrated in the same disease. It may be explained as a methodological mistake and cause certain problems in interpretation.

We analysed lipid peroxidation in cases of different experimental pathologies - induction of liver monooxygenase by phenobarbital, stimulation of mononuclear phagocytes by zimozan in the situation of the stress, called by the coldness or in cases of pathological pregnancy. We found certain stages in the development of lipid peroxidation reactivity in cell and tissues, which may influence experimental results. This hypothesis is further confirmed by the analysis of the complications of pregnancy in the ecologically altered regions.

12.10 LIPID OXIDATION IN MEMBRANE BILAYERS: EFFECT OF CARDIOLIPIN

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This study utilized various oxidant model systems in order to elucidate the formation of different conjugated dienes species during lipid peroxidation. Large unilamellar vesicles consisting of either 1-palmitoyl 2-linoleoyl phosphatidylcholine (PLPC) or PLPC + 10-20% cardiolipin were used as a model membrane, and the oxidizing system included Fe(III) - ascorbate, cupper (II) - ascorbate, lipoxydase IV, and autoxidation. Kinetics and identification of the peroxidation species was done by second derivative absorption spectroscopy. Total conjugated dienes formation was more rapid in vesicles containing cardiolipin in all oxidizing system. Electron spin resonance spectroscopy - spin trapping technique has concurrently shown the formation of carbon centered radicals. Membrane bilayer organization was investigated by multifrequency phase correlation fluorometry which measured the life time of the fluorescent probe, 1-6 diphenyl 1,3,5 hexatriene (DPH). This technique showed that oxidized cardiolipin has a more perturbing effect on bilayer organization than oxidized PLPC. It is proposed that oxidized cardiolipin may increase microeterogeneity thereby facilitating lipid peroxidation propagation.

12.11 LIPID PEROXIDATION INDUCED BY HYPERBARIC OXYGEN IN RATS AND THE ANTIOXIDATIVE EFFECT OF SOD

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Lipid peroxidation is main way of oxygen toxicity in clinic oxygen therapy. The different pressure and exposed time hyperbaric oxygen on whole animal was studied. The LPO contents and SOD activity of blood, brain and heart in rats exposed to 100% oxygen and air at 3.5, 2.0 ATA for 30 min and 2.0 ATA for 30,60, 120,240 min were investigated. The protective of extraneous SOD were observed, which injected by ip. in range of 0.25-1.25 mg/kg wt in rats exposed to 2.0 ATA for 120 min. The results showed that routine pressure (2.0 ATA) and duration of exposure (120 min) induced significant increase of LPO contents and decrease of SOD activity. There was a significant positive correlation between lipid peroxidation rate and pressure magnitude, exposed time in hyperbaric chamber. The effect of pure 0_2 was greater than air. Exposed to same pressure (2.0 ATA), the LPO content increased with exposed time, whole SOD activity increased within 60 min and further prolongation of time SOD activity on the contrary decreased significantly. It also showed brain had more sensitive to hyperoxia stress than myocardium. Extraneous SOD could inhibit lipid peroxidation which have a dose-response realationship in range of 0.25-1.0 mg/kg wt. It suggested we should pay attention to pressure and duration of exposure in hyperbaric oxygen therapy. Antioxidants could be used in protecting oxygen toxicity.

12.12 LIPID PEROXIDATION PRODUCTS IN HUMAN PLASMA

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Lipid hydroperoxides (LOOH), conjugated dienes (CD) and aldehydes were measured in healthy human plasma using sensitive, highly specific HPLC techniques to separate the various classes. LOOH were undetectable using HPLC (0.03 uM detection limit) although uM amounts of thiobarbituric acid reactive substances (TBARS) were found and levels correlated well with total plasma lipids ($P < 0.005$). The CD of hydroxyeicosatetraenoic acid was found in uM amounts and the chromophore absorbed maximally at 241-243 nm suggesting the cis-trans configuration. In females, CD and TBARS were maximal in the second half of the menstrual cycle. Human plasma contained 4-Hydroxynonenal (HNE) and alkanals with 2-10 carbons. Total aldehyde levels were 5-fold higher in males than females ($P < 0.001$). HNE, acetaldehyde propanal and butanal were present in uM amounts whereas nM levels of the more lipophilic alkanals were found. Acetaldehyde was predominant and was detected in all samples, as was pentanal. Propanal, butanal, octanal and HNE were more common in male plasma. Different classes of lipid peroxidation products can thus be detected in normal human plasma using these HPLC techniques: their application to human diseases which involve oxidative stress should prove useful.

12.13 EFFECT OF ALDEHYDES PRODUCED DURING THE LIPID PEROXIDATION ON HSPs SYNTHESIS IN HeLa CELLS.

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After our demonstration that lipoperoxidative damage in rat and human cells induces the synthesis of a subset of Heat Shock Proteins (HSPs), we have shown that 4-HNE, a major product of lipid peroxidation, stimulates the synthesis of some proteins of the HSPs family, and induces the HSF-DNA binding activity. In this work we have studied the induction of HSPs, in HeLa cells by other aldehydes produced during the lipid peroxidation. The aim of the work was to evidentiate a possible role of these compounds in the HSPs synthesis observed in the lipid peroxidation. The action of the possible metabolic products of these aldehydes was also assayed. The obtained results show that only 4-hydroxy α,β unsaturated aldehydes are able to activate HSPs synthesis and HSF-DNA binding. The different behaviour of 4-hydroxilated aldehydes compared with other assayed products could be explained considering their reactivity on thiolic groups shown by H. Esterbauer.

12.14 DETERMINATION OF MALONALDEHYDE (MDA) IN THE RAT BRAIN BY HPLC.

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The most commom method to evaluate lipid peroxidation is the spectrophotometric assay of MDA based on the reaction with thiobarbituric acid. However, this method lacks specificity and has other limitations (1). We have applied to the brain an HPLC method (2) with minor modifications. The MDA is eluted from an aminophase column with Tris buffer-acetonitrile and monitored at 267 nm. Linearity is between 2.5-1000 pmol in 20 µl. Recovery is 95%. This method was applied to the MDA assay in the homogenate of whole brain or various brain areas after incubation at 37°C either in unstressed conditions or after in vitro oxidative stress.
MDA level was below the sensitivity of the method in the non-incubated samples.
The increase in MDA was time-related at least up to 1 h either in the samples incubated without any stimulation or in those stimulated with $FeCl_2$, Fe-saccharate or xanthine-xanthine oxidase. The in vitro inhibitory effect of some antioxidants was also assessed. This method is quantitative and specific for MDA and appears to be particularly suitable for studying lipoperoxidation and antioxidants.

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12.15 "IMAGING" OF OXIDANT-STRESS-DERIVED CARBONYLS BY MEANS OF CONFOCAL FLUORESCENCE MICROSCOPY

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Biochemical methods for the detection of various aspects of oxidant stress in vivo cannot provide information on the distribution of phenomena in situ, in tissues of heterogeneous cell composition, or rather in subcellular structures of isolated cells. Nevertheless, this kind of information would be of considerable value in a number of experimental as well as spontaneous conditions of oxidant stress. Previous work from our laboratory showed that lipid peroxidation (LPO) can be revealed in tissues histochemically, by means of reagents for the carbonyl functions originating in phospholipids during the process. In tissue protein, besides the binding of LPO- derived aldehydes, carbonyls may also result from direct oxidation of aminoacid residues. The effeciency of the histochemical approach can be enhanced by the combined use of fluorescent carbonyl reagents and confocal fluorescence microscopy with image analysis. Experiments were carried out with isolated hepatocytes exposed to known prooxidants - such as haloalkanes - as well as with tissue sections obtained from animals exposed to prooxidants in vivo. In all instances, images obtained are consistent with the known patterns of the model conditions studied, and can give indications on the subcellular sites primarily involved in the oxidative changes.

12.16 IMPROVED FLUOROMETRIC DETERMINATION OF MALONALDEHYDE IN BIOLOGICAL SAMPLES USING SYNCHRONOUS FLUORESCENCE

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Lipoperoxidation is implicated in various pathological conditions. The most commonly used marker of this process is malonaldehyde (MDA) expressed as thiobarbituric acid reactive substances (TBARS). We propose a modification of the Yagi's fluorometric assay. We used synchronous fluorescence, which eliminates the interference of the Rayleigh diffusion due to the effect of solvent diffusion. Under these conditions, we increased sensitivity and particularly specificity of the assay. This procedure allowed us to point out interferences due to unknown TBARS, that lead to overestimate the results, when classic fluorescence is used for measurement. We could quantify these interferences on a second order derivative curve of the record. This improvement allowed us to avoid the long and tedious manipulations of Yagi's method, by eliminating, like in HPLC methods, the first precipitation and washing steps. The results obtained by this improved technique are well correlated with those obtained by HPLC method. Thus, this simple and rapid method seems well adaptated for the screening of a large number of samples.

12.17 FLOW-CYTOMETRIC ASSAYS OF THE AGE-RELATED RESPONSES OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES TO PEROXIDATIVE STRESS.

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In previous papers we reported that peripheral blood lymphocytes (PBL) from donors of different age exposed to exogenous peroxidative damage showed age-related, dose dependent structural and functional damages. These included modification of the membrane order and fluidity, decreased proliferative capability and lower energy metabolism, the PBL of older donor being more affected than the younger ones. However the oxidative damage of cellular lipids was chemically undetectable (1,2).
In this study human PBL from healthy donors between 18 and 85 years of age were peroxidized using increasing amounts of hypoxantine in a range between 2.5 and 100 μM. The oxygen free radical (O.R.) probe 2,7-dichlorodihydrofluoresceindiacetate (DCIFDA) was used to detect the intracellular presence of O.R. after the peroxidative stress: both the percentage of fluorescent cell and the fluorescence intensity were age-dependent: they were higher in lymphocytes from young donors, indicating an higher peroxidability of these cells. Furthermore we detected that, after peroxidative stress, there was an increase in both B and T lymphocytes. Such an increase was higher for the younger donors. Finally we followed the changes in static and dinamic properties of the membrane by means of time resolved fluorescence anisotropy of propanoyl-DPH. The measurements showed that the plasma membrane from older donors PBL was more rigid.

