Meetings

Hepatic Microsomes and Drug Oxidation

The role of hepatic microsomes in drug and steroid metabolism was the subject of a conference held at the National Library of Medicine, Bethesda, Maryland, 16–17 February 1968. A group of 49 scientists attended.

Albert Claude described studies carried out in the late 1930's which led to the isolation and characterization of microsomes, demonstrating with pictures of centrifuged liver cells the presence of a membranous phase which is now identified with the endoplasmic reticulum (E.R.). Turning his attention next to recent studies on the relation of the E.R. to other cellular constituents, Claude presented electron microscopic pictures giving a direct demonstration of smooth E.R. arising from rough E.R., and illustrating the encapsulation of mitochondria and peroxisomes by lamellar or tubular extension of smooth E.R. and the reconstitution of postmitotic nuclear envelope from segments of rough E.R. A series of electron micrographs demonstrated also the appearance, in regions of rough E.R., smooth E.R. junctions of small lipoprotein granules and their progress through smooth E.R. in Golgi-like profiles and vesicles. These different observations suggested that the various types of membranes, that is, smooth E.R., peroxisome envelopes, and the Golgi membranes, may arise from and be part of the rough E.R. system. A discussion of the morphological characterization of the E.R. and its relation to what the biochemist terms microsomes was continued in the presentation by G. Palade. Discussing the ubiquitous nature of the E.R. in eucaryotic cells and its role in establishing compartments within the cell, Palade directed his attention to the vectoral properties of membrane transport and the hypothesis of the cisternal space for segregation. Demonstrating by electron microscopic autoradiography the migration of newly synthesized protein (originating in the E.R.) to zymogen granules of the pancreas, Palade convincingly proposed the concept of E.R. compartments for segregation of cellular constituents. Turning his attention next to the dynamic nature of the E.R., Palade presented evidence that the half-life of the microsomal membrane is relatively short, that is, less than 3 days. Palade reported that a similar half-life has now been determined for the plasma membrane as well as the nuclear envelope. Pursuing the concept that the smooth E.R. originates from the rough E.R., Palade showed results of earlier studies with Siekevitz and Dallner on the development of smooth E.R. in the livers of newborn rats. The studies by Claude and Palade laid the foundation for the concept of a continuum of the E.R. membrane complex. The eventual transformation of this membrane system to Golgi apparatus or vacuoles was also indicated.

The distribution pattern of 12 enzyme activities, as well as RNA, phospholipid and cholesterol, upon isopycnic centrifugation of the microsomal fraction of liver was discussed by J. Berthet. The ability to resolve the "microsomal fraction" of liver into four subgroups, each distinguished by a chemical or enzyme marker, now establishes the groundwork for a detailed examination of membrane transformation.

Attention was next directed to the role of the endoplasmic reticulum in the expression of genetic information in a paper presented by H. Pitot. The concept of a membron was used to describe the stabilization of a membrane-bound template RNA operon. The concept was supported by the demonstration of an RNA fraction which, in smooth E.R., has a high guanine-cytosine content. In addition the demonstration of a DNA-like RNA present in membranes of the rough E.R. was suggested as a possible source of information for binding of polysomes. This session concluded with a presentation by J. Fouts on the various patterns of drug metabolism observed with subfractions of hepatic microsomes. The ability to separate by density gradient centrifugation two major fractions, one predominantly arising from smooth E.R. and the other from RNA-rich rough E.R., permits the direct demonstration of heterogeneity of enzyme activities associated with "microsomes." Exposure of animals to inducing agents, such as phenobarbital (PB) or chlordane-like materials, causes a preferential increase in several drug-metabolizing enzyme activities in the smooth-surfaced microsomes. However, treatment of animals with 3-methylcholanthrene (3-MC) had the opposite effect; drug-metabolizing enzymes which are normally highly concentrated in smooth-surfaced microsomes are more evenly distributed between the two types of microsomes after 3-MC treatment.

