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Effects of clofibric acid on porcine hepatocytes: a new model for the study of peroxisomal metabolism

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Effects of clofibric acid on porcine hepatocytes: A new model for the study of peroxisomal metabolism

by

Kenneth Wayne Turteltaub

A Dissertation Submitted to the

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TABLE OF CONTENTS

PAGE

DEDICATION

This work is dedicated to my parents, Ed and Midge Turteltaub, and wife, Brenda for showing me the value of education and making the time in the trenches tolerable.

INTRODUCTION

Peroxisomes are subcellular respiratory organelles characterized by the presence of flavin oxidases and catalase (1-3). Their existence was first reported in 1954 by Johannes A.G. Rhodin from electron micrographs of mouse kidney tissue (4,5). Rhodin called the organelle a microbody. The organelle was described in rat liver cells two years later. In the mid-1950s, after refinement of isolation techniques, P. Baudhuin and H. Beaufay described the presence of the enzyme uricase, or urate oxidase, in the organelle (4,5). They termed the organelle the uricorisome. During the following 10 years, the emphasis of studies dealing with the peroxisome focused on mammalian liver and kidney. This resulted in the discovery that the particle contains several oxidases, and most significantly, catalase. In the late 1960s, Christian deDuve proposed that the organelle be called a peroxisome to reflect the central role played by catalase (1).

Electron microscopy studies of liver reveal the peroxisome to be a spherical or dumbell shaped particle approximately 0.05 to 1.50 μm in diameter. The size and shape of the organelle will depend on the species and tissue within which it is located (6-9). The peroxisome may be connected to other peroxisomes as part of an interconnected network (10). This observation, however, has not been substantiated.

Peroxisomes are bounded by a single trilaminar membrane and possess a granular matrix. In liver, the organelle normally exhibits an electron dense anucleoid crystalline core (6,7,11). In some species, such as the rat, the core is composed of insoluble urate oxidase. In other species, such as man, it may be catalase.

The peroxisome tends to be smaller in extrahepatic tissues, approximately 0.15 to 0.25 μm in diameter. The organelle lacks a central core and has a membrane which has been reported to be continuous with the endoplasmic reticulum (12,13). To reflect the smaller size, the organelle has been referred to as microperoxisome.

The peroxisome's distribution in nature is wide. Since the mid-1960s a variety of species have been studied. This has resulted in the belief that peroxisomes are present universally throughout the eukaryotic kingdom (1,6,7). They have been described in a number of tissues as well. The list includes, but is not limited to, striated muscle, cardiac muscle, sebaceous glands, smooth muscle, brown fat, white fat, germinal tissue, epithelial tissue, bladder tissue, yolk sacs, prostate glands, schwann cells, lymphoid tissue, connective tissue, mammary tissue, intestinal tissue, lungs, chondroblasts, nervous tissue, spleen, thymus, blood vessels, kidney, adrenal tissue, brain, and liver (7,8,14).

Although the organelle is widespread in nature, astonishing differences in enzyme composition exist. The peroxisomal enzyme repertories vary, depending on the species and the tissue one is investigating (1). Variation in enzymatic composition has been noted in response to changes in substrate availability and stage of development as well (11,15,16,17,18). This diversity is totally unknown for any other kind of organelle and has made it difficult to attribute a function or assign a unified conceptual arrangement into which these variations can be placed. Thus, a function for the organelle has yet to be assigned.

β-Oxidation

The only complete metabolic pathway described in the mammalian organelle is β-oxidation. Previously, β-oxidation was believed to be associated only with the mitochondria and the glyoxisome. Comparison of mitochondrial β-oxidation and peroxisomal β-oxidation has been carried out by several investigators who have demonstrated that peroxisomes contain no energy-coupled electron transport chain (1,19-25). Peroxisomal β-oxidation is also cyanide-insensitive and results in the generation of heat, water, short-chain acyl-CoA (octanyl-CoA and shorter), reduced nicotinamide adenine dinucleotide (NADH), and hydrogen peroxide from substrates passing through the pathway.

The fate of the products of peroxisomal β-oxidation has not been established. Acyl-CoA is believed to be funneled to the mitochondria for the production of adenosine triphosphate (16,19,21). Reduced nicotinamide adenine dinucleotide may function in providing a pool of nucleotides for other cell processes and in maintaining the cell's redox potential. The NADH may be transported to the mitochondria via a specific shunt such as the dihydroxyacetone phosphate reductase-glycerophosphate dehydrogenase electron shuttle (1,16,21,26,27,28).

Physical characteristics of the enzymes are pathway-specific as well (1). This probably accounts for the different substrate specificities, pH optimum, and cofactor requirements observed for the pathways. Peroxisomes are characterized as having flavin oxidases, which require FMN or FAD as prosthetic groups (1,29). Peroxisomal enzymes have a pH optimum around 8.5 as compared to the lower pH profiles in other subcellular compartments (1,29,30). Peroxisomal enzymes have different amino acid compositions, structural arrangements, and kinetic parameters as compared to their mitochondrial counterparts (1,30,31,32,33,34,35).

As a result, the enzymes of peroxisomal β-oxidation differ in substrate specificity from those of the mitochondria. Peroxisomes prefer long-chain fatty acids and may not oxidize any smaller than octanyl-CoA (16,17,25,36,37). The peroxisomal system preferentially oxidizes fatty

acids of 14 to 16 carbons in length. Mitochondria apparently prefer short-chain fatty acids. Dicarboxylic acids and unsaturated fatty acids are oxidized in the peroxisome as well (38,39,40).

It can be proposed that since peroxisomes possess a β-oxidation chain, do not produce useful energy, prefer medium to long-chain fatty acids as substrates, and result in the production of short-chain acyl-CoA, they function to complement the mitochondria in their metabolic role. The peroxisome may be providing a substrate source to the mitochondria which would not otherwise be available. The peroxisome may also help in maintaining the cell's state of homeostasis by preventing the build-up of toxic substances such as long- and very-long chain fatty acids. These hypotheses are supported by the fact that high fat diets, prolonged vitamin E deficiency, starvation, and exposure to cold result in increased peroxisomal β-oxidation rates (17,41,42,43,44). Peroxisomal β-oxidation is very active in the perinatal period as well (45). Peroxisomal proliferation occurs in some species in response to treatment with certain drugs and exposure to some environmental toxicants. These results may indicate that the peroxisome plays a role regulating energy balance in the cell during highly stressful periods of life.

However, it has been estimated that, in rodents, the maximal contribution of the peroxisome to the cell's total capacity for β-oxidation never exceeds 10-30% (1,32). Data from other species are limited.

Cholesterol Metabolism

The ability of the peroxisome to oxidize cholesterol has recently been reported. It has been demonstrated that cholesterol can be oxidized through a hydroxylated intermediate, via peroxisomal β-oxidation, to propionate and cholic acid (46). It was further demonstrated that cholic acid can be conjugated to glycine and taurine in the organelle to form the bile acids taurocholic and glycocholic acid (47). Treatment of rodents with cholestyramine, a drug which binds bile acids in the intestines, results in an induction of peroxisomal β-oxidation (48). A peroxisomal hydroxymethyl glutaryl Co-A reductase has been identified too, suggesting that the peroxisome could produce cholesterol as well as oxidize it (49,50). A peroxisomal role in cholesterol metabolism seems likely.

Glycerolipid Synthesis

Peroxisomes may play a crucial role in the synthesis of the plasmalogens. Peroxisomes have been shown to contain a dihydroxyacetone phosphate acyltransferase (1). This enzyme is an

obligatory step in the synthesis of the ether glycerolipids, or plasmalogens (51). Much of the activity of this enzyme has been demonstrated to be in the peroxisome and it is inducible by clofibrate (51,52). The functions of plasmalogens are unknown. They are present in all cell types and are especially abundant in electrically active tissues such as nervous tissue and muscle (52). Thus, it is believed they are important in transmembrane movement of electrolytes. Individuals deficient in peroxisomes contain extremely low levels of plasmalogens (53). These individuals suffer from lack of muscle tone and mental retardation which conceivably could be due to plasmalogen deficiency (51). It appears that the peroxisome does play an important role in lipid metabolism but its full nature remains unclear .

Peroxisomes and Hypolipidemic agent

The belief of a peroxisomal role in lipid metabolism has led investigators to examine the relationship of the organelle to serum and organ cholesterol and triglyceride levels. Cholesterol and triglycerides have been implicated as possible causative agents in several pathological states, especially in this nation's number one cause of death, heart disease (43,49,50,54).

Several drugs are routinely used to control serum triglyceride levels in man and animals. Some have been noted to cause an induction of selected peroxisomal and nonperoxisomal enzymes. A proliferation of peroxisomes occurs as well as proliferation of the smooth endoplasmic reticulum (SER), and to a lesser extent, mitochondria (1). These data, however, come predominantly from the rat and mouse (55-63). Few data are available on these compound's effects in man or animals other than rodents. The number of compounds known to affect the rodent peroxisome is large and constantly increasing. They are collectively referred to as peroxisome proliferators and encompass a variety of industrial uses such as in plasticisers, pesticides, prescription drugs, and some over-the-counter analgesics. One may conclude that human exposure to these chemicals is probably high.

Clofibrate (p-chlorophenoxyisobutyric acid) is probably the most widely used hypolipidemic drug available today, and forms the core of a number of structurally related peroxisome proliferators. Clofibrate has been shown to cause peroxisome proliferation, hepatomegaly, increases in enzyme activity, and increased mitochondrial β-oxidation (56,57,58,60, 64,65,66). Fenofibrate (isopropyl[4(p-chlorobenzoyl)-2-phenoxy-2-methyl] propionate), a derivative of clofibrate, also causes a marked proliferation of peroxisomes in rats, mice, and hamsters. Hepatomegaly has been

reported in some species (56,65,66). Methyl clofinapate (2-methyl-[4-(p-chlorophenol)phenoxy]methyl propionate), a methylated derivative of clofibrate, also possesses effects similar to clofibrate, but is much more potent in rodents. Simfibate (1,3-propenediol-bis (2-p-chlorophenoxy) isobutyrate) shows little effect on hepatic lipid levels yet increases cyanide insensitive fatty acyl-CoA oxidation and carnitine acetyl transferase activity (58,65,66). Pyrinixil (BR-931; 4-chloro-6-(2,3-xylidino)-2-pyromidenylthio-(N-beta-hydroxyethyl) acetamide) behaves similarly to fenofibrate (54,63,64). A new hypolipidemic compound, POCA (ethyl-2-[5(4-chlorophenyl) pentyl] oxiran-2-carboxylate), causes a 2.15-fold increase in peroxisomal numbers and a decrease in their average size from 0.36 to 0.31 μm in the rat hepatocyte (56). It results in a 3-fold increase in the capacity for β-oxidation as well. Most importantly, it tends to inhibit mitochondrial β-oxidation at carnitine palmitoyl transferase. The compound tends to be effective only in starved rats, however, and not in fed rats. LK-903 (alpha-methyl-p-myristyroxycinnnamic acid-1-monoglycerol) also possesses hypolipidemic activity similar to clofibrate but does not result in hepatomegaly. It does increase the activity of catalase, D-amino acid oxidase, urate oxidase, and β-oxidation (60).

Acetysalicyclic acid, a common over-the-counter analgesic, also lowers serum triglyceride levels. Induction of peroxisomal enzymes and peroxisome proliferation has been reported in rats (52). Hepatomegaly has been noted. No information is available on its effect on non-rodent peroxisomes.

Certain environmental contaminants also possess hypolipidemic activity (62,63). Phthalate esters are plasticizers that have uses in different industrial processes. Large amounts of these substances have been reported to occur in water, soil, and the food chain (67). Plasticizers commonly used in industry such as di-(2-ethylhexyl) phthalate have been shown to cause proliferation of hepatic peroxisomes in the rat (62,63). Proliferation of mitochondria has been described as well (68). Increases in liver weight of up to 50% have been observed in association with the hypolipidemic action of these compounds. Phenoxyacid herbicides have been reported to result in similar effects (69).

Peroxisomes and Oxygen Toxicity

Long-term treatment of rodents with peroxisome proliferators results in the development of hepatocellular carcinomas. It has been proposed that these drugs, as a class, are carcinogenic (70). Phthalate esters have been observed to induce hepatocellular carcinomas in both F-344 rats and

B6C3F1 mice (71). Treatment with Wy14,643, an experimental hypolipidemic drug which is structurally unrelated to clofibrate, has been shown to induce hepatic carcinomas in male F-344 rats (72). These studies show that peroxisome proliferators are carcinogenic in rodents but the mechanism of carcinogenesis still remains unclear.

Investigations to elucidate the etiology of hypolipidemic drug-induced carcinomas demonstrate that peroxisome proliferating agents are not mutagenic. Studies in the lymphocyte [^{3}H] thymidine assay and mutagenesis assay show that the drugs do not form DNA adducts or cause genetic damage directly (73). The mechanism proposed to be responsible for the hepatocellular carcinoma development in rodents involves the generation of excessive hydrogen peroxide (H_2O_2). Reddy *et al.* (70) have suggested that the proliferative action of these agents on peroxisomes causes the generation of excessive amounts of reactive oxygen species. These free radicals may attack membrane lipid components, proteins, and nucleic acids -- ultimately resulting in cancer. The concentration of catalase in the peroxisome and the kinetics of the enzyme, however, do not support this.

Detoxification Capacity of the Peroxisome

Cells have evolved a number of mechanisms to deal with oxidative stress. Catalase is the standard marker enzyme for the peroxisome and accounts for approximately 60% of the total protein present in the organelle (3). Catalase functions to metabolize H_2O_2 to H_2O, O_2, and heat. Investigations into the kinetics of this enzyme show it to be very active, possessing a rate constant of 10^7 sec^{-1} $mole^{-1}$ (74).