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12.18 LIPID PEROXIDATION IN VITAMIN E DEFICIENCY IS ASSOCIATED WITH JEJUNAL HYPERSECRETION

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Malnourished infants commonly suffer from chronic diarrhoea and have reduced defences against free radical derived oxidative stress. In an animal model of chronic vitamin E deficiency, we have studied the effect of lipid peroxidation on small intestinal electrogenic anion secretion and on the biophysical characteristics of brush border membranes (BBM). Jejunal mucosae from 12 month old vitamin E deficient (E-) and sufficient (E+) male Wistar rats were studied. Vitamin E was undetectable in mucosae from E- animals. Malondialdehyde (MDA), measured in mucosal scrapings by high performance liquid chromatography, was significantly greater in E- jejuna [307 ±96 (95% confidence interval) vs 100 ±20 pmol/mg protein, n=11, P<0.01]. Membrane order determined by steady state fluorescence anisotropy using diphenylhexatriene (DPH) and 12 anthroloxy stearic acid (12-AS), showed E- BBM to be more ordered [DPH anisotropy = 0.231 ±0.004 vs 0.209 ±0.006, n = 12, P<0.01; 12AS anisotropy 0.0967 ±0.0004 vs 0.0902 ±0.0029, n=12, P<0.01]. Fatty acid composition (mol %) of BBM revealed only minor differences between the two groups. The lipid / protein ratio of BBM was the same in both groups. Basal short circuit current (Isc), representing electrogenic anion secretion measured in unstripped jejunum in vitro, was higher in E- animals [91μA/cm² ±10 vs 73μA/cm² ±6, n=40, P<0.05]. Basal Isc correlated strongly with mucosal [MDA] (r=0.87 P<0.0001) and 12AS anisotropy (r = 0.80, P<0.01). Increased lipid peroxidation in vitamin E deficiency is therefore associated with increased BBM order and increased electrogenic anion secretion in rat jejunum. This may be a mechanism in perpetuating diarrhoea in malnourished infants with diarrhoeal diseases.

12.19 TAXOL PARTIALLY PROTECTS MICROTUBULES BY IMPAIRMENT OF 4-HYDROXYNONENAL

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Lipid peroxidation produces various aldehydes: among them 4-hydroxynonenal is one of the main products. It has an antiproliferative effect, which may also be due to an interaction with the microtubular system. In fact, colchicine binding activity and polymerization of purified tubulin are decreased after incubation with the aldehyde, as far as -SH groups of tubulin, fundamental for polymerization, are reduced. Taxol is an anticancer drug which promotes tubulin assembly, stabilizes microtubules and inhibits their depolymerization; furthermore, it prevents the effect of depolymerizing compounds. Taxol does not significantly prevent the aldehyde inhibition on 'in vitro' activities of purified microtubular protein, but its effect on cultured cells is quite different. Microtubules distribution and organization in SV40-transformed 3T3 fibroblasts is completely altered after treatment with 4-hydroxynonenal, with reduction of microtubules number and length. Instead, when fibroblasts are incubated with the aldehyde after treatment with taxol, microtubular distribution remained more similar to untreated cells, probably as a consequence of the stabilizing activity of the drug.

12.20 RELATIONSHIPS BETWEEN ESSENTIAL FATTY ACIDS AND VITAMIN E DEFICIENCY IN CYSTIC FIBROSIS

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Several mechanisms have been discussed as causing essential fatty acid deficiency (EFAD) in cystic fibrosis (CF). We postulated that EFAD is due to impaired antioxidant protection, resulting in enhanced lipid peroxidation. 24 CF patients had plasma fatty acid and α-tocopherol (α-T) determinations. There was a multiplicative relationship between C20:4n-6 and α-T ($y=ax^b$, $\alpha=0.01$), and between the double bond index and the ratios C18:2n-6/C20:4n-6, C18:1n-9/C20:4n-6, C18:0/C20:4n-6, C16:0/C20:4n-6 and C18:0/C18:2n-6 on one hand and α-T on the other. A similar relationship was found between C20:3n-9/C20:4n-6 and α-T, however, this ratio, proposed as index of EFAD, was within the normal range even in the presence of extremely low α-T. A normal trien/tetraen ratio in the presence of EFAD has been reported for CF patients. These data suggest that, in contrast to findings in dietary EFAD, both numerator and denominator of this ratio may undergo increased peroxidation in the presence of vitamin E deficiency. To prove this assumption, parameters of lipid peroxidation in these patients are currently under investigation.

12.21 POSSIBLE INVOLVEMENT OF MYELOPEROXIDASE IN LIPID PEROXIDATION: STUDIES WITH NEUTROPHILS AND LOW DENSITY LIPOPROTEIN

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Myeloperoxidase (MPO) is an enzyme released by stimulated neutrophils which gives rise to the formation of highly reactive species like hypochlorous acid. The possible involvement of MPO in lipid peroxidation was studied using liposomes, neutrophils and low density lipoprotein (LDL). Exposure of liposomes to the MPO-H_2O_2-Cl^- system results in the formation of thiobarbituric acid reactive substances (TBArS) and the chemotactic aldehyde 4-hydroxynonenal (HNE). HNE formation was in the micromolar range, where chemotactic effects might be expected. Rat neutrophils stimulated by polystyrene granules oxidize liposomes as shown by the TBArS test. This process is inhibited by KCN, indicating the involvement of MPO in the neutrophil-mediated peroxidation. Human low density protein (LDL) is rapidly oxidized by the MPO-H_2O_2-Cl^- system. The kinetics of the oxidation can be monitored continuously by measuring the 234 nm conjugated diene absorption. In the absence of Cl^- LDL is mildly oxidized, but in the presence of Cl^- a propagation phase with a rapid increase of the 234 nm absorption is observed. It is concluded that these results point to an involvement of MPO both during phagocytosis by neutrophils as well as in the oxidation of LDL in the early phases of atherosklerosis.

12.22 FORMATION OF REACTIVE ALDEHYDES DURING POSTISCHEMIC REOXYGENATION AND HALOALKANE TREATMENT[1]

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Lipid peroxidation in biological systems is always combined with the formation of reactive aldehydes, e.g. 4-hydroxynonenal (HNE) and malondialdehyde (MDA).
The analytical approach for the HNE measurements included the modification of HNE by dinitrophenylhydrazine, the TLC separation of carbonyl compounds and their HPLC. MDA was coupled with thiobarbituric acid and determined by HPLC with flourescence detection.
In isolated hepatocytes exposed to haloalkane (1 mM bromotrichloromethane) or anoxia/reoxygenation the concentrations of HNE were compared with those of MDA. In reoxygenation experiments it was tried to distinguish the share of xanthine oxidoreductase inhibiting and radical scavenging effects of oxypurinol, a compound which is known to reduce the postischemic tissue injury.

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12.23 STIMULATION OF LIPID PEROXIDATION AND HYDROXYL RADICAL GENERATION BY THE CONTENTS OF HUMAN ATHEROSCLEROTIC LESIONS

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Lipid peroxidation within human arterial lesions is thought to play an important role in the development of atherosclerosis. In the presence of "catalytic" iron or copper ions, lipid peroxidation can be accelerated. Samples of the "gruel" taken from advanced atherosclerotic lesions in the abdominal aortae of human cadavers were tested for pro-oxidant properties. All samples contained bleomycin-detectable iron and phenanthroline-detectable copper. Almost all the samples stimulated peroxidation of rat liver microsomes and this was usually inhibited by the iron chelator, desferrioxamine. Some samples stimulated the formation of hydroxyl radicals from H2O2 in the presence of ascorbate, a reaction again inhibited by desferrioxamine.

We conclude that the interior of human advanced atherosclerotic lesions is a highly pro-oxidant environment and that the use of copper or iron ions to promote peroxidation of low-density lipoproteins *in vitro* may be a valid model for events in the arterial wall.

12.24 IS THERE INCREASED LIPID PEROXIDATION IN BIOLOGICAL FLUIDS FROM HYPERCHOLESTEROLAEMIC OR RHEUMATOID PATIENTS?

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It has been claimed that diabetic and/or hypercholesterolaemic (HC) patients have elevated plasma lipid peroxides and that this produces damage to the vascular endothelium and initiates atherosclerosis. We re-evaluated this claim by assessing lipid peroxidation in sera of HC patients using an HPLC-based assay for levels of TBA-reactive substances (TBARS). The assay includes the use of the antioxidant, butylated hydroxytoluene (BHT) which can slow down or prevent any further peroxidation of other lipid (or non-lipid) molecules in the sample during the assay. HPLC is used to separate the (TBA)2MDA chromogen from the other chromogens.

Since the knee joints of rheumatoid patients have also been reported to contain substantial levels of TBARS, samples of rheumatoid sera and synovial fluid (SF) were also assessed and compared with HC sera.

The addition of BHT to biological fluids with the TBA reagent decreased the detection of TBARS in all cases. The difference between TBARS in the HC patients versus controls was highly significant with our assay method. The addition of BHT to rheumatoid SF caused the greatest mean decrease in TBARS compared with SF with no BHT. The absolute levels of TBARS in HC sera were significantly higher than controls but significantly lower than rheumatoid sera. These results will be discussed.

12.25 EFFECT OF CALORIC OR CARBOHYDRATE RESTRICTED DIETS ON LIVER ANTIOXIDANT ENZYMES AND PEROXIDATION IN MICE

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Caloric restriction is the only widely recognised manipulation capable of extending the maximum life span of rodents, even though its way of action remains unknown. To be effective, it must be started before the end of the growth phase. It has been suggested that it works through a modulation of tissue free radical levels. No information about the effects of separate restriction of particular energetic components of the diet on antioxidants and peroxidation is available. 3 groups of initially 3 months old OF1 mice were maintained during eight weeks in ad libitum (controls), caloric restricted (60% of control calories) or carbohydrate restricted (60% of control calories) diets. Ingestion of minerals and vitamins was equal in the three groups. Activities of SOD, CAT, GPx, GR cytochrome oxidase and lipid peroxidation were measured in the liver. Carbohydrate restricted (but not caloric restricted) animals showed a high increase in sensitivity to in vitro lipid peroxidation.

12.26 ω-6 FATTY ACIDS AS SUBSTRATE TO 15-LIPOXYGENASE.

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Enzymatic transformation of ω-6 fatty acids by 15-lipoxygenase (LO) results in the formation of different lipid hydroperoxides. We have studied the production of 15-LO derived metabolites from linoleic acid (LA), γ-linolenic acid (γ-LA), dihomo γ-linolenic (Hγ-LA) and arachidonic acid (AA) in human neutrophils. The cells were incubated for 10 min with each fatty acid (0.01-50 μM) with and without the calcium ionophore, A23187 (1 μM). The monohydroxy fatty acids formed were determined by reversed phase HPLC.

Increasing concentrations of ω-6 fatty acids stimulated the production of the monohydroxy fatty acid. Addition of A23187 potentiated the 15-LO product of LA by 1 order of magnitude. This potentiation was not found when AA, γ-LA or Hγ-LA were used as substrate for 15-LO. These results indicate that LA was differently utilized as a substrate for 15-LO. In the presence of A23187 (1μM), eicosatetraynoic acid (ETYA) was a more potent inhibitor of 15-LO, when LA was used as substrate (pIC50=7.4), relative to if AA was used as substrate (pIC50=6.7).

The increased capability for 15-LO to utilize LA as substrate may be of importance since LA could be a major source for the enzymatic lipid peroxidation in blood cells and lipoproteins.

