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Factors Regulating Drug Metabolism in Intact Hepatocytes

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I. Introduction

REGULATION of biological systems within whole cells may occur at one or many sites within a multienzyme system. When a single site of regulation is involved, such as a specific enzyme step, data can often be interpreted on the basis of kinetic information obtained in vitro. On the other hand, regulation of a multicomponent system is best understood through the identification of one or more rate-limiting steps and the definition of interactions between them. Many biological processes involve multicomponent systems, which are often regulated by many factors. The mixed-function oxidase system is a particularly complex biological system because the components are multienzymatic in nature and are dependent on a continuous supply of reduced cofactor, nicotinamide adenine dinucleotide phosphate (NADPH), which is itself generated by other multienzyme systems. Each of these multienzyme systems in turn is regulated by concentrations of substrates and intracellular effectors. Consideration of the regulation of mixed-function oxidation and conjugation is highly important because these systems are the major routes of detoxification of drugs and many endogenous substances and they also participate in acti-

vation and inactivation of carcinogens and environmental pollutants. Approaches to control of these events in intact cells ultimately depend upon understanding factors that are rate-controlling for this process in whole cells.

Considerable progress has been made in the identification of the sequence of events involved in operation of the mixed-function oxidase system. The three major components consist of NADPH-cytochrome P-450 reductase, cytochrome P-450, and phospholipid, which are components of the membranes of the endoplasmic reticulum. Many cytochromes (e.g. at least six in rabbit liver) are present for each flavoprotein and this may account for the wide substrate specificity of the mixed-function oxidase system. Oxidation of compounds via this system involves the following sequence of events: An oxidized cytochrome binds the drug substrate, which is reduced by the flavoprotein in an NADPH-dependent reaction. This P-450^{++}-drug complex then reacts with oxygen to form a P-450^{++}-O_2-drug complex, which is further reduced by the second electron from NADPH. The breakdown of this second complex releases hydroxylated drug and water and regenerates oxidized cytochrome P-450. Work that has led to elucidation of the above sequence of events in vitro has been dealt with in a number of papers and reviews (23, 50, 63, 126, 161, 162).

In intact cells, the mixed-function oxidation system is intimately related to other cellular events involved in the generation of the reduced cofactor and the provision of activated biosynthetic intermediates needed for conjugation of oxidized products of this system. A scheme illustrating some of the interactions that may occur is presented in figure 1. NADPH is generated by highly regulated multienzyme systems that exist in several intracellular compartments. For example, the major dehydrogenases of the pentose phosphate shunt are cytosolic, whereas fatty acid oxidation and the citric acid cycle are intramitochondrial. The former enzymes provide reducing equivalents in the cytosol directly whereas the latter furnish substrates for malic enzyme and isocitrate dehydrogenase, which then form NADPH in the cytosol. Movement of reducing equivalents from the mitochondrial to the cytosolic space via specific substrates involves complex shuttle mechanisms. Until recently, scant atten-

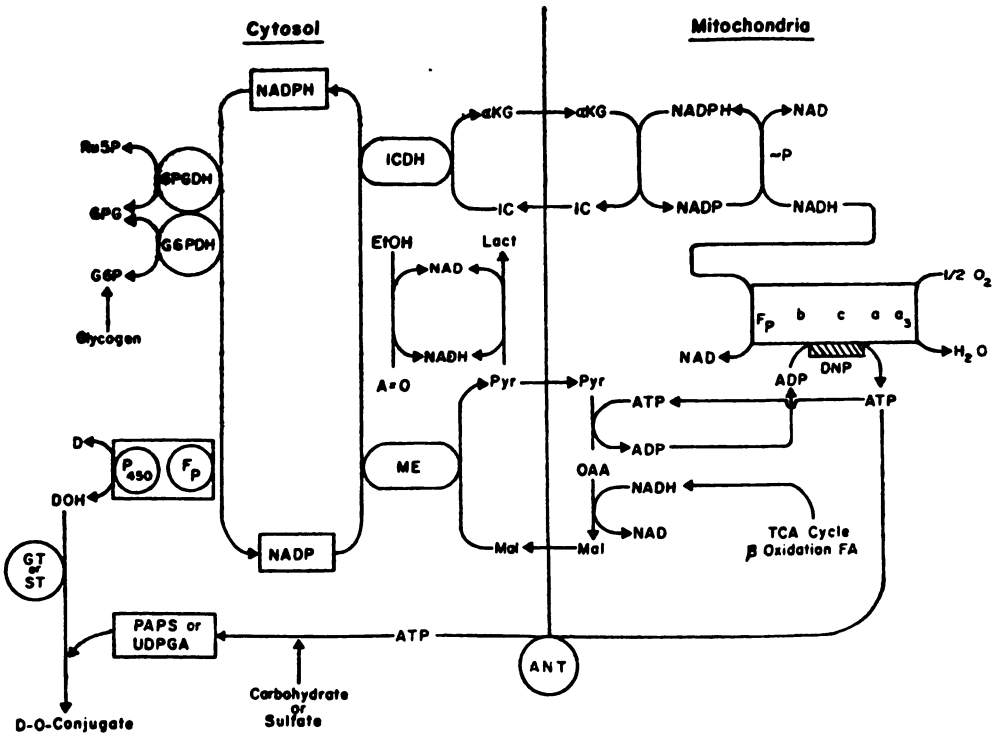

FIG. 1. Interactions between drug and intermediary metabolism in intact cells. Cytosolic reduced nicotinamide adenine dinucleotide phosphate (NADPH) may be generated by the pentose phosphate pathway in a series of reactions starting with glucose-6-phosphate (G6P) and involving the enzymes glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH). In addition, cytosolic NADPH may be generated by a malate shuttle that involves the carboxylation of pyruvate (Pyr) via pyruvate carboxylase to form oxalacetate (OAA), reduction to malate (Mal), egress of malate into the cytosol, and NADPH production via malic enzyme (ME). Alternatively, mitochondrial NADPH is generated by an energy (~P)-requiring transhydrogenase that carries out the reduction of $NADP^+$ from NADH. A shuttle mechanism involving isocitrate dehydrogenase (ICDH) transfers hydrogen from NADPH to α-ketoglutarate (α-kg) in the mitochondria and regenerates NADPH in the cytosol. Activated intermediates for conjugation reactions [e.g. adenosine 3′-phosphate 5′-phosphosulfate (PAPS) and uridine diphosphogalactase (UDPGA)] are formed from carbohydrate and sulfate in energy-dependent reactions. Other abbreviations used are: IC, isocitrate; DNP, dinitrophenol; GT; glucuronyl transferase; ST, sulfotransferase; ANT, adenine nucleotide translocase; D, drug substrate for cytochrome P-450; DOH, hydroxylated product; and D-O-conjugate, conjugated hydroxydrug.

tion has been given to interactions that occur between these systems and rates of mixed-function oxidation in whole cells.

There are at least four types of regulation that can be imposed upon mixed-function oxidation in intact cells: induction, substrate and cofactor supply, activation and inhibition by effectors, and competing reactions. Competing reactions for substrates and cofactors (e.g. fatty acid synthesis) undoubtedly play a major role in determining the availability of NADPH for mixed-function oxidation in intact cells.

Induction of enzyme components is a generally slow form of regulation; however, under some conditions it can occur in a few hours. In most cases, induction does not appear to be specific for components of the mixed-function oxidase system. For example, phenobarbital tends to induce several forms of cytochrome P-450 (49), glucuronyl transferase (164), and several NADPH-generating enzymes as well (109, 110).

A second form of regulation involves the supply of substrate and cofactor. Diffusion of oxygen, transport of a drug to binding sites on cytochrome P-450, and delivery of NADPH to the flavoprotein may be rate-controlling. Of these three possibilities, regulation via the supply of reduced cofactor may be most important since maintenance of the oxidation-reduction state of the NADP: NADPH couple is a highly regulated process in intact cells. NADPH is formed in the cytosol via oxidation of substrates for intermediary metabolism such as glucose-6-phosphate, malate, and isocitrate. This cofactor may also arise in the mitochondrial space via transhydrogenation. Reactions that compete for reduced cofactor must be taken into account in explaining regulation of mixed-function oxidation in whole cells. In addition to fatty acid synthesis, reduction of oxidized glutathione is another reaction recognized to compete for NADPH. At the cytochrome P-450 level, substrates that compete for binding sites include a wide array of xenobiotics as well as endogenous substrates such as steroid hormones, vitamin D, and bilirubin.

Modulation of rates of drug oxidation by small molecules derived from intermediary metabolism is a subject that has not been extensively studied. However, this form of regulation is suggested by strong inhibition of NADPH-generating enzymes and NADPH-cytochrome *c* reductase by ATP (109). Interactions of this nature suggest that the generation of reducing equivalents and their utilization for mixed-function oxidation and conjugation may be regulated in a concerted manner in intact cells.

Alteration of the formation of specific intermediates such as NADPH and activated substrates used in conjugation reactions provides mechanisms whereby nutritional and endocrine factors may regulate mixed-function oxidation in whole cells on both an acute and chronic basis. The review by Campbell and Hayes (41) dealt at length with effects of nutritional factors on levels of the components of mixed-function oxidases; however, acute alterations of rates of drug metabolism and conjugation by nutritional factors that involve changes in substrate and cofactor supply had not been studied extensively at that time.

Considerable work involving broken cell preparations, perfused organs, isolated cells, and cells in culture has explored the complex interactions influencing drug metabolism under conditions closely resembling those that exist in vivo. The advantages and limitations of each of these systems are considered below. The major portion of this review is devoted to the consideration of information obtained from studies with perfused organs and isolated cells.

II. Methods Used to Study Mixed-Function Oxidation in Whole Cells

A. Perfused Organs

Isolated liver perfusion has been used for many years as a model to study metabolic events in intact cells. Claude Bernard (18) described glycogen conversion to glucose in livers perfused with tap water. Other early applications of this technique have been reviewed by Miller (143). In most of the early liver perfusion studies, large animals such as the dog or cat were used, because at the time most biochemical analyses required large samples. However, the demonstration of successful liver perfusion in the laboratory rat by Trowell (239), Corey and Britton (52), Brauer et al. (28), and Miller (144), plus the development of improved biochemical methods, greatly increased interest in perfusion techniques for a wide variety of biochemical, pharmacological, and physiological applications.

A detailed description of the surgical techniques for rat liver perfusion is included in the review by Miller (144). Briefly, animals are anesthetized, the abdominal cavity is opened, and perfusion fluid is infused into the liver via a cannula placed in the portal vein. Since this procedure may involve a period of potentially deleterious anoxia, perfusion must be initiated in situ (200, 203).