Reactive oxygen species, which escape the peroxisomal catalase, can attack lipid components of the cell resulting in the generation of epoxides. These, in turn, may interact with other cell macromolecules, adversely affecting their function. Epoxide hydrase hydrates epoxides to more stable dihydro-diols. Waechter *et al.* (75) have reported that this enzyme is probably present in the peroxisome instead of the mitochondria as previously believed. Seventy percent of the enzymes activity was found to be located in the peroxisome.

Aldehyde dehydrogenase (ALDH), with a high K_m for acetaldehyde, has been partially purified from the peroxisome of rats (76). Aldehydes formed as oxidation products of lipids can also interact with macromolecules and are reduced to the more stable carboxylic acid by this

enzyme, effectively, being detoxified. The high K_m isozyme of ALDH in the rodent peroxisome has been shown to be inducible by clofibrate (77).

Superoxide dismutase scavenges the superoxide anion (O_2^-), reducing it to the more stable hydrogen peroxide which can then be destroyed by catalase or glutathione peroxidase (78). Murphy and Gee (personal communication, Dr. P.A. Murphy, Iowa State University) have found that superoxide dismutase may be associated with the peroxisome. Few studies have been conducted to examine this enzyme's response to peroxisome proliferating conditions. Some increase in activity has been noted, but decreases in activity have been reported as well.

Antioxidants are also present in normal cells. Compounds such as vitamin E, glutathione, ascorbic acid, and vitamin A are present to remove any reactive oxygen moiety generated in the metabolic processes.

It would seem that adequate controls are present in the cell to limit the amount of free radicals generated, even during periods of peroxisome proliferation. Data on the effects of hypolipidemic compounds on these cellular constituents are lacking. The exact mechanism of peroxisome proliferation and peroxisome-related tumorogenesis remains to be elucidated.

Phylogenetic Factors and Peroxisome Proliferation

The primary site of toxic action in rodents from hypolipidemic drugs is the liver. The specific lesions reported in the literature tend to depend on which species were used in the experiment. Commonly reported effects usually consist of a dose-dependent liver enlargement (hepatomegaly) with minor histological changes and elevated serum transaminase activity (3,63,65,66,79,80).

Ultrastructurally, peroxisome numbers increase and changes in organelle size have been noted after treatment with peroxisome proliferators (65,66,71,79,80). Proliferation of smooth endoplasmic reticulum and rough endoplasmic reticulum (RER) accompany these changes, with dilatation of the SER and RER membranes being common (65,66,79). Administration of hypolipidemic agents has been reported to cause depressed microsomal enzyme activities (65,79).

Some researchers have reported elevated microsomal enzyme activities as well (65,79). Slight increases in the cytochrome P-450 mixed function oxidases have been noted in some studies, but detailed data are lacking (65,79).

Lazarow *et al.* (59) have compared the response of several species to treatment with hypolipidemic drugs and have shown that induction of peroxisomal β-oxidation does not obligatorily include gross hepatomegaly or other alterations of peroxisomes. In a related study, hypolipidemic drugs resulted in peroxisomal proliferation in dogs and hamsters, but not in primates or guinea pigs (81).

Studies of peroxisomes in humans are severely limited. Human and rodent liver differ in a number of significant ways, especially in enzyme composition (3). No hepatomegaly was seen in primates or man after treatment with clofibrate, fenofibrate, or nafinopen (65,79). There did appear to be a slight tendency for the reduction of fatty infiltration of the liver, and liver glycogen levels tended to decrease also (65,79). No peroxisome proliferation has been reported in primates or man after exposure to these drugs at doses equivalent to those administered to rodents. In a separate study, isolated human hepatocytes were insensitive to levels of trichloroacetic acid which resulted in peroxisome proliferation in rat hepatocytes and mouse hepatocytes (82). Peroxisome proliferation in primates has been reported at exceedingly high dosages of these drugs, however (83,84). It would seem, then, that effects of peroxisome proliferators are not uniform across all species. "Higher animals" may be less sensitive to peroxisome proliferators than are rodents because of

intrinsic metabolic differences (85). The direct application of rodent databases to risk assessment must be questioned. Detailed data on the metabolism of the peroxisome of animals more likely to reflect human peroxisomal metabolism need to be collected.

Peroxisomes in Disease

Very little is known about peroxisomes in disease. Peroxisomes do appear to play a role in some disease states (86). Peroxisomes tend to become more abundant and assume a perinuclear configuration in human cholestatic and cirrhotic livers (86). Decreased diaminobenzidine staining, a histochemical stain specific for catalase, was common in the diseased tissue when compared to normal liver. These reactions were found in human neoplasms as well (87). Humans born with Zellweger's cerebrohepatorenal syndrome have been demonstrated to possess no peroxisomes in the liver tissue (88,89). Victims of this congenital disease usually do not survive past six months of age. Increased peroxisomal degradation has been implicated in acute hepatic failure in partial ornithine transcarbamylase deficiency also (90). An increased number of hepatic peroxisomes have been reported in several other pathological states such as alcoholic liver disease, certain infectious diseases, toxicosis, and Reye's syndrome (5,8,10,49). In addition, Sternlieb and Quintana (9) found

significant alterations in peroxisomal size and electron density in 14 out of 19 of the pathologic human livers that they examined. These livers were from patients afflicted with hepatitis induced by drug therapy, cholestasis, and Wilson's disease. Data concerning biochemical alterations are lacking.

Research Rationale

The importance of peroxisomes to the cell still remains to be elucidated. A peroxisomal role in lipid metabolism does seem likely. Studies on humans have been sparse, and the ability to extrapolate data from rodents to man is questionable. In view of the fact that peroxisome proliferators are associated with neoplasia in rodents and are affected in some disease states in humans, it would be desirable to develop a more complete understanding of the metabolic function of the organelle. It is also necessary to establish how peroxisomal metabolism differs among species, specifically if peroxisomes in phylogenetically higher animals are sensitive to agents that proliferate peroxisomes in rodents. Mechanistic differences among species in peroxisome proliferation should be elucidated to determine if a risk to humans exist.

Species specificities can arise via a number of different mechanisms. Absorption and distribution kinetics of peroxisome proliferators may vary. Sites of action may differ.

Receptors in target tissues may vary in quantity or form. A cytosolic receptor for nafinopen has been described (91).

Akbar *et al.* (92) have demonstrated that clofibrate decreases the activity of adenylate cyclase in some cell types, possibly by a receptor-mediated process. Detoxification pathways and mode of elimination may also be species specific. Intrinsic differences in sensitivity to peroxisome proliferation may also exist.

In the past, research utilizing animals other than rodents has been difficult because of spatial needs, expense, long generation-time, and handling requirements. With the advent of tissue culture methodology, these logistical problems can be overcome. It would be beneficial to apply this technology to answer questions dealing with interspecies differences in peroxisomal metabolism. New and valuable information could be contributed to the literature on this topic if tissues from animals that physiologically resemble man were studied. Primary porcine hepatocyte cultures would be such a model.

Cell culture will allow investigators to eliminate the effects of route and kinetics of absorption and distribution. Cell culture can be used to define target cells and the effects of peroxisome proliferators in those cells. Cell culture also offers a means of clarifying the contribution of the organelle to cellular lipid metabolism. Specific substrates can be added to the growth matrix for the study of carbon shunting in metabolism. Metabolic pathways can be inhibited or activated through the use of appropriate effectors. In short, cell culture offers unlimited opportunities for the study of peroxisomal metabolism and as yet has been under-utilized.

The purpose of this project then would be to examine the applicability of porcine hepatocyte tissue culture to studies involving peroxisomes, and to investigate the response of superoxide dismutase, aldehyde dehydrogenase, mitochondrial β-oxidation, and peroxisomal β-oxidation to the administration of clofibric acid, a known peroxisome proliferator.

Explanation of Dissertation Format

The dissertation is divided into two parts, each being a complete paper already submitted to a professional journal. Part I is a report of an isolation protocol for porcine hepatocyte peroxisomes and the examination of the contribution of peroxisomal β-oxidation to lipid metabolism.

It also describes the localization of an isozyme of aldehyde dehydrogenase in the organelle. Part II outlines a procedure for the isolation and growth of viable porcine hepatocytes. It describes the effects of clofibric acid on the isolated cells and the presence of a peroxisomal superoxide dismutase. Species-related factors in peroxisomal metabolism are discussed.

REFERENCES

1. Tolbert, N.E. 1981. Annu. Rev. Biochem. 50:133.

2. Connock, M.J., and S.R. Perry. 1983. Biochem. Int. 6(4):545.

3. deDuve, C., and P. Baudhuin. 1966. Physiol. Rev. 46:323.

4. deDuve, C. 1978. in Tocopherol, Oxygen, and Biomembranes. (ed. C. deDuve and O. Hayashel) Biomedical Press, Amsterdam, New York. 351pp.

5. deDuve, C. 1983. Sci. Am. 248(5):74.

6. Hruban, Z., E.L. Vigil, A. Slesers, and E. Hopkins. 1972. Lab. Invest. 27(2):184.

7. Novikoff, A.B., P.M. Novikoff, C. Doves, and N. Quintana. 1973. J. Histochem. Cytochem. 21:737.

8. Beard, M.E. 1972. J. Histochem. Cytochem. 20(3):173.

9. Sternlieb, I., and N. Quintana. 1977. Lab. Invest. 36:140.

10. Gorgas, K. 1981. N. Y. Acad. Sci. Abst P-2-16.

11. Tolbert, N.E. 1980. Chapter 9. in The Biochemistry of Plants. A Comprehensive treatse. Vol. 1.(ed. N.E. Tolbert). Academic Press, New York.

12. Masters, C.J., and R.S. Holmes. 1977. Int. J. Biochem. 8:549.

13. deDuve, C. 1973. J. Histochem. Cytochem. 21:941.

14. Schneeberger, E.E. 1972. J. Histochem. Cytochem. 20(3):180.

15. Popova, M., Ch. Ivanov, and N. Mirinova. 1979. Rus. J. Anim. Sci. Vol. XVII:2.

16. Horie, S. H. Ishii, and T. Suga. 1981. Life Sci. 29:1649.

17. Van Velthoven, P. and G. P. Mannaerts. 1985. Biochem. J. 227:737.

18. Ishii, H., N. Fukumore, and S. Horie. 1980. Biochim. Biophys. Acta 614:1.

19. Lazarow, P.B. 1978. J. Biol. Chem. 253(5):1522.

20. Manaert, G.P., L.J. Debeer, J.T. Thomas, and P.J. DeShepper. 1979. J. Biol. Chem. 254(11):4585.

21. Masters, C., and R. Holmes. 1977. Physiol. Rev. 57(4):816.

22. Osumi, T., and T. Hashimoto. 1978. Biochem. Biophys. Res. Commun. 83(2):479.

23. Thomas, J., L. J. Debeer, P.J. DeSchepper, and G.P. Mannerts. 1980. Biochem. J. 190:485.

24. Osmundsen, H., E.C. Neat, and B. Borrebalk. 1980. Int. J. Biochem. 12:625.

25. Bronfman, M., N.C. Inestrosa, and F. Leighton. 1979. Biochem. Biophys. Res. Commun. 88(3):1036.

26. Ishii, H., S. Horie, and T. Suga. 1980. J. Biochem. 87:1855.

27. Jones, C.L. and A.K. Hajra. 1980. J. Biol. Chem. 255(17):8289.

28. Hajra, A., C.L. Burke, and C.L. Jones. 1979. J. Biol. Chem. 254(21):10896.

29. Inestrosa, N.C., M. Bronfman, and F. Leighton. 1979. Life Sci. 25:1127.

30. Osumi, T., and T. Hashimoto. 1980. Arch. Biochem. Biophys. 203(1):372.

31. Furuta, S., S. Mizayawa, T. Osumi, and T. Hashimoto. 1980. J. Biochem. 88:1059.

32. Krahling, J.B., R. Gee, P.A. Murphy, J.R. Kirk, and N.E. Tolbert. 1978. Biochem. Biophys. Res. Commun. 82(1):136.

33. Krahling, J.B. and N.E. Tolbert. 1981. Arch. Biochem. Biophys. 209(1):100.

34. Connock, M.J. and S.R. Perry. 1983. Biochem. Int. 6(4):545.

35. Miyazowa, S., S. Furuta, and T. Hashimoto. 1981. Eur. J. Biochem. 117:425.

36. Osmundsen, H., C.E. Neat, and K.P. Norum. 1979. FEBS Lett. 99(2):292.

37. Hryb, D.J., and S.F. Hogg. 1979. Biochem. Biophys. Res. Commun. 87(4):1200.

38. Hiltunen, J.K., T. Karki, I.G. Hassinen, and H. Osmundsen. 1986. J. Biol. Chem. 261:16484.

39. Dommes, V., C. Baumgart, and W.M. Kunau. 1981. J. Biol. Chem. 256:8259.

40. Mortensen, P.B., N.G. Gregersen, and K. Rasmussen 1983. J. Inher. Metab. Dis. 6 suppl. 2:123.

41. Reddy, J.K., N.D. Lalwani, A.S. Dabholken, M.K. Reddy, and S.A. Qureshi. 1981. Biochem. Int. 3(1):41.

42. Nederguard, J., S. Alexson, and B. Cannon. 1980. Am. J. Physiol. 239:6208.

43. Neat, C.E., ZM.S. Thomassen, and H. Osmundsen. 1980. Biochem. J. 186:369.

44. Berge, R.K., and A. Aarsland. 1985. Int. J. Biochem. 837:141.

45. Krahling, J.B., R. Gee, J.A. Gauger, and N.E. Tolbert. 1979. J. Cell Physiol. 101:375.

46. Bjorkhem, I., B.F. Kase, and J.I. Pedersen. 1984. Biochim. Biophys. Acta 796:142.

47. Hagey, L.R., and S.K. Krisans. 1982. Biochem. Biophys. Res. Commun. 107(3):834.

48. Kase, F.B., K. Prydz, I. Bjorkhem, and J.I. Pedersen. 1986. Biochem. Biophys. Res. Commun. 138(1):167.

49. Bakke, O,M., and R.K. Berge. 1984. Biochem. Pharmacol. 33(19):3077.

50. Keller, G.A., M. Pazirandeh, and S. Krisans. 1986. J. Cell Biol. 103:875.

51. Horrocks, L.A., and M. Sharma. 1982. in Phospholipids, (eds. Hawthorne, J.N. and G.B. Ansell) Elsevier, Amsterdam. 51pp.