12.27 OXIDATION OF RAT LIVER PHOSPHOLIPIDS IN HOMOGENEOUS SOLUTION, LIPOSOME, AND HOMOGENATE

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Oxidation of biological membranes has been attracting much attention since it is a cause or a result of free radical-induced damage of the cell. The oxidizability of phospholipids in the cell has been estimated by the decrease of the phospholipids but we tried to measure it directly by detecting hydroperoxides of various membrane phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol (PC-OOH, PE-OOH, PS-OOH, and PI-OOH, respectively). Phospholipids were extracted from frozen rat liver with chloroform/methanol directly using a teflon homogenizer. This solution, liposome prepared from the same extract, and homogenate in 0.25 M sucrose were oxidized with oil-soluble radical initiator at 37°C in air. Homogenate was also oxidized by the addition of *tert*-butyl hydroperoxide. The major products were PC-OOH and PE-OOH, while little PS-OOH and PI-OOH were produced. The rate of PC-OOH formation was always faster than that of PE-OOH. In the oxidation of homogenate, significant decrease in PE and PC, and the formation of free fatty acids (FFA) were observed, while not observed in the other systems. Furthermore, hydroxy-phospholipids (PC-OH and PE-OH) and hydroxy-FFA were produced in homogenate oxidation. These results indicate the presence of phospholipase and peroxidase activities in homogenate. These activities are enzymatic since they were inhibited by the treatment of the homogenate at 100°C for 10 min. These results suggest that PC and PE are vulnerable phospholipids to oxidation and their oxidation products, hydroperoxides, are readily reduced and/or hydrolyzed. In fact, PC-OOH and PE-OOH were not observed in the oxidation of homogenate with low oxygen radical flux.

12.28 ANTIOXIDANT ACTIVITY OF all-trans- AND cis- ISOMERS OF NATURAL RETINOIDS TOWARDS LIPID PEROXIDATION INDUCED IN RETINAL MICROSOMES

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Owing to its unique metabolic characteristics and high level of long chain polyunsaturated lipids, retina is particularly susceptible to oxygen toxicity. In the retinal tissue, antiperoxidative protection is offered by enzyme systems, and low molecular weight molecules. Among the latter, vitamin E is considered the most important lipid-soluble antioxidant.

Synthetic as well as natural retinoids have been found as antioxidants and effective radical scavengers in a lipid phase (1-5). These compounds are peculiar components of retinal metabolism. We investigated their antioxidant properties against ascorbate-dependent iron-catalysed lipid peroxide formation in microsomes of retina from fresh bovine eyes. Either all-*trans*- or *11-cis*- configurations of natural retinoids, dissolved in DMSO, have been tested, in comparison with alpha-tocopherol.

Lipid peroxides formed in the membrane were determined as TBARS, and hydroxyl-radicals' formation was monitored by reaction with dimethyl sulfoxide which yields formaldehyde, then colorimetrically determined by the Hantzsch reaction (6).

All retinoids tested, assayed in a 0.1 to 100 x 10^{-5} M concentration, were able to reduce the induced peroxidation. The order of their relative potency, on the basis of the calculated IC_{50}, was *11-cis* retinol > *11-cis* retinaldehyde > all-*trans*- retinol > all-*trans*-retinaldehyde = all-*trans*-retinyl palmitate. The antioxidant effect could correlate with the increasing unstability of the chemical structure from *trans*- to *cis*- compounds.

The antiperoxidative action of all-*trans* and *11-cis* retinol was also verified when the retinoids were added to the peroxidative system as a complex with BSA. Under these conditions, the retinoid concentration to obtain the emi-maximal inhibition was three times higher with respect to the condition where retinol was dissolved in DMSO.

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12.29 EFFECTS OF FISH-OIL ENRICHED DIET ON HYPERBARIC OXYGEN TOXICITY

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Mice were fed a chow diet or diets enriched in fish oil, sunflower oil or beef tallow for 3 weeks. Fatty acid analysis was carried out in samples of plasma, brain and lungs from these animals and large changes were found in plasma and lungs with relatively small dietary induced changes in brain tissue. Bleeding times were increased very significantly in the fish oil group, and slightly increased in the sunflower oil group.

Endogenous lipid peroxidation (measured as thiobarbituric acid reactive substances) was unchanged in lung and brain, but lung tissue from fish oil fed mice produced more lipid peroxides in vitro during incubation at 37° than those of other dietary groups. Mice fed the 4 different diets were exposed to hyperbaric oxygen at 618, 585 and 515 kPa and convulsive activity and lung damage recorded. No dietary induced alterations in susceptibility to oxygen toxicity was found.

12.30 LIPID PEROXIDATION (LPO) AND VULNERABILITY OF THE FAILING HEART UPON POSTISCHEMIC REPERFUSION

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Spontaneously hypertensive rats (SHR) develop progressively with age cardiac hypertrophy. After 18 months the animals showed dilatative cardiomyopathy (LVEDP and LVEDV increased) compared to the genetic control (WKY rats). The contractile failure resulted in alterations of the mitochondria isolated from the hearts: Reduction of the release of free oxy-radicals (detection by means of the ESR-spin trap technique in the presence of antimycin A), improvement of the antioxidative activity and enhancement of the content of thiol residues.

During 30 min reperfusion (following 30 min total and global ischemia) of the hearts isolated from SHR, concentration of products of LPO (thiobarbituric acid reactive material, 4-hydroxynonenal) were higher in the perfusate compared to the control. The increase of LPO was accompanied by an increase of cell damage (leakage of CK activity) and a decrease of functional recovery (LVDP, $LVdp/dt_{max}$). The results support the hypothesis that the postischemic reperfusion injury of the heart is mediated via radical-induced LPO which, however, seems not to related to the mitochondrial radical metabolism.

12.31 PROTECTION AGAINST MYOGLOBIN-INDUCED LOW DENSITY LIPOPROTEIN OXIDATION

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Our studies demonstrate that activated ferryl myoglobin (Mb) radical causes extensive peroxidation of low density lipoprotein (LDL) by initiating peroxidation of the fatty acid side chains. We have synthesised and investigated novel hydroxamate drugs (HXD) as antioxidant compounds. The antioxidant effect of HXD occurs by hydrogen donation, reducing the ferryl Mb to the met form. The mechanism by which the HXD suppress peroxidation involves two possible modes of action: (i) reducing initiation of peroxidation by competing with LDL for the ferryl Mb radical, and (ii) removing lipid peroxyl radicals, which can propagate peroxidation, as well as the damaging lipid alkoxyl radicals. MetMb catalyses the propagation of LDL oxidation but not initiation, thus the effects are less extensive. HXD are also efficient inhibitors of this process.

12.32 HYDROPEROXIDES (HP) LEVELS IN PLASMA OF INFANTS IN RELATION WITH THE DIFFERENT MODE OF DELIVERY

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Neonates are exposed to the free radical damage which occurs by different pathways: increased oxygen consumption, hypoxia/reperfusion, phagocyte activation and exceeding free iron. The aim of this study is to investigate the occurence of oxidative injury in normal infants born by spontaneous or elective cesarean delivery, as well as in neonates with asphyxia.

Matherial and method. A controlled study was conducted on 10 normal infants (6 from spontaneous and 4 from cesarean delivery) and 4 with asphyxia (Apgar<7 at 5 min.). HP plasmatic levels (relatively stable by-products of lipid peroxidation) were assayed with Ward's modified procedure (J. Clin. Invest. 76:517, 1985) at 0 time and after 24 and 48 hours.

Results. At 0 time HP were significantly higher in asphyxiated than in normals born by cesarean section ($P< 0,01$); in infants from cesarean section HP were higher than in normals from spontaneous delivery ($P< 0.01$). At 24 and 48 hours difference remained significative only in asphyxia whereas it disappeared in the other groups.

Conclusions. From these preliminary data we can anticipate the hypothesis that in operative deliveries an increased generation of free radicals occurs, probably due to tissue distruction (with free iron release) and/or biochemical toxic effects of anaesthetics. In asphyxied infants, indipendently from the kind of delivery, the free radical generation (mostly related to hypoxia/reperfusion) is highest and more persistent.

12.33 ERYTHROCYTE LIPOPEROXIDATION AND GLICOSYLATED HAEMOGLOBIN IN DIABETES MELLITUS

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Lipid peroxidation (LP) is undoubtedly caused by free radicals (FR) action on human cells. As it was formerly postulated that the glicosylation of proteins is caused by a reaction of autoxidation, we thought of interest to report results studying action of FR in plasma and erythrocytes of 24 patients with diabetes mellitus. It was observed a significant increase of lipoperoxides (LP) in these patients as compared with controls.

Diabetics (24):
a) Plasma: 11±5.63nM/ml;
b) Red blood cells: 1061±325nM/mg Hb

Controls (20):
a) Plasma: 6,1±2.56nM/ml;
b) Red blood cells: 767.6±204nM/mg Hb
a) t=3.8; p<0.01 / b) t= 3.6; p<0.01

It was also observed a significant positive correlation between glicosylated hemoglobin (GH) and LP of erythrocytes (r=+0.91,p<0.01).
There is no doubt that there is an accumulation of malondialdehyde in erythrocytes of diabetics (it measures the degree of lipoperoxidation).
*Fellowship of National Research Council (CNPq)

12.34 THE ACTION OF EXOGENOUS ANTIOXIDANTS ON HYPERBARIC OXYGEN TOXICITY AND LIPID PEROXIDATION

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Convulsions and pulmonary damage result when animals are exposed to hyperbaric oxygen at pressures above about 300 kPa. Several hydroxyl radical scavengers (namely dimethylsulphoxide, dimethylthiourea and mannitol), the iron chelator desferrioxamine and the lipid antioxidant butylated hydroxytoluene were tested for possible protection against such hyperbaric oxygen toxicity. Dimethylthiourea and dimethylsulphoxide prolonged the latency to the first convulsion, but, surprisingly, dimethylthiourea very significantly increased pulmonary damage at both pressures used (515 and 585 kPa). Desferrioxamine also slightly increased lung damage at 585 kPa. Other antioxidants did not alter neurotoxicity or pulmonary toxicity induced by hyperbaric oxygen at 515 or 585 kPa.
The antioxidants were also tested for their ability to inhibit lipid peroxidation (TBARS formation) *in vitro*. Desferrioxamine (5 and 50 μm), and butylated hydroxytoluene (0.1 mM and 1 mM) greatly inhibited TBARS formation in brain and lung homogenates incubated at 37°C. None of the hydroxyl radical scavengers affected TBARS levels in homogenates. There was no correlation between in vitro inhibition of lipid peroxidation and in vivo protection against oxygen toxicity.