A variety of perfusion fluids have been described. Many investigators have used heparinized or defibrinated whole blood diluted with a buffer solution and supplemented under various conditions with glucose, amino acids, albumin, vitamins, or antibiotics (28, 143–145). Other investigators have used semisynthetic media with washed erythrocytes of human (94) or bovine (25, 198) origin. These fluids, as well as fluids containing fluorocarbons, allow adequate tissue oxygenation of relatively low flow rates, and may be adapted to either circulating or nonrecirculating systems (81, 94, 231). Major drawbacks of all fluids containing red blood cells include: progressive hemolysis (200), deterioration of mixed-function oxidation, utilization of substrates or production of metabolites by red cells (81), interference by hemoglobin with absorption measurements in the perfusate or intact organ or interference with fluorescence from the liver

surface (203), and the availability of red blood cells. Because of these difficulties, many laboratories now use hemoglobin-free perfusion fluids consisting of osmotically balanced and buffered electrolyte solutions (200, 203, 229). The decreased oxygen-carrying capacity of these fluids relative to blood is compensated for by high flow rates to provide adequate oxygenation of the tissue. Similar rates of oxygen uptake have been noted in rat livers perfused with blood (29) and hemoglobin-free bicarbonate buffer (234).

Perfusions of rat liver are performed with either recirculating systems (17, 28, 144, 199) or nonrecirculating systems (229). With recirculating rat liver systems, relatively small volumes of fluid, approximately 100 ml, are recirculated and reoxygenated, thus closely mimicking the situation in vivo. Nonrecirculating (open) perfusions have been developed more recently and have some distinct advantages. One advantage of this technique is that the composition of the influent perfusion medium is kept constant, in contrast to closed systems, where the arterial fluid is composed of added substrates plus metabolites produced by the liver. Other advantages of nonrecirculating perfusion systems include: ready detection of small changes in metabolites produced by the tissue, ease in establishing dose-response relationships, and the possibility of studying different metabolic conditions in the same liver. Each liver may act as its own control in nonrecirculating systems. Although open perfusion systems require large volumes of perfusion fluid, this limitation is outweighed by a number of advantages. For example, hemoglobin-free media facilitate direct optical measurement of metabolites in the perfusate, and changes in fluorescence and absorption of the tissue itself may be monitored. Techniques for the measurement of pyridine nucleotide and flavoprotein fluorescence from the liver surface have been described by a number of investigators (203, 204, 221). Flavoprotein fluorescence is primarily an indicator of the mitochondrial redox state (203), while pyridine nucleotide fluorescence reflects the cytosolic plus mitochondrial oxidation-reduction state.

Spectrophotometric measurements in the whole perfused liver have also been described. Sies and Chance (208) have developed techniques for the direct spectrophotometric measurement of catalase-H_2O_2 compound I, and the occurrence of type I and type II binding spectra of substrates to cytochrome P-450 can be measured in the perfused liver (207). In addition, direct optical methods have been used to monitor the oxidation-reduction state of cytochromes *a*, *c*, b_5, and P-450 (127, 207). Chance et al. (44) have recently described a time-sharing multichannel spectrophotometer, reflectometer, and fluorometer that may readily be applied to perfused liver studies.

The length of time that the isolated perfused liver will continue to perform its normal metabolic functions is a question of obvious concern. Tests of liver viability that have been employed include: the general appearance of the liver, the hepatic flow and vascular resistance, membrane potential, potassium levels, pH of the fluid, oxygen consumption, and the continued production of bile and metabolites. With careful control of conditions, prolonged perfusions of 8 or 12 hours have been carried out (12, 101); however, glucose and lipid synthesis are linear for only about 2 hours. Livers that have been perfused for 1 to 5 hours are viable (112).

Several comparative studies between drug metabolism in vivo and in the perfused liver have been performed, and a good correlation was found to exist between the two systems for many drugs (20, 71, 247, 248). Recent development of a direct readout system for *p*-nitroanisole O-demethylation (229) in perfused livers has facilitated the study of interactions between hepatic intermediary metabolism and mixed-function oxidation. Application of techniques such as this to a number of questions involving interactions between intermediary metabolism and mixed-function oxidation are reviewed below.

Metabolite measurements in tissue and perfusate. Samples of perfusate may readily be withdrawn for the measurement of any metabolite of interest. Liver samples can be obtained without interrupting perfusion by ligating and removing one lobe (144); however, this procedure is problematic because it changes perfusion of the remaining tissue and because metabolic events are not uniform throughout the liver. Alternatively, livers may be frozen rapidly in a particular metabolic state by freeze-clamping with tongs chilled in liquid nitrogen (110). The tissue is extracted and metabolites of interest are then measured by standard enzymatic techniques. In this way, drug metabolites (20, 75, 247, 248), proteins and amino acids (101, 145), ketone bodies (31, 257), and glycolytic intermediates (110) in the tissue may be measured conveniently. Bile content may be sampled continually and assayed after cannulation of the bile duct (28, 144).

Mitochondrial NAD^+ redox state may be determined from β-hydroxybutyrate: acetoacetate measurements. Pyridine nucleotide oxidation-reduction potentials may also be calculated from metabolites measured in tissue extracts. These calculations are based on the assumption that the oxidized and reduced substrates of some active pyridine-linked dehydrogenases are in near equilibrium with the free nucleotide in specific subcellular compartments (94, 110, 257). In this way, changes that occur during mixed-function oxidation in $NADP^+$:NADPH ratios are based on changes in substrates assumed to be in near equilibrium with malic enzyme, isocitrate dehydrogenase, and 6-phosphogluconate dehydrogenase.

A major advantage of using the isolated perfused liver is that studies may be carried out under carefully controlled conditions resembling those in vivo in which influences due to events taking place at extrahepatic sites in the intact animal are avoided. Thus, in studies of mixed-function oxidation, it is possible to administer high concentrations of drugs or toxins in perfusion fluids and detect low levels of metabolites without complications of toxicity in the whole animal.

In vitro preparations of whole cells used as alternatives

to perfused organs include tissue slices, isolated hepatocytes, and cells in culture. Discussion will be limited to the latter two systems because tissue slices have several distinct disadvantages such as loss of nutrients and dependence upon passive diffusion for oxygen, which are not uniform even in slices as thin as 0.5 mm.

B. Isolated Hepatocytes

Isolated hepatocytes have been used successfully in a number of studies of drug metabolism. In an early study, Govier (82) demonstrated that acetylation of sulfanilamide occurs in reticular rather than in parenchymal cells. More recently, there have been studies with benzo(a)pyrene (241), ethanol (133), and aminopyrine (252) metabolism in isolated liver cells. Isolated hepatocytes may be separated rapidly into cytosol and mitochondria, a distinct advantage over the perfused liver. However, isolated hepatocytes are not obtained in high yields, they are fragile, and they may deteriorate rapidly in vitro as indicated by a marked depletion of intracellular potassium. Incubation of hepatocytes with nicotinamide prevents the rapid decline in cytochrome P-450 content (168, 171). An important consideration that is often overlooked is that this preparation, as well as the liver slice, disrupts the spatial heterogeneity of cells that exists in the whole organ.

Despite these limitations, isolated hepatocytes are widely used today in studies of hepatic intermediary metabolism as well as drug metabolism. A major advantage of this system is that multiple parameters such as dose-response relationships and the effects of various inhibitors may be studied in cells obtained from the same organ. A recent application of this model to studies of benzo(a)pyrene metabolism indicated that inhibition of various pathways of conjugation leads to an increase of covalent binding of drug metabolites to tissue (38).

C. Hepatocytes in Cell Culture

Isolated hepatocytes used in short-term studies show severe degenerative changes within hours. Attempts to increase the viability of isolated hepatocytes have been made by maintaining them in cell culture. Functions such as albumin synthesis (22), bile acid conjugation (56), and porphyrin biosynthesis (84) appear to be maintained in hepatic parenchymal cells in culture for relatively long periods of time; however, processes associated with mixed-function oxidation are not. In general, two types of liver cell culture systems have been used: primary cultures and serially propagated cells. Replicated cell lines derived from hepatocytes contain little if any microsomal drug-metabolizing enzymes and therefore cannot be used as models (22); however, primary cultures of hepatocytes have been used to examine oxidative drug metabolism under well-defined conditions. Nebert and his coworkers were among the first to employ primary cultures of fetal rat hepatocytes to study mixed-function oxidation (76, 77, 153, 154, 166). The induction of aryl hydrocarbon hydroxylase (AHH), a cytochrome P-450-dependent enzyme system, by a variety of chemicals added to culture media has been studied extensively by these investigators. Both phenobarbital and 3-methylcholanthrene induced AHH activity in cultured hepatocytes. This increase was associated with an induction in cytochrome P-448 (166). The finding that both phenobarbital and 3-methylcholanthrene induce AHH in fetal hepatocytes indicates that these agents may act differentially on cells in culture vs. the intact liver in situ, since AHH is induced by 3-methylcholanthrene but not by phenobarbital in vivo.

A major problem with hepatocytes in primary cell culture is that their cytochrome P-450 declines rapidly, decreasing almost 80% during the first 24 hours in vitro (22, 29, 91). This decline can be prevented partially by cyclohexamide (89), and may be related to increases in the activity of hemeoxygenase (21). Addition of δ-aminolevulinic acid, dihydroxyacetone, ascorbic acid, and adenine to culture media partially prevents the decreases in cytochrome P-450 (90). Michalopoulos et al. (139, 140) have improved the viability of cultured primary hepatocytes by incubating them on floating collagen membranes. This technique apparently prolongs viability from a period of 3 to 4 days to more than 20 days. Supplementing growth media with relatively high concentrations of hydrocortisone (0.1 μM) slows the decline in cytochrome P-450 that occurs in hepatocytes in culture (141). Induction of a P-448 form of the monooxygenase system in cells maintained for periods of 10 days or longer in the presence of hydrocortisone has been noted (141).

By using primary cultures of nonreplicating hepatocytes on floating collagen membranes, Michalopoulos et al. (141) demonstrated that the time course of induction of components of the mixed-function oxidase system and morphological changes produced in these cells by exposure to 3-methylcholanthrene and phenobarbital resemble those seen in vivo. Exposure of cells to phenobarbital (0.1 to 2 μM) produced a dose-dependent increase in cytochrome P-450 and proliferation of membranes of the smooth endoplasmic reticulum over a 5-day period. Addition of 2 to 10 μM 3-methylcholanthrene to culture medium resulted in dose-related increases in cytochrome P-448 within 2 days. In contrast to the pattern seen in vivo, induction by 3-methylcholanthrene was greater than the induction of cytochrome P-450 by phenobarbital.

Studies with primary cultures of chick hepatocytes in chemically defined media have shown that a number of hormones potentiate the induction of heme synthesis by allylisopropylacetamide (195). In contrast to cells isolated from mammalian liver, chick embryo hepatocytes in primary monolayer culture maintained high levels of cytochrome P-450 and drug metabolizing systems (5). Amounts of these components were comparable to those in adult mammalian liver in vivo. Exposure of chick embryo hepatocytes in defined culture medium to various inducing agents increased de novo synthesis of several forms of cytochrome P-450 (5).