52. Gross, R.W. 1984. Biochemistry 23:158.

53. Haymans, H.S.A., R.B.H. Schurtgens, R. Tam, H. Vanden Basch, and P. Bast. 1983. Nature 306:69.

54. Peters, T.J., and C. deDuve. 1974. Exp. Mol. Pathol. 20:228.

55. Shio, H., G. Farquha, and C. deDuve. 1974. Am. J. Pathol. 76(1):1.

56. Reddy, M.K., N.D. Lalwani, S.A. Qureshi, and J.K. Reddy. 1982. Human Toxicol. 1(2):135.

57. Christiansen, R.Z. 1978. Biochim. Biophys. Acta 530:314.

58. Watanabe, T., and T. Suga. 1980. J. Biochem. 87:1595.

59. Lazarow, P.B., H. Shio, and M.A. Leroyhou. 1982. J. Lipid Res. 23:317.

60. Hayashi, H., S. Hino, F. Yamesaki, and T. Watanabe. 1981. Biochem. Pharmacol. 30(13):1817.

61. Lazarow, P.B., and C. deDuve. 1976. Proc. Natl. Acad. Sci. USA 73(6):2043.

62. Moody, D.E. and J.K. Reddy. 1982. Toxicol. Lett. 10:379.

63. Ganning, A.E. and G. Dallner. 1981. FEBS Lett. 130(1):77.

64. Rao, M.S., M.K. Reddy, J.K. Reddy, and D.G. Scarpelli. 1982. J. Cell Biol. 95:50.

65. Stauble, W., and R. Hess. 1978. in Hypolipidemic Agents. (ed. Kritchevsky). Springer-Verlag Press, New York. 225pp.

66. Bencze, W.L. 1980. in Mammalian Peroxisomes. (ed. L.L. Beiber). Wiley Press, New York. 108pp.

67. Autian, J. 1973. Environ. Health Persp. 4:3.

68. Ganning, A.E., G. Brunk, and R. Nilssen. 1982. J. Cell Biol. 23:225.

69. Vainio, H., K. Linnainmas, M Kahonen, J. Nickels, E. Hietanen, J. Marniemi, and P. Peltonen. 1983. Biochem. Pharmacol. 32:2775.

70. Reddy, J.R., D.L. Azarnoff, and C.E. Hignite. 1980. Nature 823:397.

71. National Toxicology Program Technical Report on the Carcinogenesis Bioassy of di(2-ethylhexyl) phthalate. No. 117-81-7.

72. Lalwani, N.D., M.K. Reddy, A. Qureshi, and J.K. Reddy. 1981. Carcinogenesis 2(7):645.

73. Warren, J.R., V.F. Simmon, and J.K. Reddy. 1980. Cancer Res. 40:36.

74. Aebi, H. 1983. Meth. Enzymol. Anal. 2:760

75. Waechter, F., P. Bently, F. Staubli, A. Volkle, and A.D. Fahimi. 1983. FEBS Lett. 158(2):225.

76. Antonenkov, V.D., S.V. Perozhkov, and L.F. Panchenko. 1985. Eur. J. Biochem. 149:159.

77. Panchenko, L.F., S.V. Perozhkov, and V.D. Antonenkov. 1982. Bull. Exp. Biol. Med. 94:1223.

78. Fridovich, I. 1974. in Advances in Enzymol. (ed A. Meister). Vol. 41. J. Wiley and Sons, New York. 35pp.

79. Cohen, A.J., and P. Grasso. 1981. Fd. Cosmet. Toxicol. 19:585.

80. Reddy, J.K., J.R. Warren, M.K. Reddy, and N.D. Lalwani. 1982. New York Acad. Sci. 386:81.

81. Svoboda, D.D., H. Grady, and D. Azaroff. 1967. J. Cell Biol. 35:127.

82. Elcombe, C.R. 1985. Arch. Toxicol. Suppl. 8:6.

83. Lalwani, N.D., M.K. Reddy, S. Gosh, S.D. Barnard, C.M. Mohello, and J.K. Reddy. 1985. Biochem. Pharmacol. 34(19):3473.

84. Reddy, J.K., N.D. Lalwani, S.A. Qureshi, M.K. Reddy, and C.M. Moehle. 1984. Am. J. Pathol. 114:171.

85. Eacho, P.I., P.S. Foxworthy, W.D. Johnson, D.M. Hoover, and S.L. White. 1986. Toxicol. Appl. Pharmacol. 83:430.

86. Goldfischer, S. 1979. J. Histochem. Cytochem. 27(10):1371.

87. Roels, F., M. Pauwels, A. Cornelis, T. Kerchaert, P. Van Der Spell, G. Goovaert, J. Versieck, and S. Goldfisher. 1983. J. Histochem. Cytochem. 31(1A):235.

88. Pfiefer, U., and K. Sandhage. 1979. Virchous Arch. Pathol. Hist. 384:269-284.

89. Goldfischer, S., C.L. Moore, A.B. Johnson, A.J. Spiro, M.P. Valsamis, H.K. Wisniewski, R.H. Rich, W.T. Norton, T. Rapin, and L.M. Gartner. 1973. Science 182:62.

90. Landrieu, P., B. Francois, G. Lyon, and F. Van Hoof. 1982. Pediatr. Res. 16:977.

91. Lalwani, N.D., W.E. Fahl, and J.K. Reddy. 1983. Biochem. Biophys. Res. Commun. 116:388.

92. Akbar, H., D.T. Witiak, H.A. Newman, R.H. Fertel, and D.R. Feller. 1982. Biochem. Pharmacol. 31(11):2125.

PART I.

SUBCELLULAR LOCALIZATION AND CAPACITY OF β-OXIDATION AND ALDEHYDE DEHYDROGENASE IN PORCINE LIVER

ABSTRACT

Porcine hepatocyte organelles were separated by isopycnic sucrose gradient centrifugation from livers of 6-month-old Yorkshire pigs. The presence of a peroxisomal palmityl-CoA oxidizing system and a peroxisomal NAD:aldehyde dehydrogenase (ALDH) with high K_m for acetaldehyde was demonstrated. Peroxisomal palmitate oxidizing capacity was found to be equal to that of the surviving mitochondria. The high K_m isozyme of ALDH was located mainly in the mitochondria (54%), with a significant portion in the peroxisome. Remaining activity was distributed among the microsomes (8.3%) and cytosol (4.6%). The low K_m isozyme was confined almost exclusively to the mitochondria. ALDH may exist in the peroxisome as a detoxification mechanism and contribute to shorter half-lives of reactive aldehydes in the cell. Species differences are discussed.

INTRODUCTION

Peroxisomes are small membrane-bound organelles. They have been identified in species ranging from yeast to man (1). The role of the peroxisome in the cell is unknown. A peroxisomal β-oxidation pathway exists in the species studied and a role in lipid metabolism has been proposed(2-4).

Perturbations in peroxisomal numbers and enzyme activity profiles, have been effected by treatment with several xenobiotics. Treatment with compounds such as the hypolipidemic agents, clofibrate and its analogs, di-(ethylhexyl) phthalate, acetylsalicyclic acid, and phenoxyacid herbicides results in profound increases in both peroxisomal numbers and selected enzyme activities. A concomitant decrease in serum triglyceride levels has been observed as well (5-12). Long-term treatment of rats with peroxisome proliferators results in development of hepatocellular carcinomas (13).

Few researchers have investigated peroxisomes in species other than rodents. An absence of peroxisome proliferation has been reported in several species (14), or may occur only at high xenobiotic concentrations (7,15). Mitochondrial β-oxidation is rarely mentioned in these studies,

thus the interaction of the two pathways is unknown in non-rodent species.

Recently, the high K_m isozyme of NAD:aldehyde dehydrogenase (ALDH) has been partially purified from the rat hepatocyte peroxisome (16). The existence of a peroxisomal ALDH in species other than rodents has not been reported. Species differences in the activity and subcellular distribution of β-oxidation, ALDH, or other detoxification systems may account for the response to peroxisome proliferators and, as yet, have not been explored. The purpose of this paper is to report the presence of a peroxisomal ALDH in the porcine hepatocyte and to report the relationship of peroxisomal β-oxidation to mitochondrial β-oxidation in the porcine liver.

MATERIALS AND METHODS

Animals

Liver sections were obtained from 6 to 8-month-old Yorkshire female and castrated male pigs immediately postmortem. Two pigs had ascarid scars in the liver. No significant difference ($P>.10$) existed between these livers and the other livers in enzyme profiles.

The central lobe from each liver was removed and perfused with an ice-cold homogenizing buffer through a central vein. The perfusion was continued until the liver was tan in color. The perfused tissue was scissor-minced and homogenized (10% wt/v) with 1 stroke of a motor-driven teflon Potter-Elvehjem tissue homogenizer at 100 RPM. The homogenization buffer consisted of 20 mM MOPS (Sigma Chemical Co.), pH 7.4, 0.25 M sucrose, and .10% ethanol. All operations were carried out on ice.

The homogenate was centrifuged for 10 min at 750 *x g* to obtain the post-nuclear supernatant. This supernatant was layered onto a nonlinear sucrose gradient, ranging in density (20°C) from 1.08 g/ml to 1.28 g/ml.

Gradients were prepared 24 hr before use. Centrifugation was carried out using a Beckman SW28Ti rotor at 105,000 *x g* (4°C) in a Beckman L-2 ultracentrifuge, for 3.5 hr.

β-Oxidation and catalase activities were assayed on gradient fractions immediately upon completion of the centrifugation run. All other assays were performed on gradient fractions stored at -20°C.

Enzyme Assays

Organelles were identified by using the marker enzymes catalase (EC 1.11.1.6) for peroxisomes, NAD:glutamate dehydrogenase (EC1.4.1.2) for mitochondria, and NADPH:cytochrome c reductase (EC 1.6.99.3) for endoplasmic reticulum (ER). Catalase (17) was assayed immediately after collection of the gradient fractions and its units correspond to mmoles H_2O_2 consumed/min. NAD:Glutamate dehydrogenase was assayed (18) as reported, with the following modifications. Fifty-five ng/ml antimycin A and 5 ng/ml rotenone were included in the assay buffer to inhibit NADH oxidase. Units correspond to nmoles NAD reduced/min. NADPH:Cytochrome c reductase was assayed as in (19). Units correspond to nmoles NADPH oxidized/min.

Mitochondrial β-oxidation was assayed (20) as palmityl-CoA-dependent oxygen consumption, using a Clark-type electrode at 35°C. The oxygen concentration of air-saturated water was taken to be 231 μM (21). The procedure was modified to include 100 μM ATP and 0.5 mM DL (+) carnitine in the assay. Units correspond to nmoles O_2 consumed/min.

Peroxisomal β-oxidation was measured (22) as μmoles oxygen consumed. The assay was modified to contain 22 ng/ml antimycin A instead of KCN. Units correspond to nmoles O_2 consumed/min.

High K_m and low K_m isozymes of ALDH (EC 1.2.1.5 and EC 1.2.1.3) were assayed as reported by Koivula and Koivusalo (23). The substrate concentration used for the high K_m isozyme was 5 mM acetaldehyde. The substrate concentration for the low K_m isozyme was 0.05 mM acetaldehyde. Units correspond to nmoles NAD reduced/min.

Protein was determined by the method of Lowry *et al.* (24).

Sucrose density was determined using a Bausch and Lomb refractometer maintained at 20°C.

Reaction rates are reported as measured for the gradients depicted in figure 1. Some data in table I were adjusted to per gram of liver in order to correct for differences in mitochondrial and peroxisomal fragility and to

make interspecies comparisons (25). For these adjustments, appropriate areas from the gradients were summed and adjusted to 100% recovery based on homogenate measurements.