12.35 STRUCTURAL MEMBRANE CHANGES BY OXIDIZED PHOSPHOLIPIDS AS A BASIS FOR THEIR PREFERENTIAL HYDROLYSIS BY PHOSPHOLIPASE A_2

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Red blood cells continuously generate and encounter oxygen radicals. It has been suggested that a phospholipid repair mechanism is active, involving among other enzymes a phospholipase A_2 (PLA_2) to remove oxidized fatty acyl chains. The molecular basis for the activation of a PLA_2 upon oxidation of membrane lipids is not well understood.
We have performed experiments to investigate the physical behavior of oxidized lipids and the penetration and activity of PLA_2 in monomolecular films of palmitoyl,linoleoyl-phosphatidylcholine (PLPC), PLPC hydroperoxide (PLPC-OOH), and PLPC hydroxide (PLPC-OH). At a surface pressure of 20 mN/m, the mean molecular area of PLPC was determined to be 65 $Å^2$. PLPC-OOH and PLPC-OH have mean molecular areas of 100 and 180 $Å^2$, respectively. This increase in molecular area is consistent with a relocation of the oxidized fatty acyl chain towards the lipid-water interface due to the increased polarity of the (O)OH-group. Monitoring hydrolysis of monolayer phospholipids by PLA_2 as change in surface pressure, bee venom PLA_2 hydrolyzed oxidized PLPC much more rapidly than non-oxidized PLPC both in the presence and absence of serum albumin. However, penetration of the PLA_2 into the monolayer was found to be considerably less when using PLPC-OOH or PLPC-OH monolayers compared to PLPC monolayers. Apparently, penetration of this PLA_2 is not required for efficient hydrolysis of oxidized PLPC monolayers. These data provide experimental support for the hypothesis that defects in membrane packing caused by oxidized lipids can serve as a recognition site for PLA_2, a necessary first step in the lipid repair mechanism.

12.36 LIPID PEROXIDATION IN LUNG, LIVER, KIDNEY, TESTES AND BRAIN AFTER ADMINISTRATION OF CADMIUM CHLORIDE IN RATS OF DIFFERENT AGES.

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Cadmium (Cd) can induce a prooxidant state in biological systems resulting, among other events, in the peroxidation of polyunsaturated fatty acids (LPO). We investigated in vivo this phenomenon in rat lung, liver, kidney, testes and brain of male Long Evans rats (12 and 36 week-old) 24 hours after being injected i.p. with 0.025, 0.125, 0.5 and 1.25 mg Cd/Kg $CdCl_2$. The measure of thiobarbituric acid reactive substances (TBARS) in the above stated tissues from 12 week-old rats demonstrated that lung and brain were the most responsive to Cd-induced LPO (up to 213% and 171% respectively as compared to control groups). LPO in liver and kidney was slightly above control levels despite the fact that these tissues accumulated the greatest amounts of Cd (up to 33% and 5% of the injected dose respectively). LPO measured in tissues of 36 week-old rats was much less pronounced than in younger animals despite higher retention of Cd in the respective tissues 24 hours after the treatment. Glutathion peroxidase and reductase and glucose-6-phosphate dehydrogenase were also differently modulated as a function of age. These changes may have a causative role in the induction of LPO following Cd exposure. Reference: Manca et al. (1991). Toxicology 67: 303-323.

12.37 GLUTATHIONE DEPLETION ENHANCES LIPOFUSCIN ACCUMULATION IN CULTURED RAT NEONATAL CARDIOMYOCYTES

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A recent hypothesis States oxidative stress results in increased formation of hydrogen peroxide (H_2O_2) that enhances lipofuscinogenesis. The diffusion of H_2O_2 to the acidic vacuolar compartments would result in intralysosomal peroxidative reactions due to Fenton chemistry (·HO formation) as lysosomes contain trace amounts of reactive iron. Cystosolic H_2O_2 is degraded mainly by the glutathione peroxidase-GSH system, and decreased GSH thus should increase the lysosomal H_2O_2 influx and ·OH formation. Cellular GSH may be manipulated by the inhibition of GSH-synthetizing enzymes. In the present study GSH biosynthesis was inhibited by cellular exposure to buthionesulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase. In cells treated with 0.1 mM BSO every second day (when growth medium was normally replaced). GSH decreased, as measured by HPLC/EC, to 45% after 2 days, to 28% after 8 days and to 18% after 14 days. Considerable higher-than-normal lipofuscin induced autofluorescence intensity was then observed. Depletion of cellular GSH by BSO thus leads to increased accumulation of lipofuscin substances in cultured cardiomyocytes which supports the hypothesis that oxidative stress is a causal factor in lipofuscinogenesis.

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Mechanism of stimulation of Fe^{2+}-supported lipid peroxidation by metals without redox capacity. 12.38

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We have reported that *in vitro* Al acts as a prooxidant depending on Al and Fe^{2+} concentrations and on membrane integrity. This study was conducted to determine if the prooxidant action of metals without redox capacity in biological systems could be exerted through changes in the physical properties of membranes rendering the fatty acids more available for oxidation. The capacity of several metals to stimulate Fe^{2+}-induced lipid peroxidation (LP) in liposomes (PC:PS, 60:40) was first characterized measuring chemiluminescence, formation of 2-thiobarbituric reactive substances (TBARS) and fatty acids profiles. Besides Al, La^{3+}, Be^{2+}, and Pb^{2+} (50 μM) increased Fe-induced LP. The stimulatory effect of Al (12.5-250 μM) on TBARS production was significantly correlated ($p < 0.02$) with its capacity to promote liposomes aggregation (r^2=0.90), 5(6)-carboxy-fluorescein release (r^2=0.87) and liposomes fusion (r^2=0.99). There was a positive correlation (r^2=0.95, $p<10^{-4}$) between the capacity of Al, La^{3+}, Be^{2+} and Pb^{2+} to induce liposomes fusion and their capacity to promote LP in the presence of Fe^{2+}. Al and Pb were found to produce estructural changes in the phospholipids head-group region in liposomes, as evaluated using Merocyanine 540. These results suggest that the promotion of changes in the physical properties of membranes rendering fatty acids more susceptible to free radical attack could be a common mechanism of non redox metals in the stimulation of Fe^{2+}-supported LP.

12.39 MOBILITY AND PERMEABILITY OF THE BILAYER IN IRRADIATED VESICLES

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Lipid peroxidation in small unilamellar liposomes was investigated by means of NMR spectroscopy in terms of its dependence on additives (including salt composition, radiosensitizer) in both the inner and outer aqueous compartments. NMR results were derived from partially relaxed proton spectra. This method is suited for the determination of differences in the lateral diffusion of lipid molecules in both lipid layers as affected by radical action. Additionally, the H-1 NMR provides a method for monitoring the stability of irradiated liposomes using Eu(3+)-ions as a shift reagent in the extravesicular solution. The time course of the penetration of this ion into liposomes indicates the degree of destabilization of the permeability barrier after irradiation.

PLASMA LIPID PEROXIDATION IN AMATEUR AND ELITE LONG DISTANCE RUNNERS. 12.40

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Several studies have reported that acute exercise in untrained animals induces oxygen free radicals generation and increase the levels of lipoperoxidation products in blood and others tissues. During exercise, bodily O_2 consumption is greatly increased and it seems likely that more O_2 and H_2O_2 form "in vivo", since O_2^- can be a product of electron leakage from mitochondrial electron transport chains. Prolonged exercise, especially in untrained individuals, produce muscle damage, as demonstrated by microscope studies or by release of muscle enzymes into the circulation. We have studied the changes of the plasma lipid peroxidation in 9 male long distance runners, 4 amateurs (28-46 years old) and 5 well trained (18-29 years old) athletes. All men were subjected to 25 km run or 30 km walk at 3'15"/km or 4'30"/km respectively. Blood samples, drawn from the cubital vein, within 30 min before exercise (basal levels), immediately after, 6 and 24 hrs after, were mixed in polyethylene tubes with anticoagulant. The plasma samples were at once obtained by differential centrifugation and stored at -80°C. The total thiobarbituric acid reactive substances (TBARS) were measured, as described by Beuge and Aust, and correlated with serum CK and LDH activity. The lipid peroxidation occurs more evidently in the amateurs long distance runners while in well trained athletes the TBARS levels are the same before and after the exercise. The results confirming the literature data, suggesting that an increase of the antioxidants supplies with the diet could be important for the million of amateurs athletes.

12.41 OXIDATIVE MECHANISMS IN IRON NITRILOACETATE TOXICITY IN ISOLATED HEPATOCYTES.

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Incubation of isolated rat hepatocytes with 0.1 mM iron nitrilotriacetate (FeNTA) caused a rapid rise in lipid peroxidation followed by cell death. The loss of hepatocyte viability was preceded by the decline of mitochondrial membrane potential and by ATP depletion. Chelation of extracellular Ca^{2+} by EGTA or inhibition of Ca^{2+} cycling within the mitochondria by $LaCl_3$ or cyclosporin A did not prevent mitochondrial damage. Conversely, a dramatic increase in the conjugated diene content was observed in mitochondria isolated from FeNTA-treated hepatocytes. Oxidative damage of mitochondria was accompanied by the leakage of matrix enzymes GOT and GLDH. The addition of the antioxidant N,N'-diphenylphenylene diamine (DPPD) completely prevented GOT and GLDH leakage, impairment of mitochondrial potential and ATP depletion, indicating that lipid peroxidation rather than a Ca^{2+}-dependent mechanisms caused of mitochondrial injury. DPPD addition also protected against hepatocyte death. Similarly hepatocytes prepared from fed rats, in spite of undergoing as well to the impairement of mitochondrial functions, were more resistant than those obtained from starved rats toward ATP depletion and cell death caused by FeNTA, indicating that the decline of ATP due to the damage of mitochondria was critical for the development of FeNTA toxicity.

Session 13

NO and Related Radicals

13.1 HYDROXYL RADICAL FORMATION FROM THE SIMULTANEOUS GENERATION OF SUPEROXIDE AND NITRIC OXIDE: MECHANISMS AND IMPLICATIONS FOR THE PATHOGENESIS OF ATHEROSCLEROSIS

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The oxidation of lipids in the artery wall has been suggested as one of the first steps leading to the development of an atherosclerotic lesion. For this reaction to be initiated abstraction of a bis-allylic hydrogen atom from an unsaturated fatty acid must occur. This initiation reaction can be achieved by reaction of LDL with the enzyme 15-lipoxygenase. We have been investigating another process which could also lead to the initiation of lipid peroxidation involving the reaction of superoxide and nitric oxide to generate hydroxyl radicals. Using a chemical system to generate these two species we have found that hydroxyl radicals are generated and their formation is associated with oxidation of LDL to a potentially atherogenic particle. We propose that if such a reaction were to occur in the artery wall it would contribute to the development of an atherosclerotic lesion.