Hepatocytes in culture offer several advantages that may be useful in future studies. For example, cell culture systems may prove useful in screening for precarcinogens whose activation is dependent on mixed-function oxidation. At least one precarcinogen, fluorenylacetamide, damages DNA of cultured hepatocytes (140). It was recently demonstrated that cellular uptake, transport, and macromolecular binding of benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene occurred in preconfluent cultures of human fibroblasts (62). Decad et al. (57) demonstrated that primary hepatocyte cultures maintained in a defined medium metabolized aflatoxin B_2 to water-soluble metabolites that bind covantly to cellular macromolecules. The successful growth of primary nonreplicating hepatocytes in culture for extended periods of time in defined media will undoubtedly lead to further use of such systems to study various aspects of hepatic function including regulation of mixed-function oxidase systems.

III. Effect of Substrates for Mixed-Function Oxidation on Intermediary Metabolism

Drug substrates that affect intermediary metabolism may have profound effects on rates and patterns of biotransformation. Substrates for mixed-function oxidation are known to alter intermediary metabolism in the liver by a variety of mechanisms including direct inhibition of specific enzyme activities, competition for cofactor, and uncoupling of oxidative phosphorylation. Examples to illustrate each of these mechanisms on gluconeogenesis, lipogenesis, and glucose utilization are reviewed below.

A. Gluconeogenesis

Drug substrates for mixed-function oxidation such as aminopyrine may divert carbon from the main pathway of glucose synthesis by inhibiting gluconeogenesis. Scholz et al. (201) studied the effect of aminopyrine on gluconeogenesis from lactate in perfused livers of phenobarbital-treated rats and found that maximal rates of gluconeogenesis were suppressed by more than 50% by 0.3 mM aminopyrine. However, low rates of gluconeogenesis from lactate or dihydroxyacetone were only slightly inhibited by aminopyrine. These data were interpreted to suggest that NADPH-utilizing processes such as the mixed-function oxidation of aminopyrine divert malate away from the pathway of gluconeogenesis toward formation of NADPH via malic enzyme. This leads in turn to a compensatory influx of substrate for the pyruvate carboxylase reaction. Thus, gluconeogenesis is suppressed only when pyruvate carboxylation is maximal. While such studies have been carried out to date only with aminopyrine, this type of interaction may be general and could occur with a wide variety of structurally dissimilar steroids, carcinogens, drugs, and chemical toxins. The carcinogenic derivatives of the polychlorinated biphenyl class reduce glucose synthesis by directly inhibiting phosphoenolpyruvate carboxykinase (136).

Gluconeogenesis is regulated in a complex manner that is not fully understood today. The supply of substrate as well as effectors of specific enzymes in the gluconeogenic sequence are under hormoral regulation. Basically, substrates for glucose synthesis can be divided into two types: those that enter the gluconeogenic sequence at the level of pyruvate (e.g. alanine, lactate, propylene glycol, pyruvate), and those that enter at the triose phosphate level (e.g. fructose, glycerol, dihydroxyacetone). The energy required to convert the former group of substrates into glucose is considerably greater than that required for the latter group. Early work suggested that regulation of gluconeogenesis from substrates such as pyruvate occurs at four key steps: pyruvate carboxylase, phosphoenolpyruvate carboxykinase, glyceraldehyde-3-phosphate dehydrogenase, and fructose-1,6-diphosphatase (204). More recently, data have been obtained that suggest that flux of carbon through the phosphoenolpyruvate-pyruvate substrate cycle is a major site of regulation of gluconeogenesis (176).

An extensive recent review on the hormonal control of hepatic gluconeogenesis focuses on the moment-to-moment regulation of this process by glucagon, catecholamines, and insulin (174). With the isolated perfused rat liver as a model, Exton and Park (66) postulated that the effect of these hormones is mediated by an elevation of adenosine 3′,5′-monophosphate (cyclic AMP). Support for this hypothesis stems from the observation that physiological concentrations of epinephrine and glucagon elevate hepatic concentrations of cyclic AMP whereas insulin lowers cyclic AMP in isolated perfused livers (65) and hepatocytes (176) exposed to the former two hormones. Within recent years it has become evident that both catecholamines and insulin also affect gluconeogenesis via cyclic AMP-independent mechanisms (175). Insulin acts via a cyclic AMP-independent mechanism since glucagon stimulation of gluconeogenesis in media lacking Ca^{++} is completely abolished by insulin in the absence of any change in cyclic AMP (47).

Under most conditions, pyruvate carboxylase is probably the rate-limiting step in glucose synthesis from 3-carbon precursors. This biotin-containing enzyme, which is located within the mitochondrial space, requires adenosine triphosphate (ATP), CO_2, and pyruvate to produce oxaloacetate. Pyruvate carboxylase is activated by the positive allosteric effector, acetyl coenzyme A (CoA) (204). This key activation is one explanation for the stimulation of gluconeogenesis by fatty acids.

Inhibitors of mitochondrial electron transport or ATP synthesis should inhibit glucose synthesis, since rates of glucose synthesis correlate directly with intracellular ATP concentrations (256). Some oral hypoglycemic agents such as biguanides [e.g. phenethylibiguanide (123)], which are respiratory chain site I inhibitors, and the sulfonylureas tolbutamide (93) and glisoxepide (215), which diminish hepatic ATP (256), also would be expected to influence a wide array of energy-dependent events in the whole liver.

B. Lipogenesis

The synthesis and release of lipids is a major hepatic function. Substrates for mixed-function oxidation interact in these biochemical pathways, often as inhibitors, leading to a net decrease in fatty acid synthesis or accumulation of triglyceride in the liver (i.e. "fatty liver"). Little work has been carried out on the effect of substrates for mixed-function oxidation on hepatic lipid metabolism. During maximal rates of mixed-function oxidation of aminopyrine, rates of synthesis of acetyl units are depressed by about 40% in the perfused liver (236). The interpretation that aminopyrine inhibits lipid synthesis by competing for NADPH is supported by the finding that the concentration of adenine nucleotides remained unaltered in livers perfused with aminopyrine.

C. Glycolysis

It is now widely accepted that under most conditions glycolysis is regulated at the level of phosphofructokinase, a key regulatory site in this sequence of reactions whereby glucose is converted to pyruvate. Rate control of this pathway may also be modulated by chemical toxins at a number of steps.

Agents that decrease cellular ATP concentrations by acting on mitochondrial function stimulate glycolysis. Several substrates for the mixed-function oxidase system may either act directly on mitochondria or be converted via mixed-function oxidation to molecules that alter mitochondrial function. For example, barbiturates and halothane represent substrates for the mixed-function oxidase system that act directly on mitochondria. Scholz et al. (202) compared the effect of several barbiturates on the hemoglobin-free perfused liver and showed that the sensitivity of respiration to amobarbital depended upon the metabolic state of the liver. Livers from fed rats, which utilize carbohydrate, were more sensitive to respiratory inhibition by barbiturates than livers from fasted rats oxidizing fatty acids. Moreover, barbiturates that were substituted at the 5-position, such as amobarbital, pentobarbital, and thiopental, all inhibited oxygen uptake by approximately 50% when present at concentrations between 0.3 and 0.4 mM.

Halothane has been shown to inhibit the oxidation of NAD^+-linked substrates specifically, but not succinate-linked substrates by isolated mitochondria. Halothane has also been shown to decrease drastically the oxygen supply to both the liver and the spleen (1).

Lardy and Phillips (119) demonstrated that 2,4-dinitrophenol stimulated respiration while decreasing motility of bull sperm. Later, Loomis and Lipmann (125) showed that this agent decreased P:O ratios in kidney preparations and concluded that dinitrophenol uncoupled oxidative phosphorylation. The mechanism of uncoupling of oxidative phosphorylation is still not well defined; however, a direct relationship seems to exist between the ability of uncouplers to carry protons into the mitochondria and their capacity to stimulate respiration and decrease energy transduction (54). Thus, uncoupling agents may act by promoting electrogenic hydrogen ion transport across the mitochondrial membrane.

Aflatoxin B_1 has marked actions on carbohydrate metabolism. It decreases hepatic glycogen content by inhibiting glycogen synthesis (180). Chronic administration of this toxin to chicks increased the activities of pyruvate carboxylase and phosphoenolypyruvate carboxykinase and decreased activities of hexokinase, phosphoglucoseisomerase, aldolase, and pyruvate kinase (180). Mechanisms that underlie these actions of aflatoxin B_1 and the relation, if any, of such changes to carcinogenic activity of the compound are unknown.

IV. Relationship between Mixed-Function Oxidation and Alteration in Intermediary Metabolism Secondary to Acute Changes in Nutrition

A. Transport of Drugs

It is clear that a wide variety of endogenous substrates, nutrients, and foreign chemicals are transported actively into hepatocytes; therefore, factors affecting these transport processes may modify rates of mixed-function oxidation in the intact organ. To date there have been relatively few studies aimed at defining possible interactions between biotransformation reactions and transport of drugs, although it is known that dietary factors as well as hormones may have marked effects on the uptake of various substances by the liver (35). It is also clear that drugs influence specific hepatic transport mechanisms. For example, carbon tetrachloride, chloroform, and halothane affect the transport of potassium by the liver (70). Organic anion transport also is altered in the perfused liver by foreign chemicals (134), and the uptake of choline by the perfused liver is decreased after pretreatment of animals with phenobarbital (203). Once drugs enter intact cells, binding to cytochrome P-450 apparently occurs extremely rapidly (87). By relating the concentration of alprenolol in the cell to changes in rates of absorbance due to binding to cytochrome P-450, it was estimated that the rate of diffusion of alprenolol to the microsomal cytochrome system was more than 500 times faster than the rate of metabolism of alprenolol (87). Thus, intracellular transport of lipophilic drugs does not seem to be rate-limiting for overall drug disposition in hepatocytes. Preincubation of liver cells with rotenone, an inhibitor of mitochondrial electron transport, did not influence the time course of binding of drugs to microsomal cytochromes (87). Thus, it appears unlikely that an active transport process is involved in the entry of drugs into hepatocytes. In accord with this possibility, treatment of rats with the antimetabolite 6-aminonicotinamide before liver perfusion diminished ATP levels but did not decrease rates of *p*-nitroanisole O-demethylation (109). In fact, a slight stimulation of the rate of mixed-function oxidation was observed in some nutritional states of rats exposed to 6-aminonicotinamide (see section IV E 2a).

B. Inhibition by Direct Binding and Inactivation

It has been recognized for many years that various chemical substrates may inhibit drug metabolism by competing for binding sites on the mixed-function oxidase system (36). In general, substrates as well as inhibitors of mixed-function oxidases are grouped into one of two major categories based on the type of spectral change (type I or II) produced when they are incubated with hepatic microsomes (196). Specific inhibitors of this nature have not been studied extensively in the perfused liver. Considerably more information than now exists is required before such compounds can be claimed to act by specifically altering the binding of drug substances to cytochrome P-450 in whole cells.

Phosphothionate insecticides such as parathion cause an irreversible inhibition of the microsomal oxidases by altering their structure (151). In addition, amines such as amphetamine and SKF-525A as well as methylene compounds such as piperonyl butoxide form adducts with cytochrome P-450 (253). Other examples of this type of inhibition have also been demonstrated recently in studies of the porphyrinogenic drug allylisopropylacetamide in the perfused liver (212). The consequences of such alteration to rates of drug oxidation in the intact hepatocyte remain to be defined.