R. Estabrook described experiments to determine the spectral and fluorometric properties of microsomal flavoproteins, indicating the failure to observe by steady-state studies distinct additive contributions by NADH-cytochrome b_5 reductase and NADPH-cytochrome c reductase. The conclusion that interactions at the level of flavoproteins might exist was suggested. In addition Estabrook described recent studies employing mixtures of NADH and NADPH for support of the enzymatic demethylation of aminopyrine. The demonstration that NADH was as competent as NADPH as a source of reducing equivalents for this type of mixed function oxidation reaction raises the possible consideration of cytochrome b_5 as a component of the enzyme complex. R. Sato described the spectral properties of a preparation of purified cytochrome P-450 obtained by digesting liver microsomes suspended in 25 percent glycerol with the bacterial protease nargarse. In addition Sato presented data on the chemical properties of "native cytochrome b_5" obtained by treatment of microsomes with deoxycholate and Triton X-100. The cytochrome b_5 purified in this manner, without exposure to proteolytic enzymes, is present as an aggregate of molecular weight 120,000 until exposed to 4.5*M* urea, when it dissociates into monomers of molecular weight 25,000. Treatment of the purified "native" cytochrome b_5 with trypsin yields a preparation of "degraded" cytochrome b_5 which is indistinguishable (molecular weight 11,500) from

that obtained by the direct proteolysis of microsomes. Sato concluded that "native" cytochrome b_5 contains a hydrophobic center, essential for its binding to the microsomal membrane and susceptible to loss when cytochrome b_5 is purified by proteolytic digestion.

J. Gillette directed attention to the postulated role of cytochrome P-450 in azo reductase and nitro reductase activity of liver microsomes. The effects of treatment of animals with inducing agents, or carbon tetrachloride, variation with sex or species, as well as inhibition of nitro reductase by substrates of microsomal mixed function oxidation reactions, supported the hypothesis of a role for cytochrome P-450 in the metabolism of *p*-nitrobenzoate and neoprontosil. Gillette also described experiments relating the rate of cytochrome P-450 reduction by NADPH with the overall rate of demethylation of ethylmorphine. He concluded that the step limiting the rate in the oxidation of drugs may be the reduction of a substrate-cytochrome P-450 complex.

M. J. Coon described the isolation from liver microsomes of three components—a reductase fraction dependent on NADPH; a fraction containing cytochrome P-450; and a heat-stable, organic solvent extractable fraction. All components are required for the reconstitution of omega oxidation of lauric acid. These studies represent the first report of the resolution of a microsomal mixed function oxidation reaction in which cytochrome P-450 is functional. Purification was achieved by treating microsomes with deoxycholate in the presence of glycerol, sucrose, citrate, potassium chloride, and dithiothreitol, and fractionation on DEAE-cellulose. The relation of the microsomal enzyme system to that isolated from bacteria was pointed out by J. Peterson. D. Ziegler then described the isolation and 40-fold purification of a flavoprotein from pork liver microsomes functional in tertiary amine oxidation for the formation of N oxide product. The broad specificity for substrates, for example, tranquilizers, tropine alkaloids, narcotics, and hallucinogens, was documented by Zeigler for this microsomal oxidation reaction system in which cytochrome P-450 does not participate.

Hj. Staudinger described results with a variety of model systems, in order to differentiate three general mechanisms of considering "activated oxygen." The role of OH-radicals, an oxene mecha-

nism, or hydroxylation by peracids (by OH^+), was evaluated in terms of current information on microsomal monooxygenase reactions. Staudinger concluded that "active oxygen" must be an electrophilic oxygen species with six electrons. S. Udenfriend discussed the significance of the "NIH shift" with respect to liver microsome hydroxylations. The intramolecular migration of a methyl group, halogen, deuterium, or tritium during hydroxylation of aromatic compounds permits the design of a variety of experiments to determine the multiplicity of microsomal hydroxylases.