INTERACTIONS BETWEEN NITRIC OXIDE (NO) AND OXYGEN RADICALS. 13.2

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NO serves as a multifunctional physiological signal. Since superoxide dismutase (SOD) potentiates the actions of NO, the reaction between NO and O_2^-, which forms $ONOO^-$, was proposed to be a significant pathway of NO decomposition. However, other reactions of SOD complicate this assessment. SOD appears to reversibly convert NO to NO^-, and appears to convert $ONOO^-$ to ONO^+ and ^-OH. Oxygen radicals can affect the actions of NO in other ways. The oxidation state of the heme moiety of guanylate cyclase, a major target for NO in vivo, may be altered. NO may react with higher oxidation state hemes, such as catalase. Some nitrovasodilators can generate O_2^- concurrently with NO. Inhibitors of O_2^- generation by NADPH: O_2 oxidoreductase can inhibit NO synthesis. NO oxidizes the reduced form of cytochrome C. These points stress both the difficulty and necessity of simultaneously considering many reactions of NO and O_2^- whenever these two radicals may be present together. (Supported by the Jung-Stiftung für Wissenschaft und Forschung and the National Foundation for Cancer Research).

13.3 THE FORMATION OF NO_2 RADICALS CAN EXPLAIN THE GENOTOXICITY OF 2-NITRO-PROPANE. A PULSE RADIOLYSIS STUDY.

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The industrial solvent 2-nitropropane (2-NP) is hepatotoxic and induces DNA damage and carcinomas in rat liver. The compound has been suggested to require metabolism by hepatic cytochrome P450 in order to become genotoxic, but the DNA damaging species has not yet been identified. Several mechanisms have been invoked, involving the initial formation of the 2-NP radical from the nitronate anion and subsequent radical chain reactions with the participation of O_2^- or peroxyl radicals.

We have used pulse radiolysis in order to produce 2-NP radicals and absorption as well as EPR spectroscopy to monitor and identify potential DNA damaging radical intermediates. 2-NP radicals could not be observed by EPR after *in situ* generation with horseradish peroxidase/H_2O_2 or after pulse radiolysis combined with spin trapping. This indicates that any 2-NP radicals or their spin adducts are too unstable to be detectable by this method.

In contrast, kinetic spectroscopy and competition studies after pulse radiolysis resulted in transient spectra which indicated the formation of NO_2 radicals and helped to explain the reported ultimate formation of NO_2^- and acetone from 2-NP. Rate constants for the reactions of NO_2 with selected nucleosides and nucleotides were determined by competition with nitroguaiacol and kaempferol. From the magnitude of the rate constants, especially for the reaction of NO_2 with deoxyguanosine and deoxyguanosine monophosphate, we conclude that the NO_2 radical is most likely the species causing the genotoxic effects of 2-NP.

Production of peroxynitrite by activated rat alveolar macrophages 13.4

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Peroxynitrite ($ONOO^-$), the reaction product of superoxide (O_2^-) and nitric oxide (NO), may be a major cytotoxic agent produced during inflammation, sepsis and ischemia/reperfusion. Bovine Cu,Zn superoxide dismutase catalyzes nitration of phenolics by peroxynitrite, which was used to assay peroxynitrite. Nitric oxide produced by rat alveolar macrophages activated with phorbol 12-myristate 13-acetate quantitatively converted to peroxynitrite. In the presence of a tyrosine analog, 4-hydroxyphenylacetic acid, Cu,Zn superoxide dismutase catalyzed the peroxynitrite-dependent formation of 3-nitro-4-hydroxyphenylacetic acid, which correspondeed to a rate of 0.1 nmol peroxynitrite$\cdot 10^6$ cells$^{-1}\cdot$min^{-1}. The inhibitor of nitric oxide synthesis, N-methyl-L-arginine, prevented the Cu,Zn superoxide dismutase-catalyzed nitration of 4-hydroxyphenylacetic acid by stimulated macrophages. Cu-depleted Zn superoxide dismutase did not catalyze the formation of 3-nitro-4-hydroxyphenylacetic acid either *in vitro* or in the presence of activated macrophages. Four other independent by indirect estimates of peroxynitrite formation by activated macrophages were consistent with the Cu,Zn superoxide dismutase-catalyzed reaction; 1) the rate of nitrite and nitrate accumulation, 2) the increase in the amount of superoxide detected in the presence of N-methyl-L-arginine, 3) the increase in the percentage of nitrate relative to nitrite following activation and 4) the decrease in oxygen consumption of phorbol 12-myristate 13-acetate activated macrophages by N-methyl-L-arginine. The formation of peroxynitrite, a relatively long lived strong oxidant, from the reaction of nitric oxide and superoxide derived from activated macrophages may contribute to inflammatory cell-mediated tissue injury.

13.5 SYNERGISM BETWEEN SUPEROXIDE DISMUTASE(SOD) AND NITRIC OXIDE IN GASTRIC MUCOSAL PROTECTION FOLLOWING ISCHEMIA-REPERFUSION IN RATS

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The effects of L-arginine, a precursor of nitric oxide(NO), combined with human superoxide dismutase (hSOD), were investigated in a ischemia/reperfusion-induced gastric mucosal injury model in rats. Ischemia/reperfusion-induced gastric injury was produced by the previously reported method (FRRC,1989,7:3). L-arginine (300 mg/kg) and/or recombinant human Cu,Zn SOD (50,000 U/kg) were administered 1 h before ischemia. 30 min ischemia and 60 min reperfusion produced gastric hemorrhagic erosions and increased TBA-reactive substances in the gastric mucosa. These injuries and increased TBA-reactive substances were significantly inhibited by hSOD and L-arginine+hSOD together but not only by L-arginine. L-arginine + hSOD together exert more significant protection on the gastric mucosa than hSOD. L-arginine may act protectively on the gastric mucosa only in the presence of SOD to prolong the action of NO by scavenging superoxide radical that inactivate NO.

STIMULATION OF TRANSCRIPTION IN MOUSE LIVER CELLS BY NITRIC-OXIDE RADICALS 13.6

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The ESR signal of nitrosyl complexes appears in mouse liver after hydroxylamine injection in vivo. This ESR signal testifies to the appearance of NO free radicals. The concomitant accumulation of tritium-labelled uridine in liver RNA evidences for stimulation of transcription. This stimulation is about the same order of magnitude as the stimulation of transcription after Gamma-irradiation of mice with lethal doses. It is assumed that NO can disorder the gene expression machinery in the cells.

13.7 EFFECT OF THE VASODILATOR ON THE SMALL INTESTINAL MUCOSAL INJURY INDUCED BY L-NITROARGININE IN RATS

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Endothelium derived relaxing factor(EDRF) has been identified as nitric oxide(NO), synthesised from the amino acid L-arginine, a process which is inhibited by the L-arginine analogue L-nitroarginine. The relaxation of the smooth muscle tissues induced by EDRF is inhibited by superoxide anion and that is recovered by superoxide dismutase(SOD)

We investigated effect of the vasodilator on the small intestinal mucosal injury induced by L-nitroarginine. Male Splague-Dawley rats were used for the small intestinal mucosal injury by the intravenous administration of L-nitroarginine and then we adoministrated papaverine or sodium nitroprusside as vasodilators. In an index of the small intestinal mucosal injury, we measured volume of the intramucosal bleeding and volume of the leaky protein from the small intestin, etc. After administration of L-nitroarginine, we found multiple erosions with bleeding in the small intestinal mucosa. The increased both in volume of the mucosal bleeding and the leaky protein was significantly inhibited by adoministration of papaverine or sodium nitroprusside. And then, TBA reactive substances(TBARS) were significantly increased. These findings suggested that ischemia and oxygen-free radical contribute to the microcirculation level in the small intestinal mucosa.

THE NEW MODEL OF THE INTESTINAL MUCOSAL INJURY INDUCED BY ENDOTHELIUM DERIVED-RELAXING FACTOR(EDRF) SYNTHETIC INHIBITOR IN RATS 13.8

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EDRF has been identified as nitric oxide(NO) derived from the amino acid L-arginine. Superoxide anions are known to rapidly destroy EDRF(NO). It has reported that EDRF synthetic inhibitor, L-nitro-arginine($Arg(NO_2)$), inhibits endothelium dependent relaxation in vitro, elevates blood pressure in vivo. It is suggested that EDRF relates to numerous disease processes associated with abnormal endothelium-dependent relaxation. We prepared the new model of the intestinal mucosal injury by $Arg(NO_2)$ infused through the superior mesenteric artery using male Wistar rats. In this model, edematosis and hemorrhage were mainly shown as gross lesions. The leakage of hemoglobin significantly increased. TBA reactive substances(TBARS) in the intestinal mucosa were significantly increased and the $NOx(NO_2^-, NO_3^-)$ in the intestinal mucosa were decreased. h-SOD, which was simultaneously treated into the jugular vein, histologically inhibited the mucosal injury. These findings suggested that ischemia and reperfusion occurred at the microcirculation level in this model induced by $Arg(NO_2)$ infusion, that SOD prevented from the injury by scavenging superoxide anions which were produced at that time.

13.9 DETERMINATION OF THE MOLECULAR CONFORMATION OF PEROXYNITRITE ANION ($ONOO^-$) BY LASER RAMAN SPECTROSCOPY

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Nitric oxide(·NO) is a secondary messenger mediating vasodilation and inhibiting platelet aggregation. Nitric oxide may also be a key injurious agent in disease states such as stroke, myocardial ischemia, sepsis and inflammation, where O_2^- is also produced. Nitric oxide rapidly reacts with O_2^- to form peroxynitrite anion ($ONOO^-$). Peroxynitrite anion is stable in 1 M NaOH, but decomposes rapidly once protonated with a half-life of 1 second at pH 7.4 to produce a hydroxyl radical (HO·) like oxidant and nitrogen dioxide (·NO_2). HO· and ·NO_2 are potent cytotoxic oxidants that will injure tissue. Cu,Zn SOD could potentially reduce ischemic injury during reperfusion by scavenging O_2^- before it reacts with ·NO, thereby preventing the formation and subsequent decomposition of $ONOO^-$ into strong cytotoxic oxidants. The molecular conformation of $ONOO^-$ is still unclear, but it is believed that the conformation of $ONOO^-$ is important in recognizing potential mechanisms of SOD-inhibitable oxidant injury to endothelium. In this project, $ONOO^-$ was studied by Raman spectroscopy in the 300-1800 cm^{-1} region. Raman spectra of $O^{15}NOO^-$ were also obtained to help with band assignments. Six bands of $ONOO^-$ in 1 M NaOH were identified, indicating the presence of only one conformation. This observation was supported by the presence of a single peak in the ^{15}N-NMR spectrum. Quantum mechanical calculations suggest that the C-shaped or *cis*-conformation should be slightly more stable than the *trans*-conformation. Normal-mode calculations based upon the *cis*-conformation also predicted the $O^{15}NOO^-$ more accurately than the *trans*-conformation. We propose that the stability of peroxynitrite in alkaline solution is related to the molecule being locked in the *cis*-conformation.