C. Effect of Oxygen

The K_m of oxygen for cytochrome oxidase has been estimated by Schindler (197) to be about 0.04 μM. Similarly, the K_m of hepatic mixed-function oxidase for oxygen is about 0.1 μM (51, 218). When oxygen was removed, both cytochromes a-a_3 and P-450 showed similar kinetics of change in perfused liver (30) and it was concluded that both cytochrome oxidase and cytochrome P-450 have similar low K_m's for oxygen. In studies in which oxygen tension has been experimentally manipulated in isolated hepatocytes or in perfused liver it has been shown that oxygen probably is not rate-limiting for mixed-function oxidation under normal physiological conditions. Oxygen also does not appear to be rate-limiting in lungs since the K_m of p-nitroanisole O-demethylase in the isolated perfused rabbit lung for oxygen is less than 1 μM (68).

Data obtained with isolated hepatocytes agree with conclusions from studies with the perfused liver. The apparent K_m's of O_2 for the mixed-function oxidation of hexobarbital, phenyromidol, and alprenolol in intact hepatocytes were determined to be 6.4, 3.6, and 9.8 μM, respectively (103). These values are similar to those determined for isolated microsomes; therefore, no detectable gradient of O_2 concentration seems to exist between the extracellular space and the endoplasmic reticulum of hepatocytes at these low oxygen concentrations (103). Metabolism of tyrosine, which occurs via another mixed-function oxidase system, is influenced by oxygen concentrations below 70 μM (104).

D. Oxidation:Reduction State of Cytochrome P-450

The degree of reduction of cytochrome P-450 has been assessed in the perfused liver (207). Under normoxic conditions in the absence of added drug, it was about 6% reduced. When NADPH supply was interrupted by the addition of antimycin A to the liver of a fasted rat (207, 234), cytochrome P-450 became more oxidized. In the presence of hexobarbital, the degree of reduction of cytochrome P-450 increased to about 30% and was accompanied by a decrease in oxygen uptake and a reduction in pyridine nucleotide fluorescence in livers from control rats (207). The decrease in oxygen consumption and reduction of pyridine nucleotides were presumably due to drug action at site I in the mitochondrial respiratory chain. Opposite results to these were obtained when hexobarbital was added to livers from phenobarbital-treated rats, a finding that has been confirmed with other drugs such as alprenolol and propranolol (87). It appears, therefore, that proliferation of the endoplasmic reticulum and increased mixed-function oxidation protect the cell against toxic agents that are both substrates for cytochrome P-450 as well as inhibitors of the respiratory chain.

E. Regulation of Mixed-Function Oxidation in Intact Cells

Work underway in several laboratories has begun to provide insight into metabolic events regulating mixed-function oxidation in whole cells. Factors that influence rates of drug oxidation in intact hepatocytes are reviewed below.

1. Rate-control by NADPH supply. There is considerable information about the catalytic properties of mixed-function oxidases in vitro that suggests that the rate-controlling step in drug oxidation via mixed-function oxidases in microsomes is probably reduction of cytochrome P-450 (23, 100). In the reconstituted mixed-function oxidase system from phenobarbital-treated rabbits, the activity of the flavoprotein, NADPH-cytochrome P-450 reductase, appears not to be rate-limiting. Experiments with stopped-flow techniques indicate that the rate-limiting step in vitro is after the introduction of the second electron (88, 100, 132). Thus, factors that influence this activity in whole cells, such as NADPH supply, must be taken into account as determinants of rates of drug oxidation. It is important to point out that experiments in vitro differ markedly from physiological conditions in intact cells, where NADPH is largely bound to various dehydrogenases.

Thurman and Scholz (234) showed that the respiration of the perfused liver from a fed rat is markedly increased upon infusion of aminopyrine, a classic substrate for the mixed-function oxidation system. However, no increase was observed when the experiment was performed in the liver of a fasted rat in the presence of an inhibitor of the

mitochondrial respiratory chain, antimycin A. On the other hand, microsomes prepared from livers in these two metabolic states oxidized aminopyrine at similar rates (230) when supplied with an active NADPH-generating system, which suggests that NADPH supply is rate-controlling for mixed-function in the intact cell. This conclusion is further supported by the observation that rates of *p*-nitroanisole O-demethylation by the perfused liver vary over a 3-fold range in different metabolic conditions even though cytochrome P-450 levels are constant (183).

Recently, more support for the hypothesis that NADPH supply is rate-controlling for mixed-function oxidation in the intact cell under some conditions has been forthcoming. When *p*-nitroanisole was infused into livers from normal well-fed rats or added to microsomes in the presence of NADPH, linear rates of *p*-nitrophenol production were observed. In contrast, high rates of *p*-nitrophenolate production by perfused livers from phenobarbital-treated rats were linear for less than 2 minutes and then declined rapidly to about 25% of the control value. In this acute experiment, amounts of microsomal components did not change during perfusion when oxygen and *p*-nitroanisole were supplied in excess. While a small part of the decline in rate could be related to conjugation reactions, a decline in NADPH supply accounts for most of the decrease in rates of mixed-function oxidation in livers from fed, phenobarbital-treated rats. Further support for this hypothesis comes from the observation that the rate of mixed-function oxidation was directly correlated with the NADPH:NADP ratios calculated from substrates assumed to be in near equilibrium with malic enzyme and isocitrate dehydrogenase in three different metabolic states (228).

2. Relationship between nutritional state, NADPH supply, and mixed-function oxidation. Campbell and Hayes (41) have stated that: "There is ample evidence that nutrition is a major determinant of drug action. First, abnormal intake of nearly every nutrient modifies the activity of the liver microsomal system. Second, modest changes in nutrient intake can produce considerable differences in enzyme activity." On a chronic basis, it is well established that nutrition can influence the rate of metabolism of foreign chemicals (see section G 1). Furthermore, comparisons of rates of mixed-function oxidation in different metabolic states show that mixed-function oxidation can be regulated acutely by diet. One explanation for this is the influence of diet on various sources of NADPH, because the K_m of cytochrome P-450 reductase for NADPH is of the same order of magnitude as concentrations of free NADPH in the cytosol (109, 245).

A. PENTOSE PHOSPHATE PATHWAY. Several studies have demonstrated that rates of generation of reducing equivalents via the oxidative enzymes of the pentose phosphate shunt are sufficient to supply the NADPH required for mixed-function oxidation of a variety of substrates in liver (106, 211). With new methods, it has been shown that aminopyrine (39) and organic hydroperoxides (210) elevate the rate of formation of $^{14}CO_2$ from 1-^{14}C-glucose, which demonstrates that substrates for mixed-function oxidation and substances that enhance cytochrome P-450 turnover accelerate flux of sugar phosphates through the pentose phosphate shunt. In one study carried out in isolated hepatocytes, it was demonstrated that rates of pentose phosphate shunt activity provide NADPH at rates in excess of rates of mixed-function oxidation (106).

It is clear that $NADP^+$ activates and NADPH inhibits activity of the pentose phosphate shunt (61). Also, the pentose phosphate shunt is stimulated by oxidized glutathione (61), which is released from perfused liver by aminopyrine (165). Thus, during high rates of mixed-function oxidation, generation of reducing equivalents via the pentose phosphate shunt in the fed state where carbohydrate reserves are high would be expected to be accelerated. This possibility is supported by the finding that oxidation of *p*-nitroanisole in perfused livers from fed animals is accompanied by significant increases in 6-phosphogluconate and ribulose-5-phosphate (110). It is noteworthy that induction of mixed-function oxidation in rat liver by phenobarbital and 3-methylcholanthrene is accompanied by significant increases in the activity of the oxidative enzymes of the pentose phosphate shunt (108, 110). Phenobarbital treatment also increased the concentration of ribulose-5-phosphate and xylulose-5-phosphate (110). When these data are considered with a rise in lactate and a decline in fructose 1,6-diphosphate, they support the hypothesis that induction of mixed-function oxidation also increases the activity and carbon flux through the pentose phosphate shunt. This effect of phenobarbital to increase the capacity of the liver to produce reducing equivalents for mixed-function oxidation occurs both by induction of various oxidative enzymes and by alteration of the oxidation-reduction state of $NADP^+$ (109). Phenobarbital and 3-methylcholanthrene pretreatment cause the NADP couple to become more oxidized and this favors increased flux through the pentose phosphate pathway. Such actions may be as significant as the well-known induction of cytochrome P-450 in enhancing rates of mixed-function oxidation in intact cells.

6-Aminonicotinamide, a diabetogen, markedly inhibited metabolism via the oxidative enzymes of the pentose phosphate pathway in the perfused liver from well-fed rats but did not diminish *p*-nitroanisole O-demethylation (109). Under these conditions, 6-phosphogluconate levels increased over 700-fold in the tissue (228). Thus, under conditions where pentose phosphate shunt activity was markedly diminished, mixed-function oxidation was not inhibited. Surprisingly, 6-aminonicotinamide caused a reduction of cytoplasmic $NADP^+$. At

present no explanation exists for this interesting and unexpected observation. One possibility, however, is that 6-aminonicotinamide treatment activates alternative sources of NADPH generation (see section E 3).

B. ROLE OF NADPH OF MITOCHONDRIAL ORIGIN. Evidence has been acquired that NADPH formed within mitochondria supports drug metabolism even in the well-fed state. Over two-thirds of the hepatocellular NADPH is located in the mitochondrial space, and is actually about −10 mV more reduced than the cytosolic pool (206). Thus, one would predict a flow of reducing equivalents from the mitochondrial to the cytosolic space based simply on thermodynamic considerations. Second, as mentioned above, inhibition of the pentose phosphate shunt in well-fed (109, 228) or fasted (183) livers with 6-aminonicotinamide did not diminish rates of *p*-nitrophenol production. Third, rates of mixed-function oxidation in perfused livers (230) or isolated hepatocytes were diminished only slightly by fasting. Fasting depleted glycogen content to less than 2% of control values in 24 hours, and diminished activity of the pentose shunt markedly.

The fasted state is characterized by a lack of substrate for the pentose phosphate shunt (220). In this condition, mixed-function oxidation as reflected by oxygen uptake upon addition of aminopyrine (234) or hexobarbital (207) is abolished by inhibitors of mitochondrial oxidations. Thus, mitochondrial oxidations must be exclusive sources of reducing equivalents in the fasted state. Both maximal rates and kinetics of *p*-nitroanisole O-demethylation differed in various metabolic states (228, 229). Maximal rates of drug metabolism were greatest in livers from fasted-refed rats, somewhat slower in livers from fed rats, and slowest in livers from fasted rats. Livers from fasted animals had the ability to sustain elevated rates of mixed-function oxidation for much longer time periods than livers from fed or fasted-refed animals. Further, the rate of decline of *p*-nitroanisole O-demethylation was less in livers from fasted rats than in livers from either of the other two groups.