S. Orrenius described recent experiments on the influence of PB treatment on the metabolism of steroids by liver microsomes. Using radioactive labeling of phospholipids as an indicator of E.R. synthesis, Orrenius proposed a timetable for the changes observed during induction of liver microsomes by barbiturates. The initial reaction between 0 to 3 hours after treatment of animals is a binding of the drug to the E.R. followed at 3 to 6 hours by an increase in phospholipid turnover. Concomitant with this change is an increase in the content of rough E.R. enzymes (4 to 6 hours) after which there is a detectable increase in the nuclear RNA polymerase (8 to 12 hours). Longer term effects are related to the increase in the enzyme content of the smooth E.R. (10 to 24 hours) and a decrease in the rate of breakdown of the E.R. (12 to 24 hours). Orrenius suggested that drug induction is a consequence of an alteration in the steroid balance of the animal, since drugs and steroids are competitive inhibitors. This concept was supported by studies with adrenalectomized and castrated animals.

A. Conney then discussed the question of the presence of a single enzyme system or multiple enzymes in liver microsomes for steroid hydroxylation. Alterations in the pattern of 6β-, 7α-, and 16α-hydroxylation of testosterone during the development of rats, as well as differential effects on enzyme activity by chlorothion, led Conney to conclude that separate rate-limiting components participate in the hydroxylation of testosterone, and that one or more CO-sensitive cytochromes (P-450) function in these hydroxylation reactions. Following the theme of the presence of multiple enzymes for hydroxylation in liver microsomes, G. Mannering presented data on two-substrate kinetics and the ability to detect, from the *p*H-dependence of the ethyl isocyanide-induced spectral changes of cytochrome P-450, the presence of a different reactive form of cytochrome P-450 in liver microsomes from animals treated with 3-MC. Studies of stability of microsomal pigments, changes in the pattern of substrate interaction as determined spectrophotometrically, and the influence of thioacetamide in causing a marked decrease in some enzyme activities, led to the conclusion that different forms of cytochrome P-450 might be present in microsomes. Another demonstration of this difference in various forms of P-450 modified by inducing agents was presented by R. Kuntzman, who described subtle spectral shifts in the location of the maximum of the CO derivative of reduced P-450 in liver microsomes from animals treated with benzpyrene, that is, a displacement from 450 nm to about 448 nm. H. Remmer and A. Hildebrandt then presented studies directly demonstrating spectral properties of the two forms of cytochrome P-450 preferentially altered by treatment of animals with barbiturates or polycyclic hydrocarbons. The ability to identify and characterize two forms of cytochrome P-450 and assess the content of each form (termed P-450 and P-446) in microsomes from various sources now opens the possibility of resolving the complexity of differences in enzyme activities which have been observed with various species, sex, age, or pretreatment of animals.

D. Nebert described studies showing the increased incorporation of amino acids in rat liver microsomes after treatment of animals with PB and the relationship of this change to the increased levels of messenger RNA. In contrast, treatment with 3-MC causes an increase in the rate of nuclear RNA synthesis and the content of RNA in the nucleus. Studies of polycyclic hydrocarbon stimulation of benzpyrene hydroxylase activity in tissue culture of embryonic cells indicate that the inducer hydrocarbon may be acting on mRNA translation, resulting in a feedback control affecting an increased synthesis of nuclear RNA. Thus it is concluded that inducing agents may exert their influence not only by causing an activation of specific genes but also by affecting the translation of mRNA. E. Bresnick then discussed the activation of chromatin by 3-MC by ascertaining the template efficacy of chromatin from livers of animals treated with 3-MC. Differences in nearest neighbor frequency in the product of RNA polymerase suggest that 3-MC causes an

activation of liver chromatin resulting in an increased number of sites available for the transcription of RNA.

I. Arias presented the results of a series of double labeling experiments designed to determine whether the increased level of hepatic smooth E.R., observed after PB treatment of animals, was a consequence of enhanced protein synthesis or decreased degradation (stabilization) of membranes. The results indicate that smooth E.R. proteins have considerably different turnover rates and that PB enhances synthesis of some microsomal proteins but not all.