13.10 Gp 120 HIV ENVELOPE PROTEIN ACTIVATES HUMAN BLOOD MONOCYTES TO RELEASE NITRIC OXIDE.

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Purpose. Nitric Oxide (NO°) was recently identified as effector of phagocytes-mediated cytostasis and/or cytotoxicity. NO°, a short-lived free radical, is synthesized by enzymatic oxidation of L-arginine and reacts with oxygen with formation of nitrite (NO_2^-) and nitrate (NO_3^-).We studied the effect of HIV gp 120 envelope on the L-Arginine Oxidative Pathway (LAOP).
Methods.Peripheral blood monocytes (PBM) were cultured for 0-4 days. Spin-trapping tecnique was used to study radical production. 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was used as spin trapping agent. NO_2^- was quantified in cell culture supernatants by using the Griess reagent.
Results As detected by the intensity of radical trapped by DMPO (adduct), gp 120 at μM concentrations was able to stimulate the production of radical by PBM (100-200% adduct increase). The adduct formation was decreased (64 $\pm$ 7%) by inhibitors of LAOP, such as N-nitro-arginine and N-methyl-arginine. The hyperfine splitting constants of the adduct suggested the formation of DMPO-OH wherease trapping of NO° is expected to gave a different DMPO adduct. Nevertheless, bubbling of NO° gas in a N_2-saturated solution of DMPO and trapping of NO° from sodium nitroprusside, an NO-releasing organic nitrite, gave a DMPO adduct with hyperfine splitting constants superimposable to that of DMPO-OH. The reaction leading to DMPO-OH formation from NO° is unclear. Consistent with the activation of LAOP, gp 120 produced about 40-150 % NO_2^- than untreated cells. NO° and NO_2^- release may contribute to cell and tissue damage in AIDS.

13.11 ROLE OF GSH IN NITRIC OXIDE SYNTHESIS BY HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS.

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Human endothelial cells cultured from umbilical vein (HUVEC) were tested for their ability to synthesize nitric oxide (NO), which has been identified as an endothelium-derived relaxing factor (EDRF). The synthesis of this free radical (measured as labeled citrulline, which is produced stoichiometrically with NO from labeled arginine) in HUVEC is Ca^{++}-dependent, is increased 6-fold by the calcium ionophore ionomycin and accounts for most basal and ionomycin-induced cyclic GMP (cGMP) production. When the cells were depleted of reduced glutathione (GSH) by incubation with 1-chloro-2,4-dinitrobenzene(CDNB),citrulline synthesis and cGMP production were inhibited in a concentration-dependent way. CDNB was not cytotoxic and did not inhibit cGMP increase elicited by sodium nitroprusside, which spontaneously releases NO. This result suggests that GSH is necessary in HUVEC for citrulline synthesis rather than for NO effect on guanylate cyclase.

Session 14

Carcinogenesis

14.1 DERANGEMENTS OF CELLULAR METABOLISM IN THE PRE-MALIGNANT SYNDROME

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Carcinogenesis is a multi-stage process involving several independent events. It has been proposed that initiation involves the generation of a clone of cells with raised mutation rate. Thus, the essential component of the pre-malignant syndrome (PMS) consists of an elevated mutation rate. Alteration in the steady-state level of mutagenesis is the consequence of metabolic alterations in the cells, affecting either the rate of DNA damage and/or the rate of repair. These derangements of cellular metabolism may permit early detection of pre-malignant cells. A number of possible biochemical abnormalities have been examined which may lead to the development of facile screening techniques with wide clinical and socio-economic implications.

14.2 ROLES OF ALDEHYDE DEHYDROGENASES IN CARCINOGENESIS

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The most effective pathway of aldehyde metabolism is oxidation to carboxylic acids by aldehyde dehydrogenases (ALDH). Three major classes of mammalian ALDHs, 1, 2 and 3, have been identified. Classes 1 and 3 contain both constitutive and inducible cytosolic forms. Class 2 consists of mitochondrial enzymes. Changes in ALDH activity occur during carincogenesis in a variety of tissues. The most common change is the appearance of Class 3 ALDH activity in tumors arising in tissues which normally do not express this form. The changes in enzyme activity occur early in tumorigenesis and are the result of permanent changes in ALDH gene expression. Various apsects of ALDH in relation to tumorigenesis will be discussed: (1) The role of ALDH in the metabolism of chemotherapeutic agents; (2) Changes in ALDH as part of an adaptive response of preneoplastic and neoplastic cells to stress due to changes in lipid peroxidation or as part of the *Ah* gene-mediated response to xenobiotic exposure. Finally, a model of inducible aldehyde dehydrogenase gene regulation will be proposed. Supported by NIH CA21103.

14.3 IRON AND COPPER INDUCED DNA DAMAGE

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The role played by free radicals in the pathology of a number of human diseases has continued to capture the imagination of scientists. Reactive oxygen species arising in reactions catalysed by iron ions and copper ions damage biological components such as DNA, proteins, carbohydrates, membrane lipids and fatty acids.

The technique of GC/MS/SIM allows definitive studies of the underlying mechanisms of free radical damage to DNA. We have used this technique to examine the reactivity of iron and copper ions. In the presence of hydrogen peroxide, copper ions were more damaging compared with equimolar iron ions in causing base modifications in DNA.

This catalytic ability suggests that the availability of 'free' iron and copper ions in biological systems should be carefully controlled. Interestingly, adult humans contain much less copper than iron. Perhaps our data provide a reason.

14.4 ROLE OF IRON IN ASBESTOS CARCINOGENICITY

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A direct implication of Active Oxygen Species (AOS) in asbestos toxicity has been recently proposed. AOS scavengers and some iron chelators both inhibit the development of the desease in experimental animals: iron appears thus involved in radical production. If the following hypothesis is correct,
Fe --> AOS --> target molecule --> DNA damage --> desease
the initial reaction will be governed by the **surface chemistry** of the fibre.
By means of the spin trap technique appplied to solid-liquid systems, we have investigated the **radical release potential** of various fibres, comparing the behaviour of well known specimens (UICC Chrysotile and Crocidolite) to that of asbestiform minerals typical of the Piedmont area. Surface modifications brought about by reducing agents, mechanical fracture of the fibre and chelators, reveal that **both Fe(II) and Fe(III) are required at the surface for the activation of molecular oxygen and hence AOS production.**

14.5 THE RELATIONSHIP BETWEEN PLASMA OXIDANT-ANTIOXIDANT STATUS AND AGGRESSIVENESS CHARACTERISTICS OF BREAST CANCER IN YOUNG AND AGED PATIENTS

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We have shown that plasma leukocyte vitamin E was significantly higher and plasma MDA was significantly lower in female breast cancer patients than in controls.Other authors have reported that tumors are characterized by a high level of antioxidants and lower peroxidation products.In this study, patients were classified as young (42 women < 47 years) and aged (49 women ≥ 70 years) because of the generally different characteristics of their tumor, and because of the eventual difference in their oxidant-antioxidant status.
Various antioxidants or element involved in antioxidant function, and malondialdehyde (MDA), as an index of lipid peroxidation, were meausured in plasma. These data were analyzed with regard to several characteristics of tumor aggressiveness.
Preliminary results show that vitamin E increase is the most constantly associated with tumor-aggressiveness characteristics in the young group, and Se increase, in the aged group. A higher level of MDA is found in the young group for negative Ki-staining (absence of cycling tumor cells) and in a peculiar pathological type of tumor characterized by a low evolutivity.Tumor size is associated with an increase of the three antioxidants in both groups.

14.6 RELEASED ACTIVE OXYGEN SPECIES AS INTERCELLULAR SIGNALS - THEIR ROLE IN REGULATION OF NORMAL AND TUMOUR CELL PROLIFERATION

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A wide variety of normal and malignant mammalian cells as well as inflammatory cells can be shown to release varying amounts of active oxygen species such as superoxide and hydrogen peroxide. Both these species can stimulate growth and growth responses in a range of normal, and particularly malignant cell types, through pathways common to natural growth factors. To assess further the significance of these observations, superoxide dismutase, or catalase, were added exogenously to the medium of growing rodent fibroblasts (immortalised or oncogene transformed). This resulted in a reduction in cell proliferation and an increased uptake of trypan blue stain, suggesting that superoxide and/or hydrogen peroxide may have important biological roles as intercellular 'messengers' of signals promoting tumour cell proliferation and maintaining cell viability. A possible signalling mechanism is suggested whereby hydrogen peroxide or superoxide may inactivate serum α-1 antitrypsin and allow cell-surface proteases to serve as mitogens for tumour cells by simply facilitating the normal action of growth factors.

14.7 ROLE OF THE RADICAL MECHANISMS IN THE OXIDATIVE STRESS DUE TO INORGANIC COMPOUNDS. EXAMPLE OF ASBESTOS CARCINOGENICITY

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The study of the relations between structure and chemical activity of inorganic compounds is a complementary and necessary contribution for a better understanding of the radical mechanisms implied in the oxidative stress.
This mechanistic approach involves a research of electrophilic species generated by inorganic compounds, capable of inducing an oxidative stress. This phenomenon implies different radical mechanisms of oxidation. In the case of several chrysotile samples, the formation of various electrophilic species is function of the nature of the different mineral contaminants of the fibers.
This approach reveals an agreement between the chrysotile surface activity in the lipid peroxidation and the data observed in the transformation assay for SHE cells, and in test of carcinogenicity in rats by inhalation.

14.8 DIFFERENT OXYRADICAL SOURCES HAVE SPECIFIC DAMAGE MECHANISMS IN NORMAL AND TUMOUR CELLS

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In order to elucidate the mechanisms of oxygen-induced toxicity, the sensitivity of normal and tumour cells to oxidative stress in different experimental conditions has been investigated. Tumour cells have an impairment of oxidative defenses being Cu,ZnSOD content 50% of normal thymocytes. Cell sensitivity, measured as LDH release and MDA production, depends not only on cellular antioxidant defenses, but also on the oxyradical source LDH release in normal thymocytes seems strictly related to MDA production.. Tumour cells are more resistant than normal cells to exogenous source of oxygen species as xanthine/xanthine oxidase system, and more sensitive to oxidative species endogenously generated by t-BOOH. t-BOOH-induced toxicity, measured as LDH release, is related to cellular metabolism, in particular to ATP depletion and directly mediated by oxyradical production, as suggested by the protection exerted by deferoxamine. Glucose addition completely reverts ATP depletion, but only partially LDH release, especially in tumour cells. This indicate that in tumour cells t-BOOH toxicity operates with a second mechanism. These cells, which have an impairment of the oxidative metabolism and a glycolitic pathway much more efficient than normal cells, are more sensitive to pyridinenucleotide oxidation. Dithiotreitol addition which protects tumour cells, but not normal thymocytes, confirms the role of the redox potential oxidation in determining LDH release in thymoma cells. Supported by MPI 60% grant.