RESULTS

Organelle Isolation

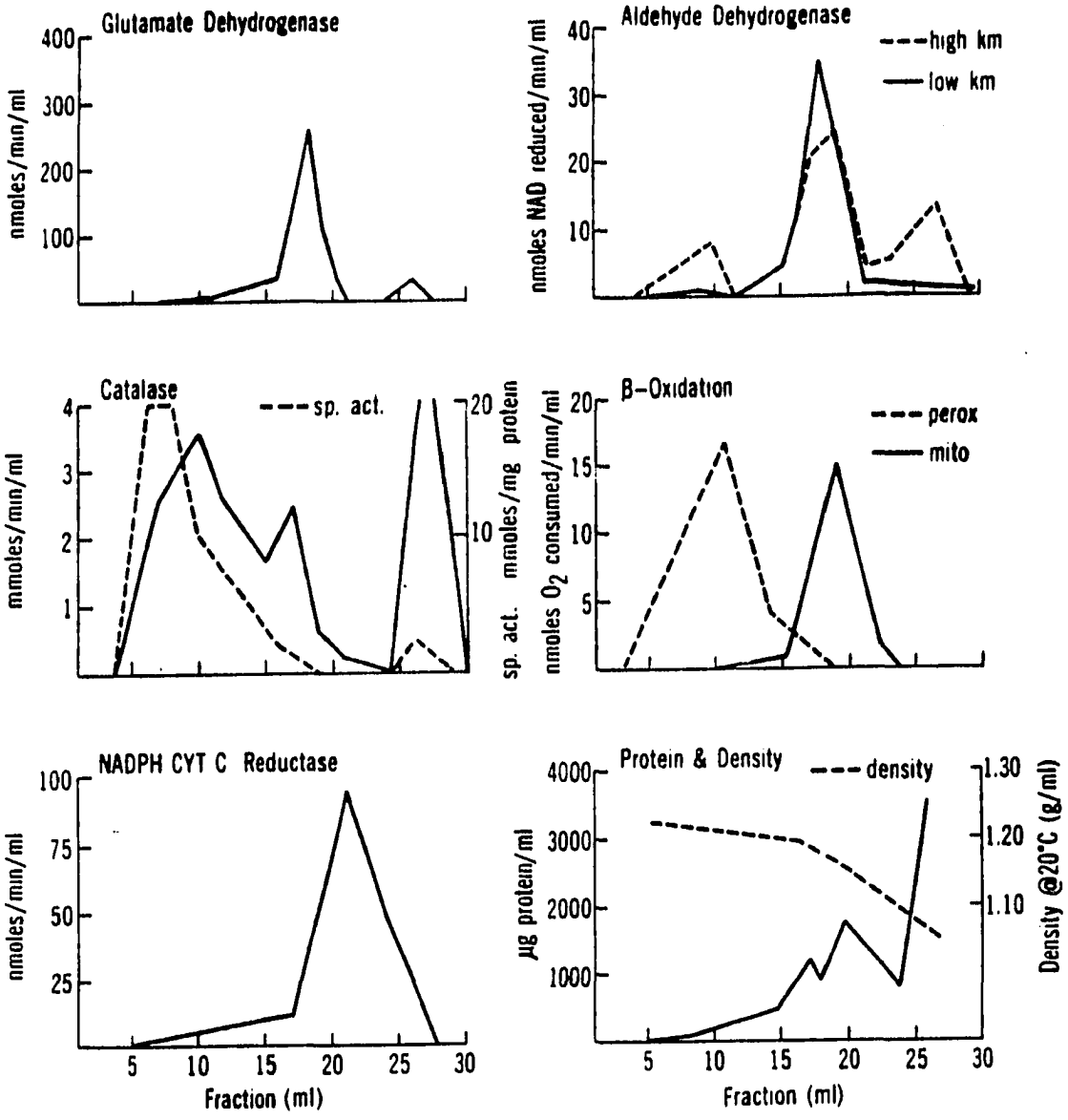
Figure 1 shows that particulate catalase banded with peak activity at a density of 1.22 g/ml. Catalase activity was spread throughout the higher-density region of the gradient and was not confined to a sharp peak. Twenty-eight percent of the catalase activity was present in the major band. On the average, supernatant catalase accounted for 57.5% of the total catalase activity. We did not ascertain how much of the supernatant catalase activity was due to broken peroxisomes. Several attempts were made to reduce the overlap of the marker peaks. Variation in buffer, pH, and gradient shape did not improve organelle resolution (data not shown). Fifty percent of the homogenate catalase activity was recovered from the gradient.

Peroxisomal β-oxidation activity corresponded with the high-density particulate catalase activity. Ninety-nine percent of the activity was isolated in this band. Fifty-two percent of the β-oxidation activity was recovered from the gradient.

NAD:Glutamate dehydrogenase sedimented in a sharp band, with maximal activity at a density of 1.18 g/ml. Seventy-nine percent of the NAD:glutamate dehydrogenase activity existed in this band. The catalase band contained 13.5% of the NAD:glutamate dehydrogenase activity. The presence of NAD:glutamate dehydrogenase activity in the supernatant (5.4%) undoubtedly corresponds to broken mitochondria. Total GDH activity on the gradient was 75% of the homogenate.

Mitochondrial β-oxidation corresponds with the activity of NAD:glutamate dehydrogenase. Mitochondrial β-oxidation sedimented to an equilibrium position, with peak activity at a density of 1.17 g/ml. A single band existed, with 94.7% of the activity present. No activity was detected in the cytosol. Forty percent of the homogenate activity was found on the gradient.

Figure 1. Distribution of enzymatic activity between peroxisomes, mitochondria, endoplasmic reticulum, and cytosol from a representative sucrose density gradient prepared from porcine liver. NAD:Glutamate dehydrogenase is a marker for mitochondria. Catalase is the marker for peroxisomes. NADPH:Cytochrome c reductase is the marker for the ER. Assay methods are described in Materials and Methods.


Glutamate Dehydrogenase
nmoles/min/ml
400
300
200
100
Aldehyde Dehydrogenase
nmoles NAD reduced/min/ml
40
30
20
10
high km
low km
Catalase
sp. act.
nmoles/min/ml
sp. act. nmoles/mg protein
4
3
2
1
20
10
β-Oxidation
nmoles O_2 consumed/min/ml
20
15
10
5
perox
mito
NADPH CYT C Reductase
nmoles/min/ml
100
75
50
25
5
10
15
20
25
30
Fraction (ml)
Protein & Density
density
μg protein/ml
4000
3000
2000
1000
1.30
1.20
1.10
Density @20°C (g/ml)
5
10
15
20
25
30
Fraction (ml)

NADPH:Cytochrome c reductase sedimented in a single large band to an equilibrium density of 1.14 g/ml. Fifty-four percent of the enzymatic activity occurred in this band. The NAD:glutamate dehydrogenase peak contained 28.9% of the NADPH:cytochrome c reductase activity, and 4.8% was present in the supernatant. Cytosolic activity probably was due to microsomal leakage during homogenization. Eighty percent of the enzymatic activity was recovered from the gradients.

No real differences in enzymatic activities and gradient distributions between castrated males and females were found (P>.10, data not shown). The castrated males were included in this study because of limited access to porcine liver tissue. Since no differences existed, all data were pooled in order to obtain better estimates of population means.

Aldehyde Dehydrogenase

Our results from pig liver demonstrate that the high K_m isozyme occurred in three major peaks corresponding to densities of the peroxisomal, mitochondrial, and cytosolic regions of the gradient (Figure 1). The majority of the high K_m ALDH activity occurred in the mitochondria (54.3%). Thirty-two percent of the activity existed in the peroxisomes, 8.3% in the ER, and 4.6% was found in the cytosol. We did not, however, find the high K_m isozyme in the microsomes as has been

reported previously (23). This may be due to overlap of the cytosol with the ER, or it may be the result of species differences. A large degree of variation was observed in the activity of the high K_m isozyme in the cytosol and ER among livers used in this study.

The low K_m isozyme of ALDH sedimented as a single peak with NAD:glutamate dehydrogenase and mitochondrial β-oxidation. Sixty-nine percent of the total activity of this isozyme occurred in the mitochondrial band. Peroxisomes contained 8.0%, and the ER possessed 8.8%. The presence of this enzyme in the peroxisomes and ER can be accounted for through mitochondrial contamination. On two occasions, however, a distinct peak of low K_m ALDH activity occurred in the peroxisomes, which could not entirely be accounted for through contamination of peroxisomes with mitochondria. Activity of the low K_m ALDH also was present in the cytosol (14.3%). Thirty-five percent and 30% of the activities of the high K_m and low K_m isozymes, respectively, were recovered from the gradients.

TABLE I

ACTIVITY[a,b,c] OF ENZYMES IN PORCINE HEPATOCYTE PEROXISOMES, MITOCHONDRIA, ENDOPLASMIC RETICULUM, AND CYTOSOL

ENZYME	PEROXISOME[b]	MITOCHRONDRIA[b]	ENDOPLASMIC[b] RETICULUM
Catalase	10±1	3±1	1±0
Glutamate Dehydrogenase	43±8	161±4	5±1
NADPH:Cytochrome c Reductase	19±5	29±5	40±10
Mitochondrial β-Oxidation	1±1	10±1	0
Peroxisomal β-Oxidation	36±6	1±1	0
Aldehyde Dehydrogenase (high K_m)	30±1	33±7	4±3
Aldehyde Dehydrogenase (low K_m)	4±4	23±3	2±2

a average of 6 livers
b units/mg protein
c mean enzymatic rates for porcine liver. Areas under the gradients were summed and adjusted to 100% of homogenate values

TABLE I (CONTINUED)
ACTIVITY[a,b,c] OF ENZYMES IN PORCINE HEPATOCYTE PEROXISOMES, MITOCHONDRIA, ENDOPLASMIC RETICULUM, AND CYTOSOL

ENZYME	CYTOSOL[b]	TOTAL LIVER[b]	MEAN LIVER RATE[c]
Catalase	12±0	6±2	190±85
Glutamate Dehydrogenase	8±4	35±14	1092±180
NADPH:Cytochrome c Reductase	4±5	40±10	4±5
Mitchondrial β-Oxidation	0	6±1	137±39
Peroxisomal β-Oxidation	0	6±1	142±37
Aldehyde	3±00	100±20	1818±88
Dehydrogenase (high K_m)			
Aldehyde Dehydrogenase (low K_m	4±4	35±16	733±67

Relationship Between Peroxisomal β-oxidation and Mitochondrial β-oxidation

β-oxidation was assayed as the rate of palmityl-CoA-dependent oxygen consumption. The area under the curves for palmityl-CoA-dependent oxygen consumption in the peroxisomes and mitochondria should indicate the relative capacities for each organelle to carry out β-oxidation (26). No palmityl-CoA-dependent oxygen consumption could be detected in the peak mitochondrial fraction by using the peroxisomal assay. Likewise, no palmityl-CoA-dependent oxygen uptake occurred by using the peak peroxisomal fraction in the mitochondrial assay.

The area for peroxisomal β-oxidation indicates an average capacity of 142 ±38 nmoles 0_2 consumed/min x g liver (Table I). The average capacity for the surviving mitochondria was 137 ±39 nmoles 0_2 consumed/min x g liver. Thus, the ratio of peroxisomal β-oxidation to mitochondrial β-oxidation is 1. This assumes that all the mitochondria and peroxisomes were respiring in the assay. We made no attempt to optimize the assay conditions nor to determine if any of the cytosolic catalase was peroxisomal in origin. Since only 5.4% of the NAD:glutamate dehydrogenase activity was in the supernatant, it seems likely that most of the mitochondria were intact. These results estimate only the maximal capacity for β-oxidation. The physiological rates occurring within the intact cell may be entirely different.

DISCUSSION

The presence of acetyltransferases in the peroxisome of porcine liver has been reported by Markwell and co-workers (27). Those authors indicated that the peroxisome may play a significant role in regulating carbon flow in the cell. The relationship between peroxisomal and mitochondrial β-oxidation was not reported, however, and the contribution of the peroxisome to carbon flow remained unknown. We have found that the porcine hepatocyte peroxisome is responsible for a significant amount of palmityl-CoA-dependent oxygen consumption. In the pig, the peroxisomal system may contribute significantly to the oxidation of palmitate.

The relative contribution of the peroxisome to lipid metabolism has been established for very few species. Data in Table II present reports of peroxisomal β-oxidation activity in various species. The rates range from 30 nmoles/min/g liver to 1766 nmoles/min/g liver. β-Oxidation in rats ranges from 280 to 1200 nmoles/min/g liver. Estimates of mouse β-oxidation range from 280 to 468 nmoles/min/g liver. The lowest estimates reported are in Rhesus monkeys and Syrian golden hamsters. Syrian golden hamsters also were reported to possess one of the highest activities per milligram of protein. The use of a light mitochondrial fraction instead

of liver homogenates may account for the anomalously high activities. Our findings for the porcine hepatocyte are similar to the estimates presented for humans.

Strain and sex differences may exist as well. A 2 -to 3-fold difference in peroxisomal β-oxidation activity between the lean C57BL/6J mouse and ICR mice has been reported (25). Differences in the ratio of mitochondrial to peroxisomal β-oxidation between males and females have been reported (20.25). It seems that the contribution of the peroxisome to cellular carbon flow may differ physiologically among different strains within a species, between sexes, and among species as well. Comparison of our data from swine to that reported for rodents supports the contention that, at the very least, species differences exist.

We have demonstrated the presence of an aldehyde dehydrogenase with a high K_m for acetaldehyde in the porcine hepatocyte peroxisome. The presence of ALDH with a high K_m toward acetaldehyde in the cytoplasm, mitochondria, microsomes, and the peroxisome of the rat has been reported previously (23,28,29). We have found two isozymes of ALDH to be present in porcine liver. The distribution of both isozymes of ALDH in porcine hepatocytes is similar to what has been reported in the rat except for our inability to discern a distinct peak of the high K_m

isozyme in the ER. Total enzymatic activity seems to be very similar also. Total rat liver low K_m ALDH activity ranges from 2.1 to 7.6 nmoles NAD reduced/min/mg protein. Total enzymatic activity for the high K_m isozyme varies from 3.3 to 34.1 nmoles NAD reduced/min/mg protein. Peroxisomal high K_m activity has been reported to be 34.5 nmoles/min/mg protein (16,30). These results are similar to what we report in porcine liver (Table I).