T. Omura then discussed his experiments designed to establish that the turnover of cytochrome b_5 was unaffected, whereas that of the flavoprotein NADPH-cytochrome c reductase of liver microsomes was affected upon treating the animals with PB. Since PB showed little effect on the incorporation of radioactive amino acid into total microsomal protein, Omura concluded that the stimulation by PB seems to be fairly specific. In addition PB was shown to influence degradation of the reductase and cytochrome b_5, indicating that the increase in smooth E.R. may be mostly attributed to a relatively nonspecific prevention of breakdown of microsomal protein components. H. Marver then discussed studies describing the role of heme in the synthesis and repression of microsomal protein. Injection of 1 to 4 μmole of heme per 100 gram body weight represses drug-induced synthesis of aminolevulenic synthestase as well as drug-induced synthesis of cytochrome P-450.

During the general discussion at the end of the meeting, J. Casida described the role of cytochrome P-450 in the metabolism of insecticides by flies pointing out the similarities between the liver microsomal hydroxylation system of mammals and the parallel enzyme system in insects. A. Conney concluded the session by discussing some preliminary studies relating the content of benzpyrene hydroxylase in placentas from women who were cigarette smokers. The ability to directly demonstrate this activity in smokers, but not in nonsmokers, represents the first direct evidence for a compensatory enzymatic mechanism by humans to detoxify carcinogenic polycyclic hydrocarbons present in cigarette smoke.

The meeting was sponsored by the Committee on Applications of Biochemical Studies in Evaluating Drug Toxicity, Drug Research Board, Na-

tional Academy of Sciences–National Research Council, and the National Institute of General Medical Sciences, National Institutes of Health. It is anticipated that a complete report of the formal presentations as well as discussions will be published in the near future by Academic Press.

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Calendar of Events

National Meetings

July

8–11. **Soil Conservation** Service and Experiment Stations, Clemson, S.C. (G. R. Craddock, Agronomy and Soil Dept., Clemson Univ., Clemson)

9–13. American **Therapeutic** Soc., Essex House, New York, N.Y. (R. T. Smith, 37 Narbrook Park, Narberth, Pa. 19072)

12. American Assoc. for the Study of **Headache**, New York, N.Y. (S. Diamond, 5214 N. Western Ave., Chicago, Ill. 60625)

13–17. American **Medical** Assoc., New York, N.Y. (F. J. L. Blasingame, 535 N. Dearborn St., Chicago, Ill. 60610)

14–16. American Inst. of **Aeronautics and Astronautics**, San Francisco, Calif. (Meetings Manager, ASME, 345 E. 47 Street, New York 10017)

21–25. American **Veterinary Medical** Assoc., Boston, Mass. (Director, Business Div., 600 S. Michigan Ave., Chicago, Ill. 60605)

22–27. American **Medical Technologists**, Dallas, Tex. (American Medical Technologists, 710 Higgins Rd., Park Ridge, Ill.)

23–26. American Soc. of **Pharmacognosy**, Iowa City, Iowa. (D. P. Carew, College of Pharmacy, Univ. of Iowa, Iowa City 52240)

25–30. American **Podiatry** Assoc., Chicago, Ill. (J. Tipton, Convention Manager, 2301 16th St., NW, Washington, D.C. 20010)

August

1–3. Conference on **Dermatology**, Aspen, Colo. (W. C. Eisele, Univ. of Colorado Medical Center, 4200 E. 9th Ave., Denver 80220)

3–9. National **Poultry** Science Assoc., Fort Collins, Colo. (R. E. Moreng, Animal Science Bldg., Colorado State University, Fort Collins 80521)

11–15. National **Medical** Assoc., Houston, Tex. (S. C. Smith, 520 W St. NW, Washington, D.C. 20001)

12–14. American Inst. of **Aeronautics and Astronautics**, Pasadena, Calif. (W. J. Brunke, Meetings Manager, 1290 Sixth Ave., New York 10019)

12–16. American **Crystallographic** Assoc., Buffalo, N.Y. (W. L. Kehl, Gulf Research & Development Co., P.O. Box 2038, Pittsburgh, Pa. 15230)