14.9 PRECLINICAL DIAGNOSIS OF THE NEOPLASTIC DISEASE: CONNEXIONS AMONG FREE RADICALS, HEINZ'S BODIES AND HISTAMINE.

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During neoplastic disease (ND) occours the appearance of circulating factors able to alter the cell membranes. To verify the ND presence we studied, both in tumor patients (TP) and in healthy controls (HC), the integrity of red cell membrane by a method based on the ability of a toxic agent for cell membranes to cause the Heinz's bodies (HB) formation after incubation with the blood. The assessment of the intoxication degree of the erythrocytes is based on the rapidity of the appearance of the HB. The results showed a highly significant difference of the appearance times of the HB mean between TP and HC. Such a reaction occurred in 92% of the TP tested.Sex, age, smoking, white and red cell number, TNM factor and histological grading did not affact the appearance times of HB. The circulating toxic factors, in our opinion, are free radicals (FR). Others showed during tumor growth an increased making of FR that damage the cell membranes producing increase of their permeability. Since it was showed that the increase of the FR provokes histamine release,we studied the appearance times of HB in allergic patients finding a direct correlation between allergic symptoms and appearance times. The effect of the FR on the histamine release can be blocked by the administration of scavengers of FR drugs, that, if administered in TP, determine an extension of appearance times of HB. Whereas the discontinuance of such drugs determine the speeding up of the reaction. These data suggest a very high correlation among tumors, Heinz's bodies appearance times, free radicals and histamine.

14.10 INCREASED LEVELS OF CuZn-SOD IN HUMAN CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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Superoxide dismutase (SOD) activity in extracts of mononuclear blood cells from chronic lymphocytic leukemia (CLL) patients was greatly enhanced as compared to healthy subjects. Normal and CLL values were 10 ± 2.5 (n=15) and 48 ± 19 U/mg protein (n=13), respectively. CuZn-SOD, as dectected by chromatographic, enzymatic and immunological methods, was found to be the isoenzyme responsible for total SOD activity increase. EC-SOD was also slightly enhanced, while no differences in Mn-SOD content were observed between normal and CLL cells. Enzyme levels appeared to be closely linked to degree of lymphocytosis in the peripheral blood. A direct relationship existed up to 70 x 10^4cell/μl, while at higher cell counts (80-400 x 10^4/μl) enzyme activity was steady around 70 U/mg protein. Further studies on both acute and chronic myelocytic and lymphocytic leukemia will be undertaken. They will confirm whether SOD increase is a general feature of hematological neoplasia and might provide an explanation for the occurrence of this peculiar biochemical trait in CLL.

14.11 INCREASED c-FOS AND c-JUN GENE EXPRESSION AND AP1 DNA BINDING ACTIVITY FOLLOWING EXPOSURE TO CROCIDOLITE ASBESTOS, TPA AND ACTIVE OXYGEN SPECIES.

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Crocidolite asbestos causes proliferative alterations in mesothelial (RPM) and tracheobronchial epithelial (HTE) cells, the progenitor cells of mesothelioma and lung carcinoma. We have shown that addition of asbestos to HTE cells in vitro causes increased [^{3}H] thymidine uptake and increased gene expression and activity of ornithine decarboxylase, an enzyme intrinsic to proliferation. These effects were ameliorated by antioxidants suggesting that active oxygen species (AOS) are mediators of asbestos-induced proliferation. To study mechanisms of cell proliferation induced by asbestos, we measured mRNA levels of c-fos and c-jun in HTE and RPM cells. In addition, we determined levels of activator protein 1 (AP-1), a DNA-binding protein which binds to a specific DNA sequence and thereby activates transcription. RPM or HTE cells were exposed to crocidolite asbestos, hydrogen peroxide or the tumor promoter 12-0-tetradecanoylphorbol-13-acetate (TPA). RNA was extracted and used for Northern blot analyses to determine mRNA levels of fos and jun. Whole cell extracts were prepared to determine levels of AP-1 activity using gel mobility shift assays. Crocidolite, TPA and hydrogen peroxide caused an increase in mRNA levels of fos and jun in HTE and RPM cells, and increased activity of AP-1 DNA binding in HTE cells. This suggests that induction of fos and jun in target cells by asbestos is intrinsic to cell proliferation.

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14.12 GENERATION OF RADICALS FROM TUMOUR PROMOTERS

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A variety of peroxides and peracids (e.g. benzoyl peroxide) are known to be tumour promoters and it is though that this activity may be due to the generation of free radicals from these materials. We have used electron paramagnetic resonance (EPR) spectroscopy in conjunction with spin trapping to investigate the production of radicals during the metabolism of these compounds by liver fractions. Incubation of a number of these compounds with rat liver microsomes in the presence of the spin traps DMPO and DBNBS results in the detection of ROO· and R· radicals. Studies using specific enzyme inhibitors, metal-ion chelators and heat denaturation suggest that cytochrome P_{450} is the major locus of metabolism. Addition to NADPH to these systems results in a stimulation of radical production via a reductive process.

Radical production can also be observed with a number of related hydroperoxides, dialkyl peroxides, diacylperoxides and peracids. The latter produce novel aryloxy radicals [RC(O)O·] which undergo decarboxylation to give aryl radicals (e.g. Ph·); both of these types of radical can be readily trapped. Radical generation has been observed with all the known tumour promoters of this type which have been tested lending support to the theory that radical generation is important in their promoting activity.

14.13 HEPATOCELLULAR GROWTH ALTERATIONS DURING MOUSE LIVER CARCINOGENESIS INDUCED BY AROCLOR 1254 AND IRON.

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Iron overload considerably enhances the hepatocarcinogenicity of Aroclor (PCBs) in C57BL/10ScSn mice. There is evidence for active oxygen species produced by the uncoupling of induced cytochrome P450 releasing Fe^{2+} from cellular and molecular compartments leading to the production of hydroxyl radicals and DNA damage [1]. The AIM of this study was to investigate growth alterations in hepatocytes throughout the carcinogenic process. METHODS: Hepatocytes were isolated from iron-loaded C57BL/10ScSn mice fed for two weeks, eight weeks and six months on Aroclor. DNA synthesis was studied by flow cytometric analysis of BdUR incorporation into hepatocytes. The ploidy of isolated nuclei was examined by staining and lysing hepatocyte suspensions in an Ethidium Bromide/RNase solution. RESULTS: At two weeks the octaploid populations in Fe-treated (22.7 ± 2.8%) and AR/Fe-treated (22.7 ± 10.3%) livers were significantly higher than control (7.8 ± 2.8%). These remained elevated at eight weeks. A dominant diploid population emerged at six months in both the AR and AR/Fe treatments. These groups also contained a significantly higher number of cells in S-phase as detected by BdUR incorporation. The octaploid population in Fe-treated livers remained elevated whilst in AR/Fe nuclei it dropped to control values. CONCLUSION: These observations suggest that Aroclor and iron both independently and in combination cause alterations in DNA synthesis and cell division. These changes may represent permanent phenotypic alterations in PCB-induced hepatocellular carcinoma.

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14.14 EVIDENCE FOR IN VIVO DNA ALKYLATION BY METHYL RADICALS. IDENTIFICATION OF C8-METHYLGUANINE IN THE HYDROLYSATES OF DNA FROM RATS ADMINISTERED 1, 2 - DIMETHYL - HYDRAZINE

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Recently, we have proposed that alkyl radicals may play a role in hydrazine-mediated genotoxicity. Accordingly, we have demonstrated the **in vitro** formation of C8-methylguanine as a result of DNA attack by methyl radicals generated during the peroxidase-catalyzed oxidation of methylhydrazine (Augusto et al, J. Biol.Chem. **265**, 22093, 1990). Now we report the identification of C8-methylguanine in the neutral hydrolysates of DNA isolated from the liver and colon tissues of rats treated with the potent carcinogen 1,2-dimethylhydrazine. In all the samples examined the biologically isolated adducts were characterized by coelution with synthetic C8-methylguanine under, at least, three different high pressure liquid chromatography conditions. The sample isolated from liver DNA was also identified by comparison of its UV spectrum with that of the standard at two different pH values. The estimated yields of C8-methylguanine obtained in hydrolysates of DNA from the liver or colon tissue were comparable to those of O^6-methylguanine. C8-Methylguanine was not detected when the spin trap alpha-(4-pyridil-1-oxide-N-tert-butylnitrone) was administered together with 1,2-dimethylhydrazine. The spin trap also inhibited N7-methylguanine and O^6-methylguanine yields, although to a lesser extent. These results constitute the first evidence that DNA alkylation by carbon-centered radicals can occur **in vivo.**

14.15 HIGH LEVELS OF GLUTATHIONE REDUCTASE-ENCODING mRNA IN FAST-PROLIFERATING LUNG TUMOURS

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Glutathione reductase (GSSG + NADPH + $H^+ \rightleftharpoons$ GR 2 GSH + $NADP^+$)is a key enzyme of the antioxidant thiol metabolism. In tumour research, GR has been found to be involved in drug resistance mechanisms and in the capacity of the cells to mitigate the oxidative stress exerted by activated macrophages.

In the present study, human lung tumours and murine experimental tumour cell lines have been investigated with respect to their expression of glutathione reductase at the DNA, RNA and protein level. The mouse tumour cell lines (EAT, LL, SAB) showed high levels of GRmRNA in comparison to (nearly undetectable) GRmRNA in normal mouse lung tissue.

In a series of 11 human lung tumours obtained from surgery we found five fast proliferating tumours with high levels of GRmRNA and a 3-fold higher specific enzyme activity in comparison with control tissues. Four tumours with low proliferation rates did not show elevated levels of GR. The failure of two other obviously fast proliferating tumours to express high levels of GRmRNA may be due to inhomogeneity of the tumour material or to a delayed onset of GR-expression.

The hybridization pattern in the Southern blots of all tumours and control tissues were identical and gave no indication for gene rearrangements. The implications of high glutathione reductase levels for the metabolism of a proliferating cell are discussed.

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14.16 H_2O_2 INCREASE, CATALASE ACTIVITY IMPAIRMENT, DNA REPAIR-DEFICIENT ABILITY AND CARCINOGENESIS.

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Xeroderma pigmentosum (XP) and Trichothiodystrophy (TTD) are two human autosomal recessively-transmitted diseases characterized by DNA repair deficiency. Trichothiodystrophy is not a cancer-prone disease. Therefore, unpaired UV-induced lesions do not appear to be enough to give rise to tumours.

Results : In 21 different XP diploid fibroblast lines, catalase was decreased by a factor of five as compared to controls, while XP heterozygote lines exhibited intermediary reponses. All seven TTD lines were deficient in UV-induced lesion repair and exhibited a high level of catalase activity. UV-irradiation induces 5 times more H_2O_2 compared with TTD or controls. Two results indicated that (i) catalase decrease and H_2O_2 increase are not in direct relation with the lost of one allele 11p, (ii) molecular analysis of catalase transcription showed no difference between normal, XP and TTD lines.