The function of ALDH in the peroxisome may be detoxification. ALDH converts aldehydes into more water-soluble carboxylic acids, which could contribute to shorter half-lives of reactive aldehydes in the cell. Aldehydes, epoxides, and other lipid oxidation products may contribute to the development of the hepatocellular carcinomas (13). Species differences in the response of ALDH to peroxisome proliferation, as well as in the abiity ofALDH to clear aldehydes, may exist. Interspecies variation in aldehyde metabolism and peroxisomal/mitochondrial β-oxidation ratios may explain the variation in susceptibilities to develop hepatocellular carcinomas during long-term treatment with peroxisome proliferators. ALDH has been reported to be inducible in the rat during treatment with peroxisome proliferators and may occur in response to the increased oxidizing conditions caused through peroxisomally produced H_20_2. ALDH may also contribute reducing equivalents and carboxylic

acids for β-oxidation. Correlation of peroxisomal ALDH activity to peroxisomal β-oxidation rates and catalase activity may lend insight into the function of these enzymes in the organelle.

TABLE II
RELATIONSHIP OF HEPATOCYTE PEROXISOMAL B-OXIDATION[a] AMONG SPECIES

SPECIES	β-OXIDATION RATE	SP. ACT[b]	REFERENCE
Rat[c](Fischer F344, Female)	800		11
Rat[c]	810±50		31
Rat[d]	280		20
Rat[c](male Wistar)	585±178		32
Rat[c](6 wk old male Wistar)	660120	5.800±1.100	33
Rat[c](27 wk old male Wistar)	430±111	4.040±1.240	33
Rat[c](male Wistar)	380±110		34
Rat[c](male Wistar)	840±41	1.44±0.07	35
Rat[e](male F344)	1200±0300		36
Syrian Golden Hamster[cf](male)	106.7±15.4		9
Syrian Golden Hamster[d](male)	30±10		37
Mouse[c](lean)	263±143	9.3	25
Mouse[c](obese)	468±59		25
Mouse[c](DDY Strain)	1766±307		32
Pigeon[c]	380±130		7
Chicken[c]	300±20		7
Cat[c]	450±10		7
Cynomolgus Monkey[c]	570±120		7
Rhesus Monkey	140±30		7
Human Biopsy[c]	240±60		31

[a] Rates are reported for non-drug treated control groups.
[b] Reported as nmoles NAD reduced/min/mg protein.
[c] Rate reported as nmoles NAD reduced/min/g liver.
[d] Rate reported as nmoles O_2 consumed/min/g liver estimated from figure.
[e] Rate reported as soluble ^{14}C acyl CoA released/mim/g liver.
[f] NADH was estimated using rezazurin and NADH oxioreductase. Rates Were determined in an enriched peroxisomal fraction.

REFERENCES

1. Masters, C. and R. Holmes. 1977. Physiol. Rev. 57(4):816.

2. Masters, C. and D. Crane. 1985. Mol. Cell. Biochem. 65:23.

3. deDuve, C. 1983. Sci. Am. 248(5):74-84.

4. Tolbert, N.E. 1981. Annu. Rev. Biochem. 50:133-175.

5. Kawashima, Y., H. Katoh, H. Watanibe, M. Takegihi, and H. Kozuka. 1985. Biochem. Pharmacol. 34(3):325.

6. Reddy, J.K., J.R. Warren, M.K. Reddy, and N.D. Lalwani. 1982. Ann. N.Y. Acad. Sci. 386:45.

7. Reddy, J.K., N.D. Lalwani, S.A. Qureshi, M.K. Reddy, and C.M. Moehle. 1984. Am. J. Pathol. 114:171.

8. Reddy, J.K., D.L. Arzarnoff, D.J. Svoboda, and J.D. Prasad. 1974. J. Cell Biol. 61:344.

9. Pourbaix, S., F. Heller, and C. Harvengt. 1984. Biochem. Pharmacol. 33(22):3661.

10. Sakurai. T., S. Miyazawa, T. Osumi, S. Furuta, and T. Hashimoto. 1981. Toxicol. Appl. Pharmacol. 59:8.

11. Lazarow, P.B., H. Shio, and M.A. Leroy-Houyet. 1982. J. Lipid Res. 23:317.

12. Moody, D.E. and J.K. Reddy. 1978. Toxicol. Appl. Pharmacol. 45:497.

13. Reddy, J.K., D.L. Azarnoff, and C.E. Hignite. 1980. Nature 283:397.

14. Cohen, A.J. and P. Grasso. 1981. Food Cosmet. Toxicol. 19:585.

15. Lalwani, N.D., M.K. Reddy, S. Ghosh, S.D. Barnard, J.A. Molello, and J.K. Reddy. 1985. Biochem. Pharmacol. 34(19):3473.

16. Antonenkov, V.D., S.V. Pirozhkov, and L.F. Panchenko. 1985. Eur. J. Biochem. 149:159.

17. Tolbert, N.E. 1974. Meth. Enzymol. 31(pt.A):734.

18. Leighton, F., B. Poole, H. Beaufay, H. Baudhuin, J. Coffe, S. Fouler, and C. Deduve. 1968. J. Cell Biol. 37:482.

19. Masters, B.S., C.H. Williams, and H. Kamen. 1967. Meth. Enzymol. 10:565.

20. Krahling, J.B., R. Gee, J.A. Gauger, and N.E. Tolbert. 1979. J. Cell. Physiol. 101:375.

21. Battino, R. and H.L. Cliver. 1966. Chem. Rev. 66:395.

22. Lazarow, P. and C. deDuve. 1976. Proc. Natl. Acad. Sci. U.S.A. 73:2043.

23. Koivula, T. and M. Koivusalo. 1975. Biochim. Biophys. Acta 397:9.

24. Lowry, O.H., N.J. Rosebough, A.L. Farr, and R.J. Randall. 1951. J. Biol. Chem. 193:265.

25. Murphy, P.A., J.B. Krahling, R. Gee, J.R. Kirk, and N.E. Tolbert. 1979. Arch. Biochem. Biophys. 193(1):179.

26. Krahling, J.B., R. Gee, P.A. Murphy, J.R. Kirk, and N.E. Tolbert. 1978. Biochem. Biophys. Res. Commun. 82(1):136.

27. Markwell, M.K., E.J. McGroarty, L.L. Bieber, N.E. Tolbert. 1973. J. Biol. Chem. 218(10):3126.

28. Siew, C., R.A. Deitrich, and V.G. Erwin. 1976. Arch. Biochem. Biophys. 176:683.

29. Nakayasu, H., K. Mihara, R. Sato. 1978. Biochem. Biophys. Res. Commun. 83(2).

30. Panchenko, L.F., S.V. Pirozhkov, and V.D. Antonenkov. 1982. Bull. Exp. Biol. Med. 94:1223.

31. Bronfman, M., N.C. Inestrosa, and F. Leighton. 1979. Biochem. Biophys. Res. Commun. 88(3):1030.

32. Horie, S. and T. Suga. 1985. Biochem. Pharmacol. 34(9):1357.

33. Kawashima, H., H. Katoh, H. Watanuki, M. Takegishi, and H. Kozuka. 1985. Biochem. Pharmacol. 34(3):325.

34. Ishii, H. and T. Suga. 1979. Biochem. Pharmacol. 28:2829.

35. Bone, A.J., H.S.A. Sherratt, D.M. Thurnball, and H. Osmundsen. 1982. Biochem. Biophys. Res. Commun. 104(2):708.

36. Reddy, J.K., N.D. Lalwani, A.S. Dabholkar, M.K. Reddy, and S.A. Qureshi. 1981. Biochem. Int. 3(1):41.

37. Reddy, M.K., N.D. Lalwani, S.A. Qureshi, and J.K. Reddy. 1982. Human Toxicol. 1:135.

PART II.

EFFECTS OF CLOFIBRIC ACID ON ISOLATED PORCINE HEPATOCYTES: A NON-RODENT MODEL FOR THE STUDY OF PEROXISOMAL METABOLISM

ABSTRACT

The effects of clofibric acid on primary porcine hepatocytes from 6-month-old female Yorkshire hogs were investigated. Isolated hepatocytes were exposed to clofibric acid concentrations ranging from 0 to 3.0 mM. Catalase, NAD:glutamate dehydrogenase, mitochondrial β-oxidation, and peroxisomal β-oxidation were not affected in the range of drug concentrations used in this study. Drug treatments resulted in a 40% increase in cellular protein, a 900% increase in NADPH:cytochrome c reductase, a 600% increase in high K_m and low K_m isozymes of aldehyde dehydrogenase (ALDH), and a 40% increase in superoxide dismutase (SOD) activity. Regression analysis of enzyme activity and protein content onto drug concentration was significantly quadratic for all increases. The response of the low K_m isozyme of ALDH had a threshold at 0.37 mM clofibric acid. Separation of organelles by isopynic sucrose-gradient ultracentrifugation resulted in a distinct peak of SOD activity in the peroxisomal region of the gradients. Peak SOD activity corresponds to the peroxisomal markers and was found to be approximately 70 standard SOD units/ml gradient. Contamination of peroxisomes with mitochondria did not account for the presence of SOD in the isolated peroxisome. Interspecies differences in metabolism are discussed.

INTRODUCTION

Peroxisome metabolism and proliferation have been extensively studied in rodents. Although data exist on the distribution, composition, and response to environmental stimuli of the organelle in rats and mice, little information can be found for other mammalian species. In addition, the enzymatic profile of the organelle within a species can vary, depending on the tissue examined and substrate availability (1,2,3). It is evident that treatment with peroxisome-modulating drugs may not have the same effects in all species nor all tissues (4,5). Thus, interspecies variation in peroxisomal metabolism seems likely.

The void in interspecies metabolism data is of concern. Treatment of rodents with several structurally diverse xenobiotics results in the proliferation of peroxisomes and the induction of selected peroxisomal and non-peroxisomal enzymes (4). Long-term treatment of rodents with these agents results in the development of hepatocellular carcinomas and has prompted the theory that peroxisome proliferators, as a class, are carcinogenic (6). Structurally diverse agents such as di-(ethyhexyl) phthalate, phenoxyacid herbicides, acetylsalicyclic acid, valproic acid, and various hypolipidemic drugs are peroxisome proliferators (7-11). Some of these chemicals are widespread contaminants of the environment (12).

The lack of interspecies metabolism data, coupled with the observation of proliferation in some species, absence in others, or occurrence only at unacceptably high dosages, raises questions concerning the application of rodent data to the risk assessment of peroxisome proliferators (4,11,13,14).

One must question whether the contribution of the organelle to metabolic homeostatis differs among species. In rodents, various nutritionally-related states alter peroxisomal metabolism. States such as prolonged fasting, high-fat diets, and vitamin E deficiency result in peroxisome proliferation (15-17). Little is known about peroxisomal metabolism other than in rodents. We previously demonstrated that the capacity of the peroxisome to contribute to cellular β-oxidation is much greater in the pig than in the rat. The maximum capacity to carry out β-oxidation in the peroxisome is approximately 5 times lower for porcine liver than the rat. Rates which we obtained from porcine liver are more similar to human liver (18). What seems clear is that detailed data on peroxisomal metabolism in species other than the rat and mouse are needed. These data will help in unraveling the metabolic role of the organelle in the cell.

We have undertaken this study to evaluate the use of porcine cell culture in the study of peroxisomal metabolism. Porcine liver is a

convienent source of tissue to researchers and possibly a better model for human metabolism than is rodent liver. Porcine tissue can be collected from local slaughter operations and cultured *in vitro*. The *in vitro* model can be used to study the effects of xenobiotics on peroxisomal metabolism. We have attempted to determine the sensitivity of β-oxidation to peroxisome proliferators for porcine hepatocytes grown in culture. We are also interested in ascertaining what differences exist, if any, in selected detoxification pathways associated with the peroxisome. We have chosen clofibric acid since clofibrate is hydrolyzed to clofibric acid by esterases in the digestive tract and the blood.

MATERIALS AND METHODS

Tissue Dissociation

Porcine liver was obtained from 6-month-old female Yorkshire hogs. Livers were removed within 30 minutes post-mortem as soon as the body cavity was opened. Livers were handled aseptically. The central lobes of the livers were dissected free and placed in a sterile hood for further processing.

The tissue was dissociated by using a three-step procedure similar to that described by Seglen (19). Livers were first perfused for 4 minutes with a calcium magnesium-free balanced salt solution (19). The medium was modified to contain 0.5 mM EGTA and 1 g/L D-glucose. Perfusion was carried out using a large central vein. The tissue was then perfused with approximately 100 ml of the suspension solution (BSS), described by Seglen, to remove remaining EGTA from the tissue. This was followed by perfusing with a digestion buffer for 25 minutes. The digestion buffer of Seglen was modified to contain 0.12% collagenase (type I, Sigma Chemical Co.), 125 mg/L taurine, 910 mg/L mannitol, and 1 g/L D-glucose. The buffers were maintained at 37°C throughout the perfusion.

Perfusion was conducted by using a peristaltic pump maintained at a rate that kept the liver tissue firm. Liver effluent was collected, filtered through cheesecloth, gassed with oxygen, and reperfused by using a sterile apparatus.

Upon completion of the perfusion, the tissue was scissor-minced and placed in 100 ml of Hams F-12 medium (Sigma Chemical Co.) with 0.10% collagenase for an additional 10 minutes. Gentle shaking was applied to dislodge the cells. The resulting cell suspension was placed on ice and filtered through cheesecloth to remove tissue debris and cell clumps. The cell suspension was then centrifuged at 50 *x g* for 2 minutes, resuspended in BSS and pelleted as above. The pellet was washed once more with Hams F-12 medium containing 25% fetal calf serum suspended in the equilibration medium, counted, and plated to a density of 2.5 x 10^6 viable cells/ dish (60 x 15 mm Falcon cell culture dish). Viability was determined by trypan-blue dye exclusion.

Culture Conditions.