Carbohydrate reserves differ in the three metabolic states compared above. Fasted-refed livers contain large stores of glycogen, fed livers contain intermediate stores, and the fasted liver contains only 1% to 2% of the glycogen present in the fed state (220). It is unlikely that an intermediate of carbohydrate metabolism such as glucose-6-phosphate has a predominant role in sustaining high rates of mixed-function oxidation because there was an inverse relationship between the duration of high rates and carbohydrate reserves. Thus, it is difficult to ascribe a major role for the pentose phosphate shunt in providing reducing equivalents for drug oxidations. Further, rates of mixed-function oxidation declined in the presence of high rates of glucose production (229). The failure of glucose to stimulate *p*-nitroanisole O-demethylation in livers from fed animals, even though ATP levels were sufficient to allow phosphorylation of the sugar (110), suggests that generation of reducing equivalents from carbohydrate oxidation is not rate-limiting for mixed-function oxidation in the fed state.

It is not altogether clear how reducing equivalents generated in the mitochondrial space move into the extramitochondrial space to support mixed-function oxidation. First, mitochondrial membranes are impermeable to pyridine nucleotides (120). Also, the citric acid cycle and β-oxidation generate NADH from the oxidation of acetyl-CoA and acyl-CoA, respectively. However, the mixed-function oxidation system functions with highest efficiency when NADPH is the cofactor. Mitochondria can transform NADH into NADPH via an energy-dependent transhydrogenase. Energization of the mitochondria has a profound effect on this reaction and drives it in the direction of NADPH while inhibiting the reverse reaction. Energization drives the apparent equilibrium constant for the transhydrogenase from near unity to about 500 in the direction of NADPH formation (97). The transhydrogenase is inhibited by acyl-CoA compounds (194). The possibility that mitochondria serve as sources of reducing equivalents is supported by the finding that oxidation of aminopyrine by liver slices was stimulated by citric acid cycle intermediates (45, 46).

Little information on the role of the transhydrogenase in supplying reducing equivalents for mixed-function oxidation is available. Hoek and Ernster (97) demonstrated that the energy-linked transhydrogenase was highly sensitive to carbonyl cyanide *p*-trifluoromethylphenylhydrazone (FCCP), and uncouplers of oxidative phosphorylation (dinitrophenol and FCCP) have also been shown to inhibit the mixed-function oxidation of *p*-nitroanisole in perfused livers from fasted rats while not affecting activity of isolated microsomes (15).

Another, and perhaps more important, mechanism for generating cytosolic NADPH from mitochondrial oxidations involves substrate shuttles. The subject of anion transport systems for moving reducing equivalents across the mitochondrial membrane has been reviewed extensively (258). Two major hydrogen shuttle mechanisms have been proposed to move mitochondrial hydrogen into the cytoplasmic space. One such shuttle mechanism involves $NADP^+$-dependent isocitrate dehydrogenases in the mitochondrial and extramitochondrial spaces. $NADP^+$-dependent enzymes are present in both spaces, whereas an NAD^+-dependent enzyme is present only as a component of the citric acid cycle in mitochondria (177). This latter enzyme is a highly regulated essentially nonequilibrium system (79). Thus, mitochondrial NADPH but not NADH may be transported by the isocitrate:2-oxoglutarate shuttle. Isocitrate dehydrogenase is predominantly cytosolic in location (174), and this activity in liver is higher than other $NADP^+$-dependent dehydrogenases (110). The participation of the isocitrate:2-oxoglutarate shuttle in providing reducing equivalents for mixed-function oxidation is supported by the finding that when ureogenesis was stimulated in isolated hepa-

tocytes, rates of mixed-function oxidation were diminished slightly (206). The authors concluded that NADPH of mitochondrial origin was diverted from the isocitrate:2-oxoglutarate shuttle to ureogenesis. There is further support for the involvement of the isocitrate:2-oxoglutarate shuttle mechanism in mixed-function oxidation. First, ethanol inhibits the citric acid cycle and markedly lowers the intracellular concentrations of 2-oxoglutarate and isocitrate (182). Ethanol also causes over a 50% inhibition of the O-demethylation of *p*-nitroanisole in the fed and fasted state that could be partially reversed with addition of asparate or glutamate (182). Second, in isolated hepatocytes from fed rats, the transaminase inhibitor aminoxyacetate had no effect on $^{14}CO_2$ production from ^{14}C-aminopyrine; however, the production of $^{14}CO_2$ was decreased about 30% in hepatocytes from fasted rats (252).

Another energy-sensitive shuttle mechanism based on mitochondrial pyruvate carboxylase and malate dehydrogenase and an extramitochondrial malic enzyme has been proposed (234). This shuttle mechanism differs from the isocitrate:2-oxoglutarate shuttle in that mitochondrial NADH rather than NADPH is the hydrogen donor. Thus, the energy-linked transhydrogenase is not necessary for reducing equivalents to be produced by this mechanism. With this shuttle mechanism, mitochondrial oxaloacetate accepts reducing equivalents from NADH. The malate formed diffuses into the cytosol where it reacts with malic enzyme to form NADPH and pyruvate. Pyruvate then enters the mitochondria and an energy-requiring carboxylation regenerates mitochondrial oxaloacetate to complete the cycle. Substrates for mixed-function oxidation such as aminopyrine may divert malate carbon from the pathway of glucose synthesis into NADPH generation for drug oxidation (236). As discussed above, aminopyrine was found to suppress maximal rates of glucose synthesis from lactate over 50% in perfused liver from phenobarbital-treated rats; however, submaximal rates of glucose formation with lactate or maximal rates with dihydroxyacetone were only slightly affected. These data suggest that active NADPH-utilizing processes such as the mixed-function oxidation of aminopyrine diverts malate away from the pathway of gluconeogenesis for formation of NADPH via malic enzyme and this leads to a compensatory influx through the pyruvate carboxylase reaction. Thus, gluconeogenesis is suppressed only when pyruvate carboxylation is maximal, indicating that the malic enzyme shuttle is functioning under gluconeogenic conditions in the presence of substrates from mixed-function oxidation.

3. Relationship between energy metabolism and mixed-function oxidation. There is a small body of information that suggests that an indirect and possibly regulatory role exists between energy metabolism and mixed-function oxidation. For example, a number of drug substrates or products of mixed-function oxidation can act as inhibitors or uncouplers of oxidative phosphorylation in the perfused liver [for review, see Thurman et al. (233)]. β-Adrenergic blocking agents (propanolol and alprenolol) have been shown to block mitochondrial respiration at the level of NADH oxidase (87). Moreover, benzphetamine and its product via mixed-function oxidation, norbenzphetamine, are potent uncouplers of oxidative phosphorylation. Conversely, substrates for mixed-function oxidation that decrease the ATP/adenosine diphosphate (ADP) ratio in the cell may actually stimulate their own metabolism.

Hexobarbital (100, 209) and aminopyrine (110, 252) oxidize cytosolic NADPH; however, oxidation of NADPH with *p*-nitroanisole probably does not occur, even though high rates of mixed-function oxidation of this compound were observed in phenobarbital-treated rats. The oxidation of NADPH by aminopyrine could be blocked by addition of an uncoupling agent of oxidative phosphorylation, dinitrophenol.

Both *p*-nitroanisole alone and aminopyrine plus dinitrophenol uncouple oxidative phosphorylation as reflected by decreased ATP/ADP ratios (110). However, aminopyrine alone or hexobarbital alone had no effect. Lowering of the ATP/ADP ratio may be a critical event, because both dehydrogenases of the pentose phosphate pathway (2, 8, 111, 172) as well as isocitrate dehydrogenase and malic enzyme (109) are strongly inhibited by ATP. Inhibitor constants of ATP for the various NADPH-generating enzymes range from 9 μM for malic enzyme to 1.8 mM for glucose-6-phosphate dehydrogenase (109). Thus, a decrease in the concentration of ATP in the liver by an agent that uncouples oxidative phosphorylation activates metabolism of hexose phosphate, malate, and isocitrate, leading to enhanced NADPH generation. During the mixed-function oxidation of a drug such as hexobarbital, the rates of NADPH oxidation probably exceed the capacity of the liver to regenerate NADPH; therefore, the calculated $NADP^+$/NADPH ratio increases. When an uncoupling agent is either present (e.g. dinitrophenol) or generated (e.g. *p*-nitrophenol), the high capacity of the liver to form NADPH in livers from phenobarbital-treated rats via pentose phosphate, malic enzyme, and isocitrate dehydrogenase is greater, possibly due to a decline of ATP. The rate of NADPH generation may then be equal to or even exceed the rate of NADPH utilization. Thus, mixed-function oxidation of *p*-nitroanisole to *p*-nitrophenol leads to reduction of $NADP^+$ rather than to the expected oxidation of NADPH.

There are at least two examples of agents that lower ATP/ADP ratios and stimulate mixed-function oxidation. First, fructose is an active substrate for ketohexokinase and causes a rapid lowering of the ATP/ADP ratio (109, 128, 259). The addition of fructose to perfused liver from phenobarbital-treated rats stimulated the O-demethylation of *p*-nitroanisole, which was accompanied by a decrease in the $NADP^+$/NADPH ratio (109). Second, pretreatment with 6-aminonicotinamide elevated *p*-nitroanisole O-demethylation slightly and lowered the calculated $NADP^+$/NADPH ratio (228). 6-Aminonicotin-

amide treatment also caused a decrease in the ATP/ADP ratio (109). Thus, these examples support the conclusion reached with the in vitro systems and suggest that toxic agents that alter the ATP/ADP ratio by inhibiting or uncoupling oxidative phosphorylation stimulate their own metabolism acutely and rapidly by increasing NADPH for the mixed-function oxidation system. Alteration in steady state concentrations of pyridine and adenine nucleotides occurs rapidly over a period of a few minutes and thus represents a means whereby rates of mixed-function oxidation may be changed long before enzyme components of the system are induced.

4. Effect of ethanol. Ethanol is an agent that has long been known to affect drug metabolism. Most reports indicate that ethanol inhibits drug metabolism. To understand ethanol-drug interactions clearly, it is important to relate ethanol oxidation in the cell to microsomal oxidations. Several papers and reviews have dealt with the controversy surrounding the mechanism of microsomal ethanol oxidation (189, 226, 231). In summary, Orme-Johnson and Ziegler (163) first demonstrated that microsomes incubated with NADPH and oxygen were capable of converting ethanol into acetaldehyde. However, it is not known whether this is due to the direct oxidation of ethanol via cytochrome P-450 or to the action of H_2O_2 with catalase. It has been demonstrated that microsomes can generate H_2O_2 (227). A number of workers have failed to observe ethanol oxidation in purified, reconstituted mixed-function oxidases (231, 235, 244) whereas others have observed it (149, 160). In these latter studies, however, the production of acetaldehyde was not accompanied by increases in consumption of either NADPH or oxygen, common requirements for classic mixed-function oxidation reactions.