Conlusions :Our work is now engaged in (i) possible defect in catalase tetramerisation (the only active form of this enzyme) or nucleotide (NADPH) absence in the active sites of catalase; (ii) catalase deficiency reparation by *in situ* enzymatic overexpression, and/or H_2O_2 scavengers addition; (iii) *in vivo* H_2O_2 measurements (ultra-microelectrodes) in only one cell;

Aim of our work thus concens as fondamental research of mechanisms, as clinical use .

14.17 IN VITRO CHARACTERIZATION OF FERRIC NITRILOTRIACETATE-MEDIATED DNA SINGLE AND DOUBLE STRAND BREAKS: RELATIONSHIP TO RENAL TUBULAR CARCINOGENESIS

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Fe*** bound to a chelator, nitrilotriacetate (NTA), has been shown to induce a high frequency of adenocarcinoma localized to the proximal tubule of the kidney in rodents. NTA is widely used as a detergent in households and hospitals, and FDA limited the presence of NTA to 5 ppm in boiler feedwater of food processing plants. In order to examine possible mechanisms for carcinogenic activity, we investigated the *in vitro* production of single and double strand breaks and 8-hydroxydeoxyguanosine in DNA mediated by iron alone or Fe-NTA chelate using supercoiled plasmid pz189. Neither Fe*** nor NTA alone broke DNA. Fe*** plus NTA together mediated the oxidative production of DNA single and double strand breaks in the presence of reducing agents (L-ascorbate >> H_2O_2 > L-cysteine). The number of double strand breaks was proportional to the square of the number of single strand breaks (r=0.75). The Fe***/NTA ratio (1:4) that was found to be optimal for DNA strand breakage was similar to the ratio that produced carcinogenesis in animals. Maximal Fe-NTA-mediated DNA damage in vitro was found under conditions of neutral pH, low ionic strength, presence of reducing agent, and absence of albumin. These conditions are present exclusively in the cortical proximal tubules of the kidney, the only location where toxicity and carcinogenicity of Fe-NTA has been observed. Thus, localized DNA strand breakage may explain the proximal tubular renal localization of carcinogenesis induced by Fe-NTA.

*This work was done while S.T. held a National Research Council-FDA research associateship.

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14.18 MECHANISMS OF ACTION OF AN OXIDANT TUMOR PROMOTER: *IN VIVO* AND *IN VITRO* STUDIES WITH BUTYLATED HYDROXYTOLUENE HYDROPEROXIDE

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The food antioxidant butylated hydroxytoluene (BHT) has several known toxic and carcinogenic properties, including activity as a tumor promoter in a number of tissues initiated with diverse agents. The extensive metabolism of BHT and its secondary products may mediate these activities. The oxidative metabolite BHT hydroperoxide (BHTOOH), a free radical-generating compound, is a tumor promoter in mouse skin. Structure-activity studies performed *in vivo* demonstrated that the metabolite BHT-quinone methide, a potent oxidant, mediates promotion by BHTOOH. The cellular effects of BHTOOH on keratinocytes have also been explored *in vitro*, wherein BHTOOH enhances growth and induces genes critical to proliferation. At higher doses, BHTOOH is also toxic to keratinocytes; this toxicity may provide the basis for the differential effects of BHTOOH on a heterogeneous cell population *in vivo*. Initiated cells have developed phenotypic attributes which favor their survival and growth, such as elevated glutathione levels, which afford greater resistance to the toxic effects of tumor promoters such as BHTOOH. Thus, in addition to directly stimulating the target cells in skin, BHTOOH may also create a selective advantage for the initiated cell population *in vivo* through its toxic effects on normal keratinocytes. Studies of the mechanisms of action of BHTOOH at the cellular level may lead to the elucidation of oxidant-mediated pathways for the alteration of gene expression and tumor promotion. Supported by PO1 CA44530 and NIEHS Training Grant ES07141.

14.19 QUANTITATIVE IMMUNOFLUORESCENCE ASSAY OF SINGLE-STRANDED DNA BREAKS CAUSED BY BENZOYL PEROXIDE IN MURINE KERATINOCYTES.

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A number of organic peroxides and hydroperoxides mediate tumor promotion and malignant conversion in mouse skin and are useful probes for defining the role of free radicals in multistage carcinogenesis. Benzoyl peroxide, for example, undergoes copper-dependent activation to benzoyloxyl and phenyl radicals which in turn produce protein adducts and single strand breaks in DNA. *In vitro* plasmid DNA nicking assays with ϕX174 DNA suggest a predominant role for the benzoyloxyl radical in this action. Non-radical metabolites, principally benzoic acid, are inactive. To address whether similar mechanisms are involved in benzoyl peroxide-mediated DNA strand breakage in cells, an assay for the *in situ* quantitation of single-stranded DNA (ssDNA) in individual keratinocyte nuclei has been developed using computer-assisted microfluorometry. The assay uses a polyclonal antibody specifically recognizing cytidine breaks in denatured DNA. A fluorescein-labeled secondary antibody (FITC) and a fluorochrome (DAPI) which binds to DNA are then used to quantitate ssDNA and total DNA, respectively, in the same nucleus. Preliminary experiments with benzoyl peroxide utilizing this immunofluorescence assay indicate DNA damage occurs at non-cytotoxic concentrations as low as 300 nM. Formation of ssDNA by benzoyl peroxide can be blocked by metal chelators, spin traps, and thiols while glutathione depletion potentiates damage. These results suggest that benzoyl peroxide produces direct, radical-mediated damage to DNA in keratinocytes. This technique may ultimately prove useful in the measurement of DNA damage and repair *in vitro* and *in vivo* following exposure to a number of environmental, occupational and iatrogenic agents. Supported by PO1 CA 44530, NIEHS Center Grant ES 03819, K04 CA 01230 and The Proctor and Gamble Company.

14.20 GENERATION OF ACTIVE OXYGEN SPECIES BY NEUTROPHILS AT HODGKIN'S DESEASE

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The study of human peripheral blood neutrophils functional state at Hodgkin's disease (HD) characterized an expressed immunological homeostasis disturbance is of great interest so far as these cells play an important role in the development of organism antitumor protective reactions. The ability to activate molecular oxygen is the main criterion of the neutrophils functional activity. A comparative study of oxygen activation by the healthy donors and HD patients neutrophils has been carried out. Significant differences in quantitative parameters of luminoldependent chemiluminescence induced by neutrophils adhesion on glass surface of these samples are determined. The cells of HD patients generate less effectively active oxygen species after stimulation by arachidonic acid and concanavalin A than healthy donors neutrophils. These distinctions are supposed to be the consequence of different capacity of HD patients and healthy donors neutrophils for metabolic activation. The ability of HD leukocytes to generate active oxygen species after a course of medical treatment has also been studied. The results obtained suggest that the method of luminoldependent chemiluminescence permits the control of HD patients neutrophils functional state.

14.21 AN INCREASE IN CELLULAR DEFENSES AGAINST OXYGEN RADICALS IS ONE OF THE FEATURES DEVELOPED BY HL-60 CELLS RESISTANT AGAINST DOXORUBICIN

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An increase in cellular defenses against oxygen radicals seems to be one of the features developed by human leukemia cells resistant against doxorubicin (HL-60R), since the homogenate from this cell line had only 65% of the ability of the original cell line to reduce oxygen to superoxide radicals during doxorubicin reduction. This result may be explained in part by the slight increase in superoxide dismutase and DT-diaphorase enzymatic activities. However, this increase in defenses against oxygen radical formation in HL-60R cells, which are cross resistant to daunorubicin, epidoxorubicin and 4-demethoxy daunorubicin, is not sufficient to prevent the cytotoxic effects of quinones which are more reactive with oxygen. Incubation of HL-60R cells with 5-OH-1,4-naphthoquinone and 5,8-diOH-1,4-naphthoquinone, two quinones highly reactive with oxygen, resulted in complete cytotoxicity at clinically relevant concentrations.

14.22 THE ACTIVITY OF ANTIOXIDANTS ON CELLULAR IMMUNITY IN ALVEOLAR ENVIRONMENT COULD PROTECT FROM LUNG CANCER.

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The oxidant stress may alter cell immunity and has been associated with lung cancer. A premedication with antioxidants protect from the experimental tumor. The reduction of alveolar macrophages activity and CD4/CD8 lymphocyte ratio in bronchoalveolar lavage (BAL) is related to lung cancer and smoke habit (an important source of oxidants). In our Institute we are testing the activity of antioxidants on cellular immunity administering aerosolic reduced Glutathione (600 mg bid for 6 days) in patients affected with chronic obstructive pulmonary disease in steady state. We found a significant increase in CD4 lymphocyte subset (32.1 vs 39.6; $p < 0.03$) and a significant improvement of alveolar macrophages phagocytosis (33.2 vs 50.7; $p < 0.04$). Our data show, after administering aerosolic Glutathione, immunological alterations opposite to lung cancer ones,and they might point out that the protection of antioxidant compounds from lung cancer could be also produced by an improvement of the immunological surveillance in alveolar environment.

14.23 FREE RADICALS-INDUCED Na-PUMP ACTIVITY CHANGES UNDERLAYING THE TUMOR DEVELOPMENT

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Ion gradients play important role in the inter- and intracellular signal transduction and ionic imbalances can lead to several pathological processes. The Na-pump (Na^+/K^+ ATPase), an integral plasma membrane lipoprotein, is involved in the maitenance of Na^+ and K^+ balance in cell. Earlier we have shown that the Na-pump is severely changed in brain tumor cells and in fibroblasts, transformed by papillomaviruses: its ion-transport efficiency, Na^+-binding nature, lipid-protein interaction etc. are changed. Also we have described changes in lipid peroxidation (LP) in these cells (glioma and transformed fibroblasts) and we have suggested that the LP changes can possibly explain the Na-pump changes.

In our last experiments using another model to study the role of free radicals in tumorigenesis ($KBrO_3$ induced renal carcinoma) we found that the 8-hydroxydeoxyguanosine producing agent, $KBrO_3$, also changes the activity of the Na-pump. The possible mechanism of this effect will be discussed.

14.24 ENHANCING EFFECT OF HIGH FAT DIET ON DIESEL EXHAUST PARTICLES (DEP)-INDUCED LUNG TUMORIGENESIS IN MICE

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High fat diets have been reported to modify carcinogenesis in various organs including lungs. It is well known that diesel exhausts cause lung carcinogenesis. Diesel engine powered cars are steadily increasing year by year. Therefore, it is anxious about increments of lung carcinogenesis by diesel exhausts and high fat diets.

We examined the effects of dietary high fat on diesel exhaust particles (DEP)-induced lung tumorigenesis. 0.1mg of DEP was instilled intratracheally to ICR mice 10 times (once/week). Tumor incidences in low and high fat diet groups without DEP were 20% and 30%, respectively. Tumor incidences in low and high fat diet groups with DEP were 12% and 48%, respectively. Significant difference was observed between both groups. There was also significant difference between low fat group without DEP and high fat group with DEP.

These results show that high fat diet have enhancing effects on lung carcinogenesis by DEP, although tumor incidence by only DEP instillation was not high.