Cells were equilibrated in a 95%/5% oxygen/carbon dioxide atmosphere at 37°C. Equilibration medium consisted of Hams F-12, pH 7.5, supplemented with 25% fetal calf serum, 125 mg/L taurine, 8.3% brain heart infusion (Difco Laboratories), 910 mg/L mannitol, 50 mg/L L-

ascorbic acid, 460 mg/L Na pyruvate, 10^{-4} M hydrocortisone, 10^{-6} M insulin, and 122 mg/ L additional L-glutamine. The cells were equilibrated in the incubator for 4 hours post-isolation whereupon the medium and dead cells were removed. Treatments were initiated at this time in a medium similar to that described above, but containing only 10% fetal calf serum, pH 7.5. Media and solutions were sterilized by filtration through a 0.2 micron filter. All solutions contained 60 units/ml penicillin base, 100 mcg/ml streptomycin sulfate, and 0.25 mcg/ml Fungizone (Gibco Laboratories).

Drug Treatments

Treatments consisted of a no-drug control, 0.19 mM, 0.37 mM, 0.75 mM, 1.5 mM, and 3.0 mM clofibric acid (Na salt). Clofibric acid was dissolved in distilled water and added to the medium immediately prior to beginning the treatments. The experiment was replicated 5 times. Cells were exposed to the drug for 72 hours. Medium was changed at 24 hour intervals. Lactate dehydrogenase release into the medium was monitored over the duration of 2 of the replications. At the end of the treatments, the plates were washed 2 times with ice-cold BSS. The cells were detached from the plates by using a rubber policeman and centrifuged at 50 *x g* for 2 minutes. Cells were resuspended in a homogenizing buffer

consisting of 20 mM MOPS, pH 7.4, 0.25 M sucrose, and 0.10% ethanol. Cells were homogenized with a motor-driven teflon Potter-Elvehjem homogenizer. The resulting homogenate was centrifuged at 750 *x g* for 10 minutes to remove debris. All operations were carried out on ice. Catalase and β-oxidation activities were determined immediately after isolation. The remaining homogenate was stored at -20°C until needed.

Statistical analysis was conducted using the General Linear Models procedure of SAS (20). T-tests were used where appropriate.

Enzyme Assays

Catalase (EC 1.11.1.6) activity was assayed immediately after homogenization as in (21). Units correspond to μmoles H_2O_2 consumed/minute. NAD:Glutamate dehydrogenase (EC 1.4.1.2) activity was assayed (22) with 55 ng/ml antimycin A and 5 ng/ml rotenone included in the buffer to inhibit NADH oxidase. Units correspond to mmoles NAD reduced/min. NADPH:Cytochrome c reductase (EC 1.6.99.3) activity was assayed as in (23). Units correspond to nmoles cytochrome c reduced/min. Lactate dehydrogenase (LDH; EC 1.1.1.27) activity was assayed (24) in 80 mM Tris-HCL, final concentration. Medium was dialiyzed overnight at 4°C against the assay buffer before

use. Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed as in (25). SOD units are reported as amount of protein, in mg, to inhibit cytochrome c reduction by 50%. The high K_m and low K_m isozymes of aldehyde dehydrogenase (ALDH; EC 1.2.1.5 and EC 1.2.1.3) were assayed as reported by Koivula and Koivusalo (26). The assay buffer was modified to contain 0.1% bovine serum albumin and 0.1% Triton X-100. Assays were monitored over a 48-hour period against a blank at 4°C. The blank reaction was similar to the above but contained no aldehyde. The substrate concentration for the high K_m isozyme was 5.0 mM acetaldehyde and 0.05 mM acetaldehyde for the low K_m isozyme. Units correspond to nmoles NAD reduced/min.

Mitochondrial β-oxidation activity was assayed (27) as palmityl-CoA-dependent oxygen consumption by using a Clark-type electrode at 35°C. The oxygen concentration of air-saturated water was taken to be 231 μM (28). The procedure was modified to include 100 μM ATP, and 0.5 mM DL-(+)-carnitine. Units correspond to nmoles O_2 consumed/ min. Peroxisomal β-oxidation (28) activity was recorded as μmoles oxygen consumed using a Clark-type electrode as above. The assay mixture was modified to contain 22 ng/ml antimycin A instead of KCN. Units correspond to nmoles O_2 consumed/min.

Ornithine transcarbamylase (EC 2.1.3.3) activity was assayed as reported (30).

Protein concentration was determined using the method of Lowry *et al.* (31).

Isopynic Sucrose Density Centrifugation

Intact liver tissue from 3 Yorkshire female pigs, age 6-months, was taken as described in tissue preparation. The tissue was immediately perfused with ice-cold homogenizing buffer which consisted of 20 mM MOPS pH 7.2, 0.25 M sucrose, and 0.10% ethanol . The tissue was scissor-minced, diluted 1:10 (W/V) with the homogenization buffer, and homogenized with 1-stroke of a motor-driven teflon Potter-Elvehjem homogenizer at 100 RPM. The homogenate was then filtered through cheesecloth and centrifuged at 750 *x g* at 4°C in order to remove nuclei and cell debris. The post-nuclear supernatant was layered onto a nonlinear sucrose gradient, ranging in density (20°C) from 1.08 to 1.28 g/ml. Gradients were prepared 24 hr. before use. Centrifugation was carried out at 105,000 *x g* for 3.5 hours using a Beckman Ti28 rotor and a Beckman L-2 ultracentrifuge. At the completion of the run, the gradients were collected in 1-ml fractions and assayed as described.

TABLE I

Comparison of Enzyme Parameters[a] between Intact Tissue and Isolated Hepatocytes

ENZYME	INTACT TISSUE	ISOLATED HEPATOCYTES
Catalase	6±2 mmole	615±29 μmole
Glutamate dehydrogenase	35±14 nmole	31±3 nmole
Mitochondrial β-oxidation	6±1 nmole	5±2 nmole
Peroxisomal β-oxidation	6±2 nmole	8±1 nmole
Aldehyde dehydrogenase (high K_m)	101±20 nmole	74±8 nmole
Aldehyde dehydrogenase (low K_m)	35±16 nmole	59±9 nmole
NADPH:cytochrome c reductase	34±6 nmole	10±1 pmole
Superoxide dismutase	70±5 unit	7±2 units
Ornithine transcarbamylase	N.A.	53 μmole

a Activities are expressed per mg protein/min. Assay methods are described in methods section.

RESULTS

Viability and Culture Characterization

Cell viability was determined immediately post-isolation by trypan-blue dye exclusion. Viability routinely ranged between 80-97%. Any amount of dye uptake was taken to be a sign of cell death. Cells attached to the plates within four hours after isolation. Morphologically, cells were large, appeared pleomorphic, and were epithelial in nature. Distinct cell membranes and nuclei could be seen. Cytoplasm tended to appear slightly granular. A number of binucleated cells were randomly distributed throughout the cultures.

Lactate dehydrogenase (LDH) release into the medium was monitored over the 72-hour culture period for 2 of the replicates (data not shown). After the first medium change, LDH activity in the medium decreased to low levels (180 nmoles NAD reduced/min/ml medium) and remained constant throughout the culture period. Drug concentration did not affect LDH release into the medium. Under standard culture conditions, cell division did not occur although random colonies could occasionally be observed. Cell division could be stimulated with 25% fetal calf serum and 20 ng/L liver cell growth-factor (glycyl-L-histidyl-L-lysine) (32). After 72

hours, random colonies of 40-50 cells could be seen. Cultures never grew to confluency, however. Ornithine transcarbamylase activity was high (Table 1). These data indicate that, although the cultures were heterogeneous, most of the cells were parenchymal and viable.

Comparison of Intact Liver and Isolated Hepatocytes

Activities of selected enzymes were determined in intact porcine liver and isolated cells not exposed to clofibric acid (Table 1). Data are expressed per milligram of protein. Total catalase activity was less in the isolated cells. This result is somewhat perplexing. Catalase activity may be decreasing as a result of culture aging, or it may have been overestimated initially due to residual red blood cell catalase. NAD:Glutamate dehydrogenase activity, the mitochondrial marker enzyme, was similar for both the intact tissue and isolated cells. β-Oxidation for both the peroxisomal and mitochondrial pathways were similar both *in vivo* and *in vitro*. Aldehyde dehydrogenase rates were also similar between the intact tissue and isolated cells. NADPH:Cytochrome c reductase activity decreased in the isolated cells, as did the SOD activity.

Effects of Clofibric Acid on the Enzymatic Activity in Isolated Hepatocyte Organelles

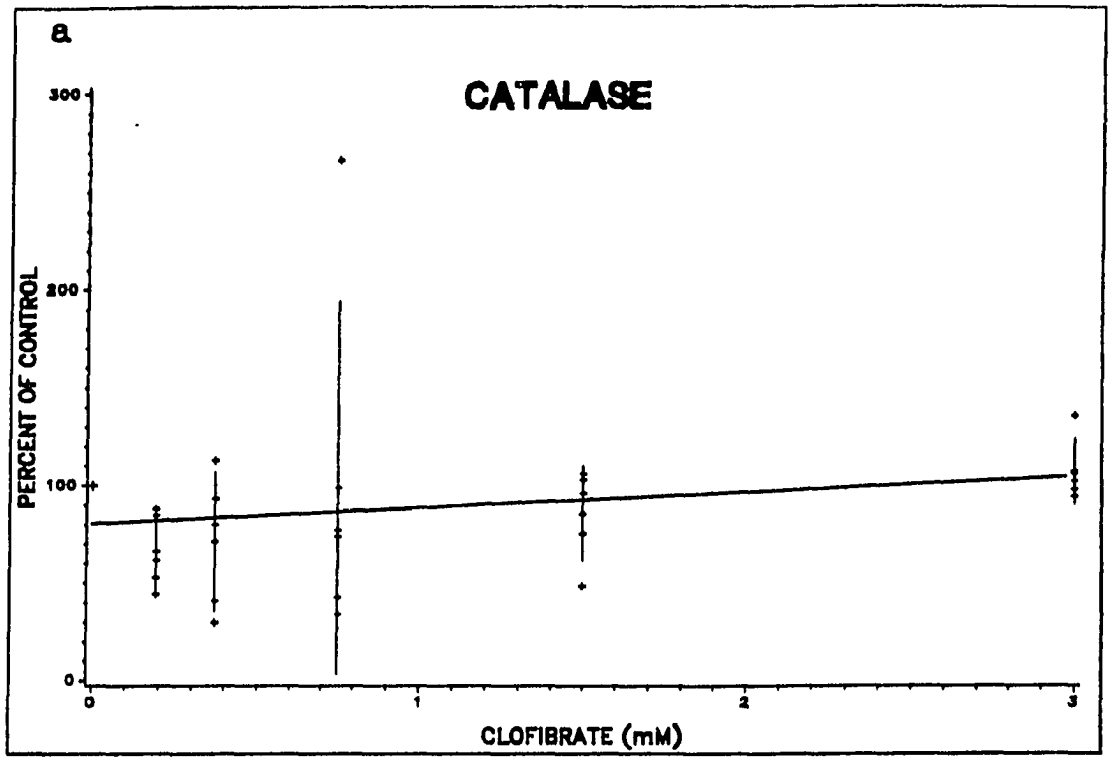
Isolated hepatocytes were exposed to clofibric acid concentrations ranging from 0 to 3.0 mM. Concentrations were selected to represent those used in studies utilizing isolated rodent hepatocytes (33). Rates are expressed as % of control rates/10^6 cells.

Figure 1a portrays the response of catalase to treatment with clofibric acid. Catalase activity was not affected by any of the treatments. Treatment means did not differ significantly ($P>.52$) from the control and the slope of the regression was not significantly different from zero ($P>.75$).

NAD:Glutamate dehydrogenase activity was not affected either (Fig. 1b). No significant ($P>.64$) differences existed amoung treatments and the slope of the best-fit regression was not different from zero ($P>.76$). A slight decreasing trend is visible. This was caused by high GDH activity at the low drug dosages in one replicate, possibly a result of contamination.

Clofibric acid did effect changes in total cell protein, however (Fig. 1c). Drug treatment resulted in a 40% increase in cellular protein.