While the mechanism of microsomal ethanol oxidation remains controversial, the effect of ethanol on mixed-function oxidation in vitro is clearer. In a review of this subject, Mezey (138) cited several reports in which it was shown that ethanol inhibited mixed-function oxidation in vitro. This action may be dependent on the substrate for mixed-function oxidation because ethanol has been reported not to affect the metabolism of hexobarbital (192). In general, very high concentrations of ethanol are required to inhibit drug metabolism in vitro. The mechanism for this inhibition is, in all likelihood, direct binding of ethanol to cytochrome P-450 and subsequent displacement of the drug substrate. This mechanism is unlikely to account for inhibition of drug metabolism in whole cells because the binding constants of ethanol in vitro range between 0.5 and 1.3 M for microsomes (99) and the purified cytochrome P-450 (244), respectively. Concentrations of ethanol above 0.1 M are lethal in man. Moreover, *p*-nitroanisole O-demethylation in the perfused liver is half-maximally inhibited by 1 to 2 mM ethanol (186). Rubin et al. (192) observed that meprobamate metabolism was inhibited by 10 mM ethanol in liver slices and Grundin (86) reported that low concentrations of ethanol stimulated alprenolol oxidation in isolated hepatocytes, whereas 10 mM inhibited it. Similar effects of ethanol have been noted with *p*-nitroanisole O-demethylation. Reinke et al. (183, 186) observed that low concentrations of ethanol first stimulated then inhibited *p*-nitroanisole O-demethylation in perfused rat livers. The stimulation was observed only in livers from fasted rats whereas the inhibition was present in both fed and fasted livers. Stimulation by ethanol corresponded directly with the effect on reduction of NAD^+. Other agents that reduced NAD^+, such as glucose, sorbitol, and xylitol (185), stimulated *p*-nitroanisole O-demethylation in a manner similar to ethanol. These findings along with the observation that inhibition of alcohol dehydrogenase by 4-methylpyrazole prevented stimulation by ethanol suggest that NADH produced from ethanol in all likelihood interacts with NADH-cytochrome b_5 reductase to stimulate mixed-function oxidation in the whole organ in a fashion similar to that described for "NADH-synergism" in isolated microsomes (53, 130, 186, 96). The high amount of NADH formed from glycolysis in the fed state probably explains why infusion of ethanol does not stimulate mixed-function oxidation in this metabolic state. NADH-dependent mixed-function oxidation is probably activated maximally in the fed state.

In another series of experiments, Reinke et al. (183, 187) showed that inhibition of mixed-function oxidation of *p*-nitroanisole by ethanol (K_i = 1 to 2 mM) was apparently due to acetaldehyde generated from ethanol oxidation. Acetaldehyde apparently enters the mitochondrial space, generates reducing equivalents via aldehyde dehydrogenase, which, in turn, inhibits the citric acid cycle and depletes key intermediates needed for the movement of reducing equivalents from the mitochondria into the cytosolic space. Evidence for this hypothesis is listed as follows: first, the ethanol inhibition was abolished by 4-methylpyrazole, an inhibitor of alcohol dehydrogenase. This indicates that the ethanol molecule per se is not responsible for the inhibition, but either NADH, acetaldehyde, NADH from acetaldehyde, or acetate is the causative agent. Since sorbitol and xylitol, agents that elevate the cytosolic NADH redox state, do not inhibit mixed-function oxidation, NADH of cytosolic origin can be ruled out as well. Changes in pyridine nucleotide and flavin oxidation-reduction state correlated well with the inhibition of mixed-function oxidation as the concentration of ethanol was increased. Acetaldehyde also inhibited *p*-nitroanisole O-demethylation at concentrations one to two orders of magnitude lower than those necessary to inhibit microsomal *p*-nitroanisole O-demethylation in vitro. During ethanol inhibition, levels of α-ketoglutarate and isocitrate declined markedly, possible below the K_m values for transport of NADPH into the cytosol from the mitochondria via the isocitrate substrate shuttle mechanism, which suggests that ethanol acts to deplete intermediates necessary for this substrate shuttle. This conclusion is supported by the

observations that aspartate and glutamate, amino acids that give rise to oxalacetate and α-ketoglutarate in the cell, both partially reversed the inhibition of *p*-nitroanisole O-demethylation by ethanol. Taken together, these data strongly support the concept that ethanol interrupts the flow of NADPH of mitochondrial origin into the cytosol. Implicit in this conclusion is that mitochondrial NADPH plays an important role in the normal regulation of mixed-function oxidation in the hepatocyte.

Ethanol can also interact with mixed-function oxidation by inducing components of the drug-metabolizing system. Several groups (121, 225, 237) have demonstrated that chronic exposure of animals to ethanol leads to an increase in cytochrome P-450 levels. Often, this is cited as evidence that ethanol is a substrate for the mixed-function oxidase; however, other compounds that bind to cytochrome P-450 (e.g. barbital) but are not metabolized also induce microsomal components.

It has been suggested that the increase in the activity of the microsomal ethanol-oxidizing systems accounts for the well-documented (121) increase in ethanol metabolism after chronic exposure to ethanol in rodents or in man. This argument should be viewed with caution, however, since considerable evidence has accumulated to support the concept that microsomal ethanol oxidation, irrespective of its molecular mechanisms, does not operate in whole cells. For example, carbon tetrachloride treatment markedly diminishes microsomal drug and ethanol metabolism but does not affect the rate of ethanol elimination in vivo (114). Similar conclusions can be drawn from experiments with menadione. Menadione stimulates microsomal H_2O_2 production but does not alter the rate of ethanol uptake by the perfused rat liver (235). Finally, Mezey (137) treated rats chronically with ethanol to induce cytochrome P-450 and accelerate the rate of ethanol elimination. However, when the treatment was terminated, ethanol elimination rates returned rapidly to control levels in 48 hours, whereas two weeks were required for the decay in elevated cytochrome P-450 levels as well as rates of microsomal ethanol oxidation. Thus, there appears to be no relationship between levels of microsomal mixed-function oxidase components and rates of ethanol elimination in intact cells.

F. Conjugation Reactions

The ability of cells to conjugate xenobiotics, products of mixed-function oxidation, and endogenous compounds such as estradiol and bilirubin, represents a major mechanism of detoxification (3). Such reactions have been studied extensively in vitro, but only a few studies have been performed in vivo or in isolated cells or perfused organs.

1. Glucuronidation. Conjugation with glucuronic acid represents a major form of removal of a majority of products of mixed-function oxidation. Glucuronidation of drug metabolites has been studied primarily in isolated hepatocytes (164) and in the perfused rat liver (184, 232).

At least five rate-controlling factors have been identified for glucuronidation in whole cells: substrate supply, uridine diphosphate (UDP) glucuronic acid, NAD^+/NADH redox state, the supply of carbohydrate, and the activity of a group of glucuronyltransferases. In isolated hepatocytes (38, 164, 254) as well as in the perfused liver, (184) rates of glucuronidation of *p*-nitrophenol, 4-methylumbelliferone, harmol, phenolphthalein, biphenyl, and 2-naphthal were accelerated with increases in drug substrate concentration. Thus, a major factor regulating glucuronidation is substrate supply. Maximal rates of conjugation of 7-hydroxycoumarin (164) and *p*-nitrophenol could be enhanced up to severalfold by pretreatment of the experimental animal with 3-methylcholanthrene or phenobarbital, respectively. In large part, these increases are probably due to enhanced rates of production of hydroxylated products of mixed-function oxidation (i.e. more substrate for conjugation reactions). Alteration of pathways of conjugation with specific inhibitors change markedly the pathway of benzo(a)pyrene binding in isolated hepatocytes (38).

Cellular energetics influence glucuronidation via formation of uridine triphosphate (UTP) required to form UDP-glucose. In a recent study, Reinke et al. (184) demonstrated that rates of *p*-nitrophenol glucuronidation varied over 10-fold between livers of fasted and fasted-refed, phenobarbital-treated rats but that ATP/ADP ratios were identical. Thus, it appears that cellular energetics do not normally limit glucuronidation. On the other hand, agents that drastically diminish the energy state (e.g. potassium cyanide (KCN) or dinitrophenol) inhibit glucuronidation of *p*-nitrophenol in the perfused rat liver (L. Reinke and R. G. Thurman, unpublished observations). Moreover, salicylamide (0.5 mM), an agent that interacts with the mitochondrial respiratory chain, markedly inhibits sulfation without diminishing glucuronidation (164). One possible interpretation of this interesting finding is that sulfation is more dependent on ATP than glucuronidation. By diminishing the ATP/ADP ratio, carbohydrate supplied for glucuronidation may be enhanced. Data acquired with isolated hepatocytes suggest that conjugation reactions may be more susceptible to depletion of cellular ATP than hydroxylation. Wiebkin et al. (255) examined the effect of menadione, rotenone, and dinitrophenol, inhibitors of mitochondrial function, on the metabolism of biphenyl, 7-ethoxycoumarin, and benzo(a)pyrene in hepatocytes from normal and phenobarbital-treated rats. Although menadione depressed drug hydroxylation to a great extent, possibly via oxidation of NADPH and inhibition of cytochrome P-450 reduction, the other inhibitors of mitochondrial function had little effect on drug hydroxylation. In contrast, the conjugation of all three drugs was depressed markedly by the inhibitors of mitochondrial function.

UDP-glucose dehydrogenase (E.C. 1.1.1.22) is a NAD^+-requiring enzyme; therefore, alteration in redox state of

NAD^+ could influence the rate of glucuronidation by regulating the intracellular concentration of UDP-glucuronic acid. This is exemplified in experiments with ethanol, an agent that markedly increases the intracellular NADH content. Ethanol causes at least 50% inhibition of glucuronidation of morphine (59) and *p*-nitrophenol (L. Reinke and R. G. Thurman, unpublished observations). However, while redox inhibition of conjugation reactions is easily demonstrated, it does not appear that glucuronidation can be stimulated by increasing the NAD^+ supply with infusion of pyruvate into the perfused liver.

One major regulating factor for glucuronidation appears to be carbohydrate reserves. This is not surprising since glucuronic acid is derived from glycogen and glucose. In phenobarbital-treated rats, Reinke et al. (184) showed that large differences in rates of conjugation of *p*-nitrophenol, essentially due to glucuronidation, paralleled carbohydrate reserves and uridine diphosphoglucuronic acid (UDPGA) levels in the livers of animals in different nutritional states. For example, maximal rates of conjugation were observed in livers that had the highest levels of glycogen (184). Addition of phenobarbital to hepatocytes increased UDPGA levels (158); however, chronic phenobarbital treatment did not alter UDPGA levels in the perfused rat liver (F.C. Kauffman and R. G. Thurman, unpublished observations).

Glucuronyl transferases are essential but are probably not rate-controlling factors in intact cells except under extreme conditions. After nutritional manipulations, maximal rates of glucuronidation in the perfused liver did not correlate with the V_{max} of glucuronyl transferase activities measured in vitro (184). Thus, it appears that factors that regulate rates of mixed-function oxidation and carbohydrate reserves are primary rate-controlling steps in glucuronidation. Interestingly, rates of mixed-function oxidation are also correlated with carbohydrate reserves, suggesting that mixed-function oxidation and glucuronidation are in some manner coordinately regulated. One mechanism whereby rates of mixed-function oxidation and glucuronidation may be coupled in intact cells involves removal of oxidized products that inhibit the microsomal oxidase system. Fahl et al. (67) observed that addition of UDP-glucuronic acid to isolated microsomes resulted in a marked stimulation of benzo(a)pyrene oxidation. The authors interpreted this as removal of a hydroxylated product that inhibited mixed-function oxidation. A similar stimulation of benzo(a)pyrene oxidation (24) and glucuronyl transferase (19) has been observed with the addition of UDP-N-acetylglucosamine to an isolated microsomal system. Whether such a mechanism operates in intact cells remains to be determined.

2. Sulfation. Much less is known about factors that regulate sulfation in tissues. Rates of sulfation in the liver exceed those of other tissues such as gut and the lung (179). In general, when sulfation is decreased, glucuronidation is increased (152).

Elizabeth and James Miller (129, 142) demonstrated that 2-acetylaminofluorine (AAF), a potent carcinogen, is hydroxylated via mixed-function oxidation and subsequently sulfated in the liver to a compound that reacts covalently with critical sites in the cell. The formation of the N-sulfate requires the presence of an active sulfotransferase and β-phosphoadenosine-5′-phosphosulfate (PAPS). The sulfate is much more carcinogenic than the parent compound. Thus, sulfation can increase the toxicity of some foreign compounds.

Sulfation in intact cells, in analogy with glucuronidation, could be regulated by supply of substrate, the ATP/ADP ratio, the supply of inorganic sulfate, and the activity of a group of sulfotransferases. Sulfation may be much more sensitive to changes in cellular energetics than glucuronidation because agents that uncouple oxidative phosphorylation inhibit sulfation. Salicylamide (164), *p*-chlorophenol, and 2,6-dichlorophenol (152), all of which decrease sulfation in intact cells, either inhibit or uncouple oxidative phosphorylation.

3. Glutathione. Because of its well established role in decreasing acetaminophen toxicity and participating in conjugation of carcinogens, much attention has been given to glutathione conjugation (7). However, few studies have been designed to identify rate-controlling factors of glutathione conjugation in intact cells. Mercapturic acids have long been recognized in detoxification reactions. For example, naphthalene mercaptide was discussed over 70 years ago. Thirty years ago, Boyland (26) argued that polycylic aromatic hydrocarbon metabolism proceeds via an epoxide intermediate. Recently, in an elegant series of experiments, Mitchell et al. (147, 148) have demonstrated that glutathione is involved in protecting the liver against necrosis due to acetaminophen metabolism. Covalent binding of acetaminophen metabolites to tissue occurred only after 60% to 70% of the intracellular glutathione had been depleted. Metabolites of acetaminophen were conjugated to glutathione and were responsible for this depletion. Subsequently, metabolites of glutathione bound covalently to critical intracellular sites leading to tissue necrosis.

Agents that increase intracellular glutathione levels, e.g. cysteine and methionine, decrease both covalent binding of acetaminophen metabolites and tissue necrosis (27, 148). Glutathione depletion via conjugation or inhibition of glutathione synthesis has also been implicated in the toxicity of benzene (124) and brombenzene (224).

Glutathione conjugates have been described for several oxides of benzo(a)pyrene (73) and styrene oxide (242). However, little is known about regulation of glutathione conjugation in intact cells. Whether ATP, which is required for glutathione synthesis (135), substrate supply, or the activity of glutathione-S-transferases are controlling factors in intact cells will also require further work.

Isolated perfused organs have been used in a series of studies of conjugation by Fouts and his colleagues. In perfused livers, ^{14}C-styrene oxide was excreted mainly as the glutathione conjugate (193). They have studied the

oxidation and conjugation of benzo(a)pyrene and the conjugation of styrene oxide. In the isolated, perfused rat liver, the rates of formation of glutathione conjugates and styrene glycol were investigated. At low concentrations of styrene oxide, the glycol conjugate predominated (243). In the perfused rabbit lung, methylcholanthrene treatment had no effect on AHH activity or glutathione-S-transferase, but did enhance the activity of epoxide hydrase (214). Similar results were obtained with benzo(a)pyrene-4,5-oxide in the perfused liver (213).

G. Chronic Influence of Diet, Inducing Agents, and Age on Drug Metabolism

1. Chronic influence of diet. The abnormal chronic intake of nearly every nutrient evaluated experimentally has been shown to modify the activity of drug metabolizing systems in vitro via alterations in the structure and composition of microsomal membranes (107). The relationship of such data to actual rates of drug metabolism in intact cells is difficult to assess in the absence of information concerning the effects of altered nutritional status on concentrations of reduced cofactor and substrates that modulate the activity of mixed-function oxidation in intact cells. As discussed above, nutrients are known to affect steady-state concentrations of NADPH and supply active intermediates utilized in conjugation reactions.

The long-term influence of diet on drug-metabolizing systems has been related mainly to alteration in concentrations of the various components of these systems. For example, Dixon et al. (60) were the first to show the effect of dietary restriction on drug metabolism in isolated microsomes. Although there is a vast literature concerning the influence of nutrition on intermediary metabolism, there is very little information about the consequences of such alterations on oxidative drug metabolism or whether the influence of diet on drug-metabolizing systems is related to either nutrients or non-nutrient chemicals contained in food. Non-nutrient chemicals include endogenous inducing agents such as indoles, steroids, and flavones as well as certain chemical residues that may be added in the processing of foodstuff. The influence of specific nutrients and general nutritional status on mixed-function oxidation measured in vitro has been covered in several excellent reviews (40–42, 250) as have the effects of micronutrients (14, 260) and non-nutrients (251). A major problem in assessing the influence of chronic dietary manipulations on drug metabolism is the difficulty in discussing actions that are secondary to altered endocrine or immunological status. Although patterns of dietary intake are known to have marked effects on the metabolism of foreign chemicals in whole organisms and in broken cell preparations in vitro, the cellular basis for these effects remains poorly defined. Material derived mainly from studies with isolated enzyme systems or whole animals is reviewed briefly below since structural alterations produced by chronic dietary manipulation are important determinants of drug metabolism in intact cells.

The type and quantity of lipid ingested in the diet can affect mixed-function oxidation in intact cells via alterations in the structure of the endoplasmic reticulum (43, 131, 157, 250) as well as by competition for substrate binding sites (e.g. omega oxidation), competition for electron transfer reactions (e.g. lipid peroxidation) and, as discussed above, by serving as a substrate for the generation of reducing equivalents within mitochondria. Variation in the incidence of 7,12-dimethylbenzanthracine-induced mammary tumors in laboratory animals by diets of different lipid compositions corresponds directly with the influence of dietary lipid on cytochrome P-450 content and NADPH-cytochrome *c* reductase in liver (188). Highest activity of the monooxygenase system and increases in the incidence of mammary tumors were noted in animals fed a diet high in polyunsaturated fat. In addition, diets containing cholesterol produced a doubling of *p*-nitroanisole O-demethylase without altering cytochrome P-450 content or NADPH cytochrome *c* reductase activity (95).

Alterations in the structure of the endoplasmic reticulum due to dietary lipid may be related to antioxidants (78), as well as to peroxidized lipids and steroids contained in experimental diets (41, 48). Peroxidation of lipids within intact cells is also known to alter rates of mixed-function oxidation since lipid peroxidation damages membranes of the endoplasmic reticulum (219). Lipotropes, compounds that serve as methyl group donors or participate in methyl group transfer during the synthesis of phospholipids, have been implicated as important determinants of mixed-function oxidation in intact cells. Lipotrope-deficient diets are associated with enhanced hepatoxicity of aflotoxin and nitrosamines (156, 191) that do not appear to be due to elevated activities of microsomal drug metabolizing enzymes measured in vitro. In fact, *p*-nitroanisole O-demethylase and ethylmorphine demethylase were decreased in microsomes isolated from lipotrope-deficient rats (190). Lipotropes are involved in the synthesis of phosphatidyl choline required in the microsomal electron transport chain (41).

The effect of varying the dietary protein on microsomal drug metabolism is well documented beginning with the work of Kato (107) and has been reviewed extensively (4, 6, 41, 42). Quality of dietary protein, as well as the quantity of this nutrient, markedly influences the amounts and types of mixed-function oxidase activity (146) and a high protein intake has recently been shown to enhance rates of metabolism of aminopyrine in man (6). Decreases in rates of mixed-function oxidation in animals receiving protein-restricted diets may be related to altered interactions between the three major components of the microsomal electron transport chain (155). Substitution of either cytochrome P-450 or NADPH-cytochrome *c* reductase isolated from livers of normal or protein-restricted rats with its counterpart from the other

dietary state modified the kinetic properties of benzphetamine N-demethylase in the reconstituted microsomal drug-metabolizing systems. Drug metabolism was inhibited approximately 80% in reconstituted systems made up of NADPH-cytochrome P-450 reductase from protein-restricted animals and cytochrome P-450 from normal animals. Less inhibition was observed when cytochrome P-450 from the low protein group was used in combination with the reductase from normal animals and no inhibition was noted when the phospholipid fraction from restricted animals was added to the cytochrome P-450 and the reductase from normal animals (155).

In considering the influence of proteins and other nutrients on rates of mixed-function oxidation in intact cells, it is necessary to determine rates obtained at physiological concentrations of drug substrates. Employing calculations of rates of ethylmorphine demethylase activity from apparent K_m's and maximal velocities determined in vitro, Campbell (40) estimated that rats fed a low protein diet have 63% lower activity of this enzyme than control animals when exposed to high concentrations (3 mM) of ethylmorphine but 83% less when exposed to low concentrations of the drug (0.001 mM). When similar estimates were made for epoxide hydrase activities, a low protein intake caused a 61% depression at the higher substrate concentration and 66% depression at the lower concentration of substrate. Estimates such as these underscore the difficulty of utilizing data obtained from in vitro experiments, which employ saturating concentrations of drug substrates and reduced cofactor, to estimate rates of drug metabolism and identify rate-limiting steps in whole cells.

There is considerable information to show that dietary vitamins and minerals affect mixed-function oxidase systems. Vitamin A is of particular interest since deficiency of dietary retinoids enhanced susceptibility of epithelial cells to chemical carcinogens (216, 217). Epidemiological studies have shown a high correlation between vitamin A deficiency and the incidence of lung cancer in Norwegian men (4) and retinoid deficiency has been found to cause a 4-fold increase in binding of benzo(a)pyrene to tracheal epithelial DNA in the hamster (74). Information concerning interactions of vitamin A with the mixed-function oxidase systems indicates that the deficiency is associated with lower hepatic N-hydroxylase and N-demethylase activities as well as diminished levels of cytochrome P-450 (13). These findings suggest that enhanced binding of the precarcinogen, benzo(a)pyrene, to DNA in epithelial cells may not reflect an action of vitamin A and synthetic retinoids on the mixed-function oxidase system; however, additional information is needed in whole cell preparations.

2. Inducing agents. Exposure of laboratory animals and man to the environment is an important factor determining the activity of mixed-function oxidation in whole cells. Information presented above indicates that the effect of inducing agents may not be limited to altered rates of synthesis and degradation of microsomal electron transport components but also involves alterations in NADPH supply (110). The induction of drug metabolism was the subject of a recent symposium in which a number of studies involving whole cell preparations were described (64).