Figure 1. Effect of clofibric acid. All rates expressed as % of control rate/10^6 cells. Assay methods described in materials and method section. (a) Catalase activity. Mean control rate =35.41±6.95 μmoles/min/10^6 cells. (b) NAD:Glutamate dehydrogenase activity. Mean control rate =16±4 nmoles/min/10^6 cells.


a
CATALASE
PERCENT OF CONTROL
300
200
100
0
0
1
2
3
CLOFIBRATE (mM)

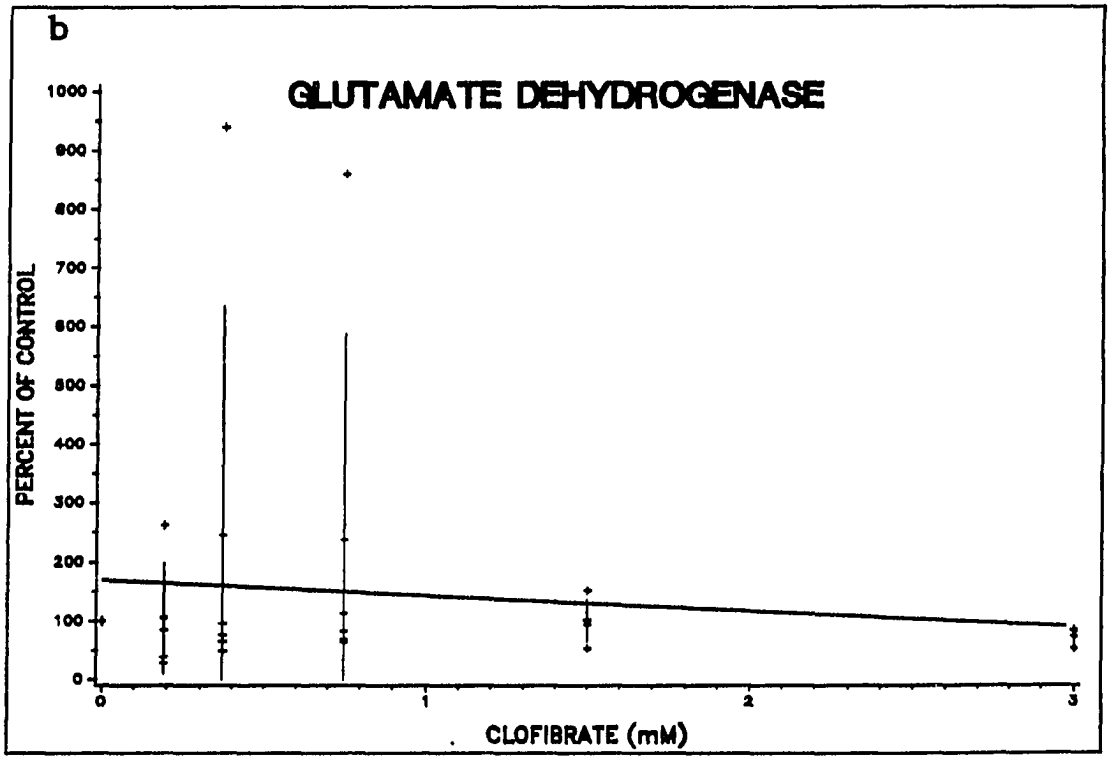
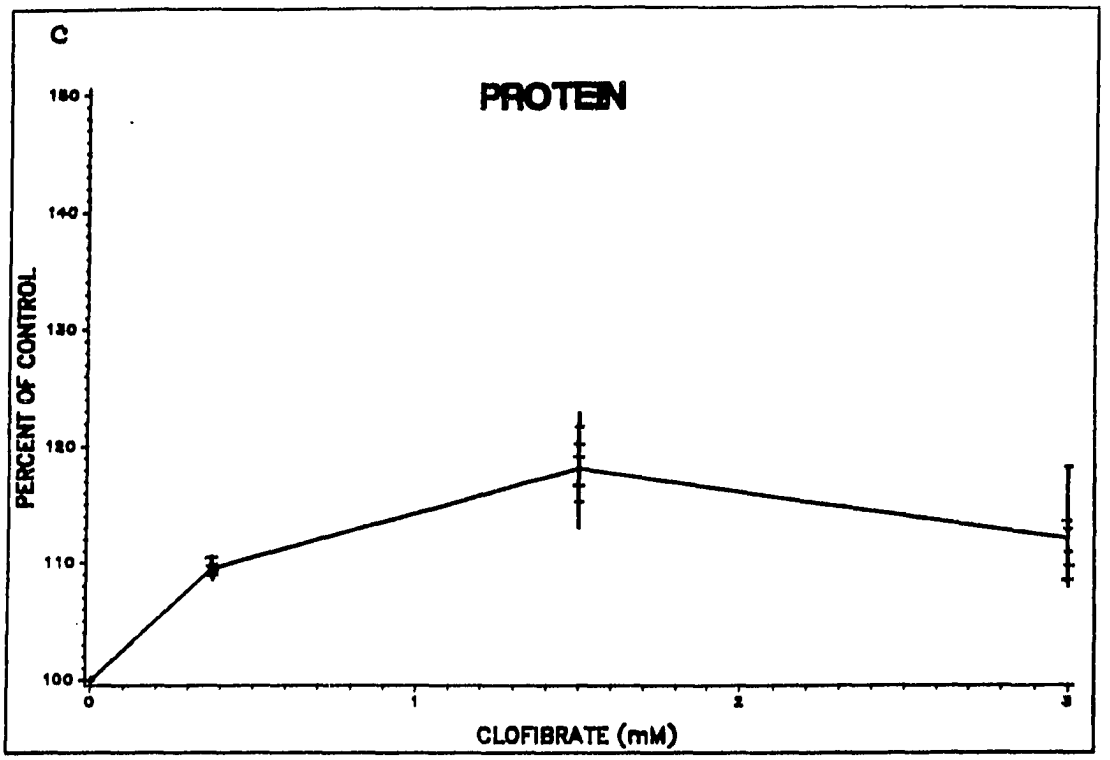
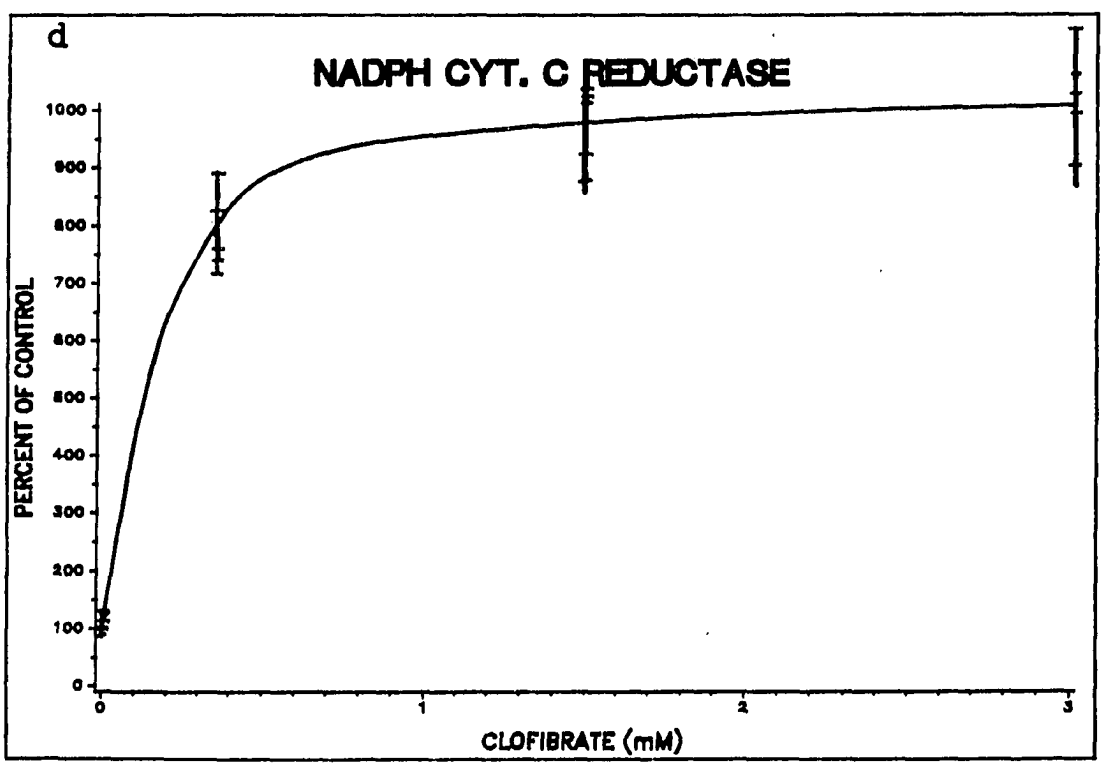

b
GLUTAMATE DEHYDROGENASE
PERCENT OF CONTROL
1000
900
800
700
600
500
400
300
200
100
0
0
1
2
3
CLOFIBRATE (mM)

Figure 1 (continued). (c) Protein content. Mean control rate = 485±239 μg protein/10^6 cells. (d) NADPH:cytochrome c reductase. Mean control value = 7±4 nmoles cytochrome c reduced/min/10^6 cells. Assay methods are described under Materials and Methods.


c
PROTEIN
PERCENT OF CONTROL
160
140
130
120
110
100
0
1
2
3
CLOFIBRATE (mM)

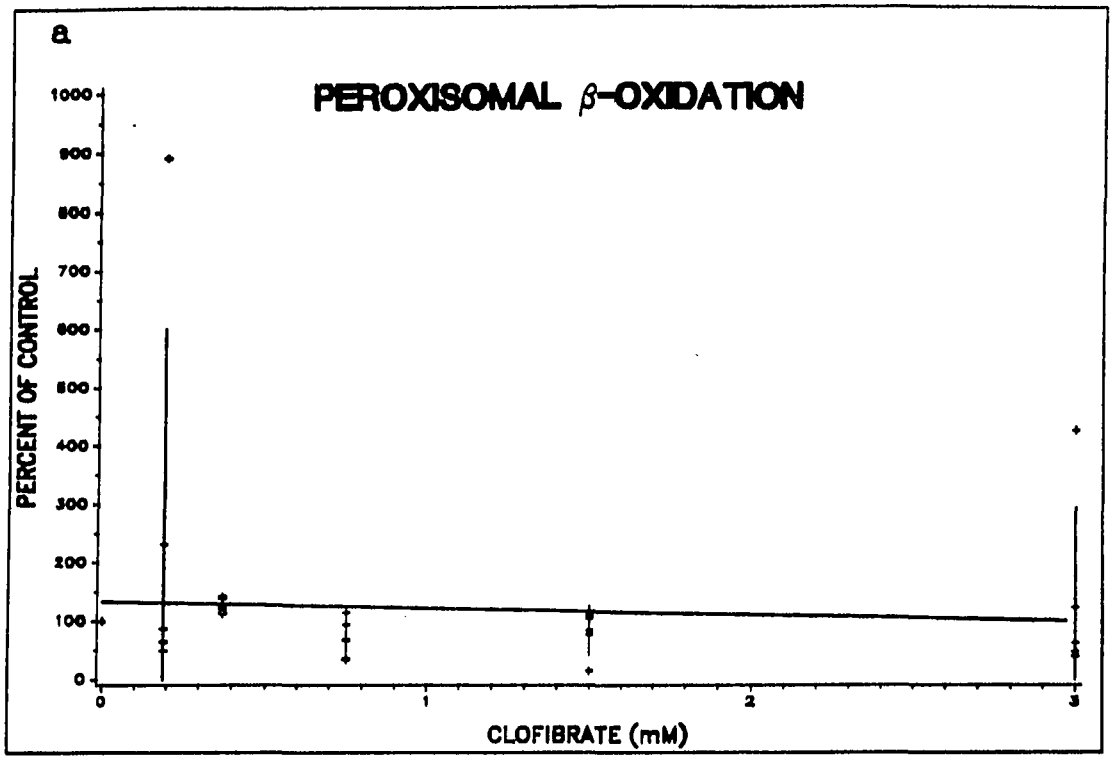

d
NADPH CYT. C REDUCTASE
PERCENT OF CONTROL
1000
900
800
700
600
500
400
300
200
100
0
0
1
2
3
CLOFIBRATE (mM)

A significantly quadratic (P>.01) response existed that appeared linear to 0.37 mM. Maximal protein content occurred at the 0.75 mM level and remained constant at the higher dosages.

Most of the increase in protein was probably due to induction of the microsomes as depicted in Figure 1d. NADPH:Cytochrome c reductase activity, a marker for microsomes, was dramatically induced by clofibric acid treatment. The response was significantly (P>.01) quadratic with an increase of 900% over that of the control. A plot of the rates expressed per mg protein is similar to the rates expressed per 10^6 cells indicating the activity is not being diluted by induction of other proteins in the cell (data not shown).

The response of peroxisomal and mitochondrial β-oxidation to clofibric acid will indicate the sensitivity of the pathways to clofibric acid. These responses are shown in Figures 2a and 2b, respectively. Mitochondrial β-oxidation is not affected in the range of drug concentrations used in this study. No significant (P>.66) induction occurred. The best-fit regression was linear but the slope was not different from zero (P>.26). A slight increasing trend can be seen which is probably due to anomalous behavior by one replicate. Likewise, peroxisomal β-oxidation activity is not affected by clofibric acid as no significant differences (P>.72) were found amoung the treatments.