As discussed above, isolated hepatocytes in culture have been used to study induction of microsomal components by various chemicals. These systems have the advantage of being removed from neural and hormonal events that may influence the induction process in vivo. Use of primary hepatocytes in culture to study induction has not proved to be very satisfactory because of rapid declines in microsomal components in vitro. Although several workers (91, 141) have demonstrated induction of cytochrome P-450 by phenobarbital in primary hepatocytes in culture, the levels achieved were far below those observed after phenobarbital induction in vivo. To date, most successful studies of induction in vitro have employed either cell lines derived from hepatoma or fetal cells because such cells are generally more viable in culture than primary hepatocytes (34). Arylhydrocarbon hydroxylase has been shown to be inducible in fetal liver cells by polycyclic aromatic hydrocarbons, phenobarbital (76, 77), biogenic amines (170), and by tryptophan (167). The latter compounds apparently do not induce the enzyme in vivo. Based on studies with fetal liver cells in culture, Paine and Legg (169) have advanced the interesting hypothesis that induction of arylhydrocarbon hydroxylase in intact cells is due to the formation of active oxygen species such as superoxide or singlet oxygen, or to reactive oxygen-containing molecules. Benzo(a)pyrene metabolism has also been studied in isolated hepatocytes (33, 102) and in the perfused liver (105).

Hepatocytes isolated from phenobarbital-treated rats displayed marked decreases in reduced glutathione when exposed to bromobenzene (223). This decrease was accompanied by marked declines in pyridine nucleotides and coenzyme A (222). Phenobarbital and 3-methylcholanthrene produced different effects on the conjugation of 4-hydroxybiphenyl in isolated hepatocytes (252). Phenobarbital pretreatment caused large increases in levels of 4-hydroxybiphenyl. This increase in substrate for glucuronidation affects the yield of specific conjugates.

The use of isolated whole cell preparations to examine the influence of inducing agents on intermediary metabolism has been discussed in section II. Inducing agents such as phenobarbital and 3-methylcholanthrene have been shown to enhance the capacity of the isolated perfused rat liver to form NADPH (108, 110). Induction of NADPH-generating enzymes as well as alterations in the oxidation-reduction state of the tissue contribute to altered cofactor supply. Both phenobarbital and 3-methylcholanthrene treatment elevate the activities of several $NADP^+$-dependent dehydrogenases (32, 205). Phenobarbital pretreatment also decreased the total amount of NADPH (207) as well as the free NADPH/

free $NADP^+$ ratio in perfused livers. Similar effects have also been noted in isolated hepatocytes (150). This action of phenobarbital could enhance the activity of several $NADP^+$-dependent dehydrogenases in intact cells (109). Examples of altered interactions between energy metabolism and routes of drug conjugation in intact cells brought about by inducing agents are beginning to be studied (184).

Intracellular as well as extracellular binding proteins have a major influence on drug distribution and metabolism. Binding proteins within cells are undoubtedly important determinants of concentrations of free drug substrates and metabolites and may also serve a transport function from the extracellular compartment to sites of metabolism. A specific glutathione transferase is of particular interest because it not only serves a catalytic function in forming glutathione conjugates but also binds a wide array of chemicals of diverse structure (113). This protein is referred to as *ligandin* and is located in the soluble fraction of cells (7). Ligandin is apparently the same protein as the azocarcinogen-binding protein (122), cortisol binding protein II (7), and glutathione transferase B (92). It has been observed to change in parallel with components of the mixed-function oxidase system under different nutritional states and in the presence of various inducing agents.

Extracellular binding proteins may also have marked effects on rates of mixed-function oxidation and conjugation in intact cells. A recent study with isolated hepatocytes demonstrated that a significant proportion of the O-deethylation product of ethoxyresorufin first diffused out of hepatocytes and then reentered them to undergo conjugation (37). Extracellular bovine serum albumin inhibited the rate of conjugation by binding resorufin that had left the hepatocytes.

3. Effect of age. Age-related changes in components of microsomal mixed-function oxidase systems, as well as enzymes associated with hepatic energy metabolism, have been known for a number of years; however, the relationship of these changes to altered rates of drug metabolism and altered control of this system in intact cells has not been established. Kuenzig et al. (117) studied the hepatic metabolism of benzo(a)pyrene, *p*-chloro-N-methylamine, and chlorcyclizine as well as cytochrome P-450 content and cytochrome P-450 reductase in guinea pigs, pre- and postnatally. These authors showed that although the content of P-450 increased relatively rapidly during early development, the oxidation of the above compounds and reduction of cytochrome P-450 developed much more slowly. A direct correlation between microsomal mixed-function oxidation and proliferation of the smooth endoplasmic reticulum was observed in hepatocytes from normal and phenobarbital-treated animals. There are clear differences in the types of enzymes that are induced in intact hepatocytes at various developmental stages. Further, induction of microsomal drug-metabolizing systems by various chemicals is influenced markedly by aging (80, 115). Polycyclic aromatic hydrocarbons induced hepatic epoxide hydratase and monooxygenase activity in adult rats; however, these same compounds induced only the monooxygenase in fetal rat liver when administered to pregnant rats (159). Whether this difference was due to transplacental modification of inducing agents or to inherent differences in fetal and adult liver cells is not known. Oesch (159) has suggested that this selective transplacental induction of monooxygenase activity may be one reason why polycyclic hydrocarbons induce liver tumors transplacentally in fetal rats but not in adult rats where both monooxygenase and epoxide hydratase are induced. Elevated monooxygenase activity relative to epoxide hydratase activity may underlie the well-documented high susceptibility of newborn rats to chemical carcinogens (58, 238). For example, the higher susceptibility of rats between weaning and sexual maturity to the carcinogenic effects of dimethylnitrosamine may be due to increased dimethylnitrosamine demethylase activity during this period of development (55).

The human fetus appears to be unique in comparison to those from various animal species in having relatively high activities of oxidative drug-metabolizing enzymes; cytochrome P-450 content ranges from 60% to 70% of levels reported in human adult liver microsomes obtained by surgical biopsy (181). Although the mixed-function oxidase system is active in human fetal liver, conjugation reactions appear to be very low or absent (181). Thus, fetal tissue may be particularly sensitive to injury by substrates activated by the mixed-function oxidase system. Alterations that occur in drug metabolism in the aged have been reviewed (240). An extensive review on the disposition of drugs in the fetus with a detailed discussion of the properties and appearance of oxidative drug-metabolizing enzymes has also been published recently (249).

Activity of the mixed-function oxidase system during early stages of development has been suggested to influence the development of other metabolic events in hepatocytes. For example, the concentration of ligandin in liver of mutant mice lacking cytochrome P-450-dependent monooxygenase activity after radiation was twice that of liver of heterozygous littermates and newborn controls (72), suggesting that substrates normally metabolized by the cytochrome P-450 oxidative system accumulate and induce ligandin synthesis. NADPH-dependent lipid peroxidation and superoxide anion formation represent other processes that are influenced by changes in mixed-function oxidation that occur with aging (11, 178).

Mechanisms for the elimination of drugs do not develop directly in proportion to the body weight (9, 69). The low rates of drug metabolism in neonatal rats may be explained in part by small liver weights relative to total body size. At 3 days before term, fetal rat liver represents 8% to 9% of the body weight and only 3% at

7 days postpartum, which compares with 5% to 6% at 35 days (16). The early development pattern of drug-metabolizing enzymes in liver appears to parallel cellular RNA and protein contents (118); however, changes in rates of drug metabolism that occur with aging involve qualitative as well as quantitative changes in enzyme activity. The apparent K_m's for a number of oxidative drug-metabolizing enzymes change during development (16, 83, 116, 173). Superimposed on these apparent qualitative changes in enzyme protein are changes in concentrations of inhibitory (98) and stimulatory substances in whole cells as well as possible changes in the supply of NADPH with development.

Changes in interactions that occur between intermediary metabolism and mixed-function oxidation with early development and aging have not been explored. Interactions described above are likely to vary with age because glucose, amino acid, and fatty acid metabolism via various metabolic pathways change with age. For example, mixed-function oxidation in fetal liver may be strongly influenced by NADPH supply via the pentose pathway since this route of metabolism is particularly active early in development (246). Changes in other enzymes associated with hepatic carbohydrate metabolism (10) and gluconeogenesis (85) with aging have also been described.

V. Conclusion

Work considered in this review indicates that regulation of mixed-function oxidation of drugs in whole cells is related intimately to intermediary metabolism. The concentration of NADPH, cellular energy state, and transport of reducing equivalents across intracellular membranes have been shown to influence rates of mixed-function oxidation and conjugation in whole cell preparations. A partial list of the multiple interactions that influence drug metabolism in intact cells is presented in table 1. In understanding and predicting how acute and chronic changes in the nutritional state of the whole organism alter rates of drug metabolism and activation of toxic chemicals it is important to consider such events. Thus, in addition to considering changes in amounts and kinetic properties of components of the mixed-function oxidase system that occur in the presence of various inducing agents and altered nutritional states, it is necessary to consider changes that take place in the capacity of the liver to generate and transport reducing equivalents. Depending upon the nutritional state of the whole organism, reducing equivalents may arise from either cytoplasmic or mitochondrial sources. Alterations in mechanisms associated with movement of reducing equivalents within cells represent important interactions between intermediary metabolism and drug metabolism that warrant further study. Agents that affect the capacity of cells to maintain ATP may have acute effects on rates of mixed-function oxidation since at least one component of this system as well as several enzymes associated with the formation of NADPH are inhibited by ATP. Work outlined above indicates clearly that consideration of factors that regulate rates of mixed-function oxidation and conjugation in intact cells is important for our ultimate understanding and rational manipulation of chemical carcinogenesis and drug-induced toxicity as well as the metabolism of a variety of commonly used drugs.

TABLE 1

A partial list of factors that influence rates of drug hydroxylation and conjugation in intact cells

Factors	References
I. Drug hydroxylation	
A. Content and kinetic properties of microsomal enzymes	16, 23, 50, 63, 83, 116, 126, 173, 162
1. Inducing agents	76, 77, 121, 167, 70, 225, 237
2. Activators	53, 96, 98, 109, 110, 130, 185, 186
3. Inhibitors	36, 86, 99, 114, 138, 151, 170, 183, 186, 192, 212
B. Availability of drug substrate	
1. Transport from extracellular sites	35, 62, 70, 134
2. Intracellular binding	7, 37, 87, 92, 122
C. Cofactors supply	
1. Cytoplasmic sources	39, 53, 96, 108–110, 150, 210, 228, 230
2. Mitochondrial sources	15, 23, 87, 182, 206, 107, 234, 252
D. Competing reactions	
1. Fatty acid synthesis	11, 178, 194
2. Reduction of oxidized glutathione	
II. Conjugation	
A. Content and kinetic properties of enzymes	24, 164
B. Availability of drug substrate	
1. Supply via mixed-function oxidation	67, 184
2. Transport between cellular compartments	37, 38, 164, 184
C. Supply of activated intermediates	
1. Via carbohydrate reserves	27, 148, 184, 223, 254
2. Influence of energy state	59, 135, 152, 164, 184

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