Figure 2. (a) Peroxisomal β-oxidation activity and (b) mitochondrial β-oxidation as influenced by clofibric acid treatment for 5 representative replicates. Content expressed as % of control value/min/10^6 cells. Mean control value = 7±6 nmoles O_2 consumed/min/10^6 cells for peroxisomal activity and 5±1 nmoles O_2 consumed/min/10^6 cells for mitochondrial activity. Assay method described in Materials and Methods section.


a
PEROXISOMAL β-OXIDATION
PERCENT OF CONTROL
1000
900
800
700
600
500
400
300
200
100
0
0
1
2
3
CLOFIBRATE (mM)

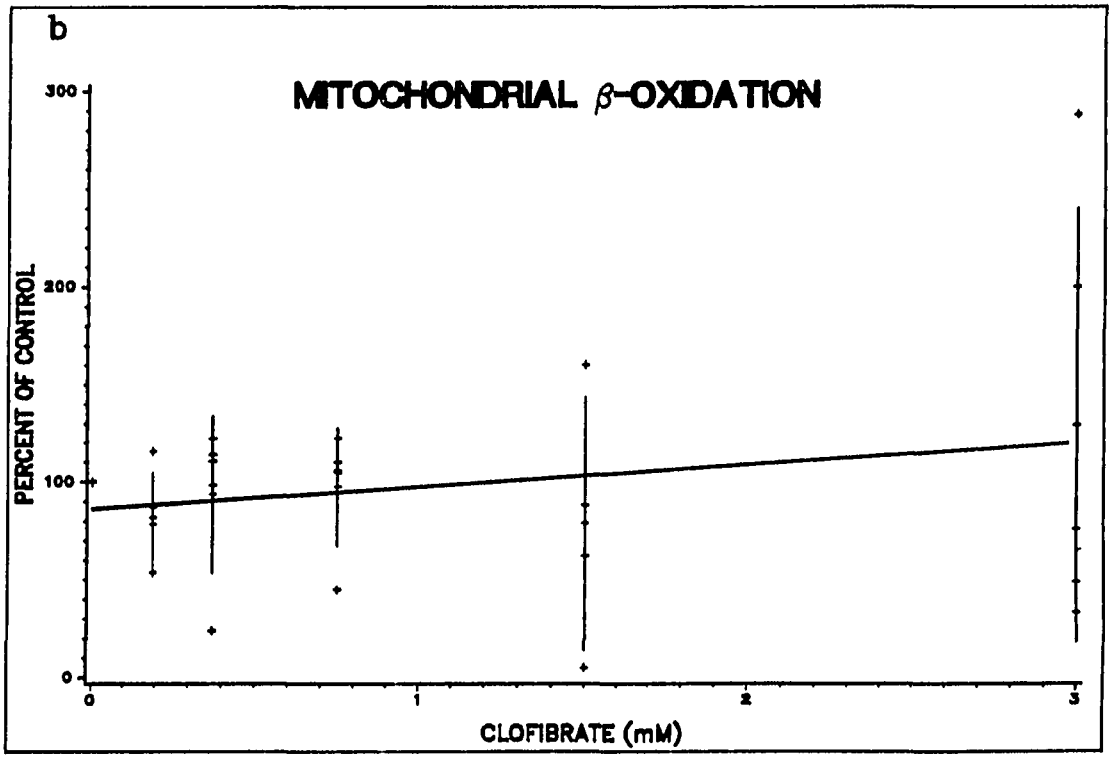

b
MITOCHONDRIAL β-OXIDATION
PERCENT OF CONTROL
300
200
100
0
0
1
2
3
CLOFIBRATE (mM)

Figure 3. Distribution of superoxide disutase activity through a sucrose gradient for a representative replication. Organelle location, identified through enzyme markers, are: (a) catalase for peroxisomes, (b) glutamate dehydrogenase for mitochrondria, (c) mitochondrial β-oxidation, and (d) NADPH:cytochrome c reductase for microsomes. Assay methods described in Materials and Methods section.

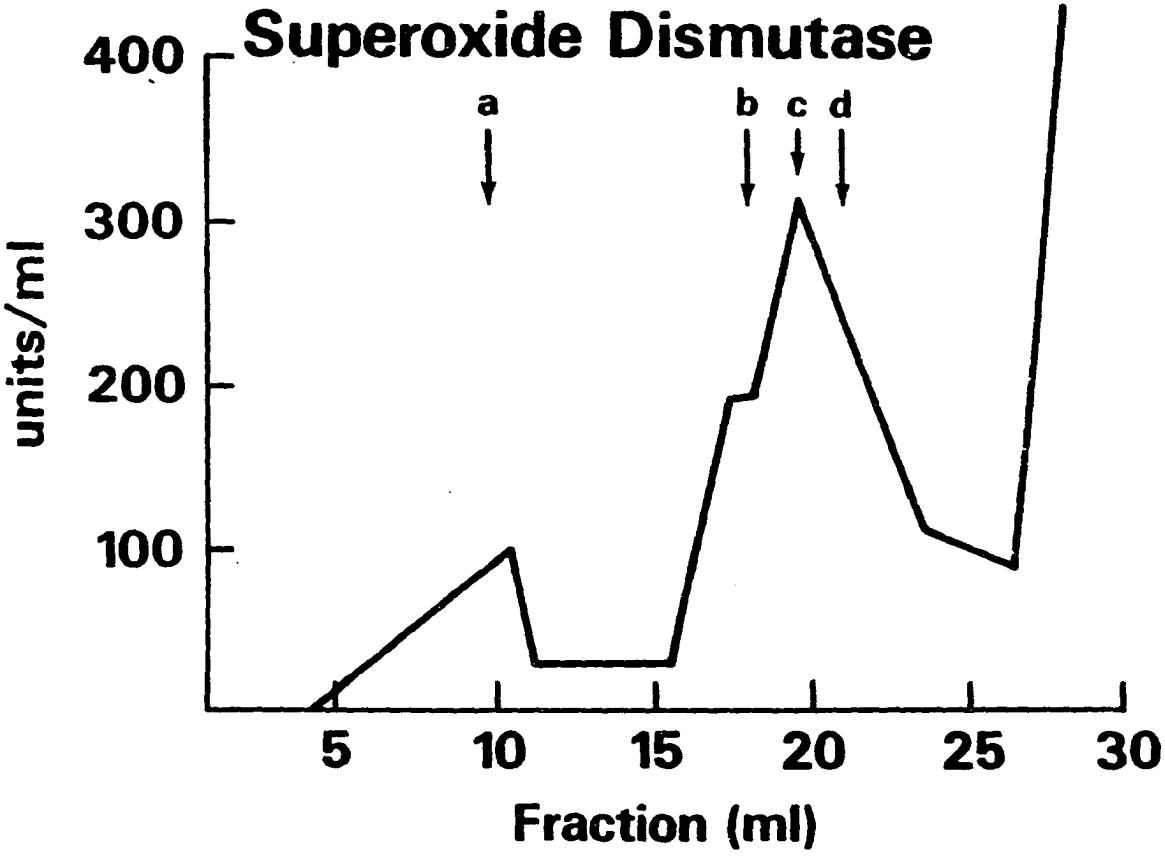
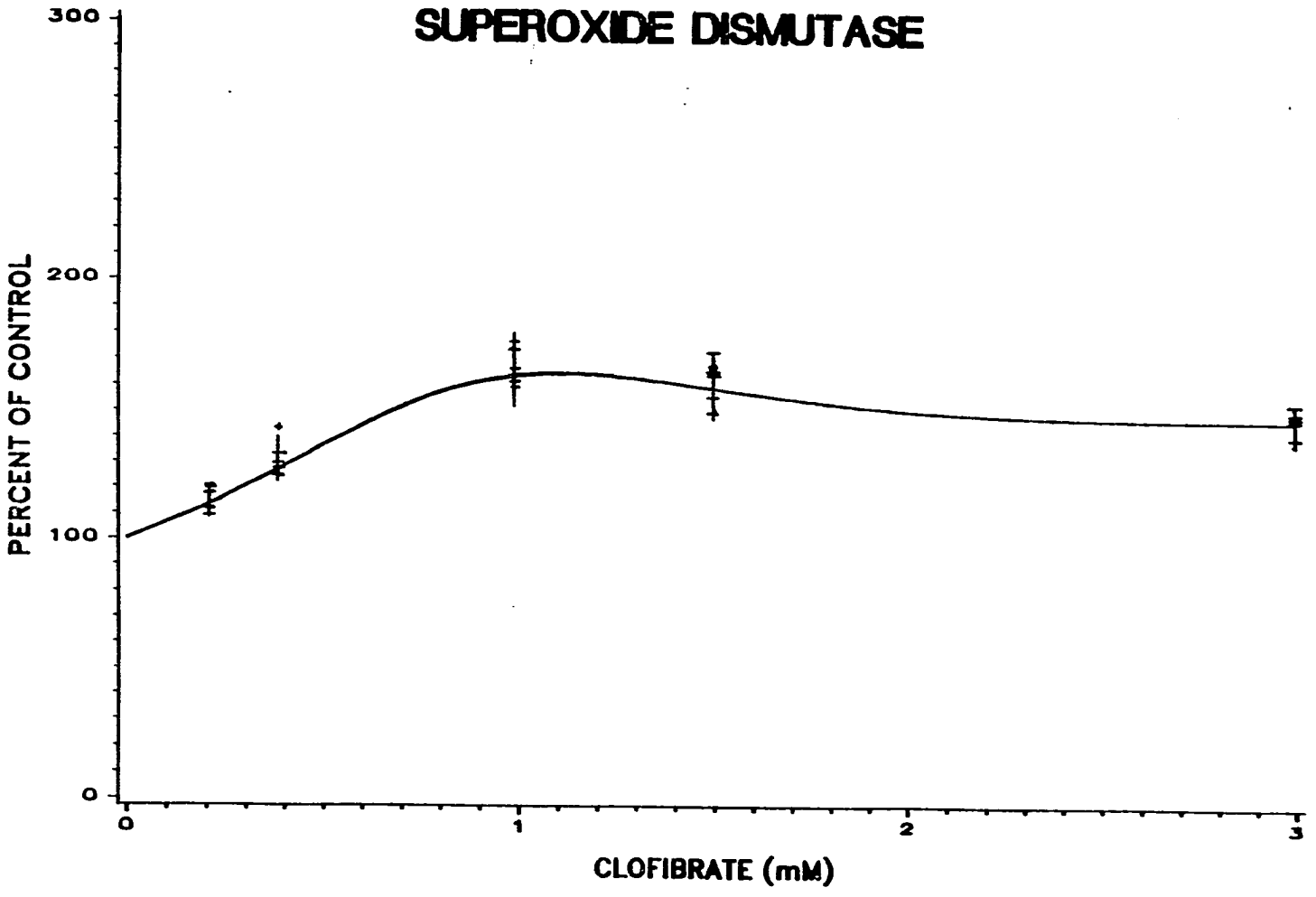

Superoxide Dismutase
400
300
200
100
units/ml
a
b
c
d
5
10
15
20
25
30
Fraction (ml)

Figure 4. Superoxide dismutase activity as influenced by clofibric acid treatment. Content expressed as % of control value/min/10^6 cells. Mean control value = 31±7 units/10^6 cells. Assay method is described in Materials and Methods section.


SUPEROXIDE DISMUTASE
PERCENT OF CONTROL
300
200
100
0
0
1
2
3
CLOFIBRATE (mM)

These data indicate porcine peroxisomal β-oxidation is insensitive to clofibric acid. In rodents, a 4- to 30-fold increase in activity can be expected in the range of drug concentrations we have chosen. One replicate did show an increase in activity at the 0.19 mM level but was not reproducible.

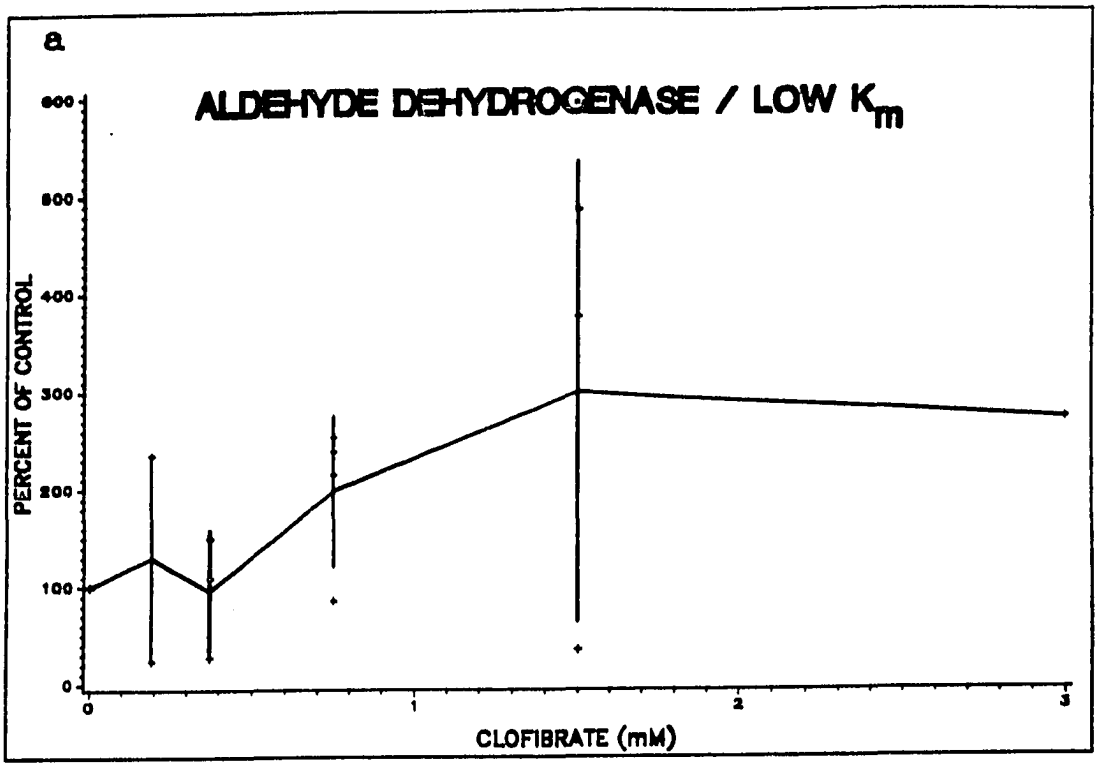
Separation of organelles by isopynic sucrose-gradient ultracentrifugation is depicted in Figure 3. Distribution of the marker enzymes catalase for peroxisomes, GDH for mitochondria, and NADPH:cytochrome c reductase for microsomes shows that adequate resolution of the organelles was obtained. A distinct peak of SOD activity can be seen in the peroxisomal region of the gradient (10% of total SOD activity) with maximal activity corresponding to that of catalase and peroxisomal β-oxidation. Peroxisomal SOD activity was found to be approximately 70 units/ml gradient. Contamination of peroxisomes with mitochondria did not account for all the activity of SOD found in the peroxisomal region of the gradient. Thus an isozyme of SOD seems to be present in the organelle. Organelle separations were carried out on intact liver tissue becaus we were unable to obtain enough material from the cell cultures to perform organelle isolations. Enzymatic activity was very low in the gradients because of dilution of the peroxisomal SOD isolated from the porcine cell cultures. We were able to measure SOD activity in the

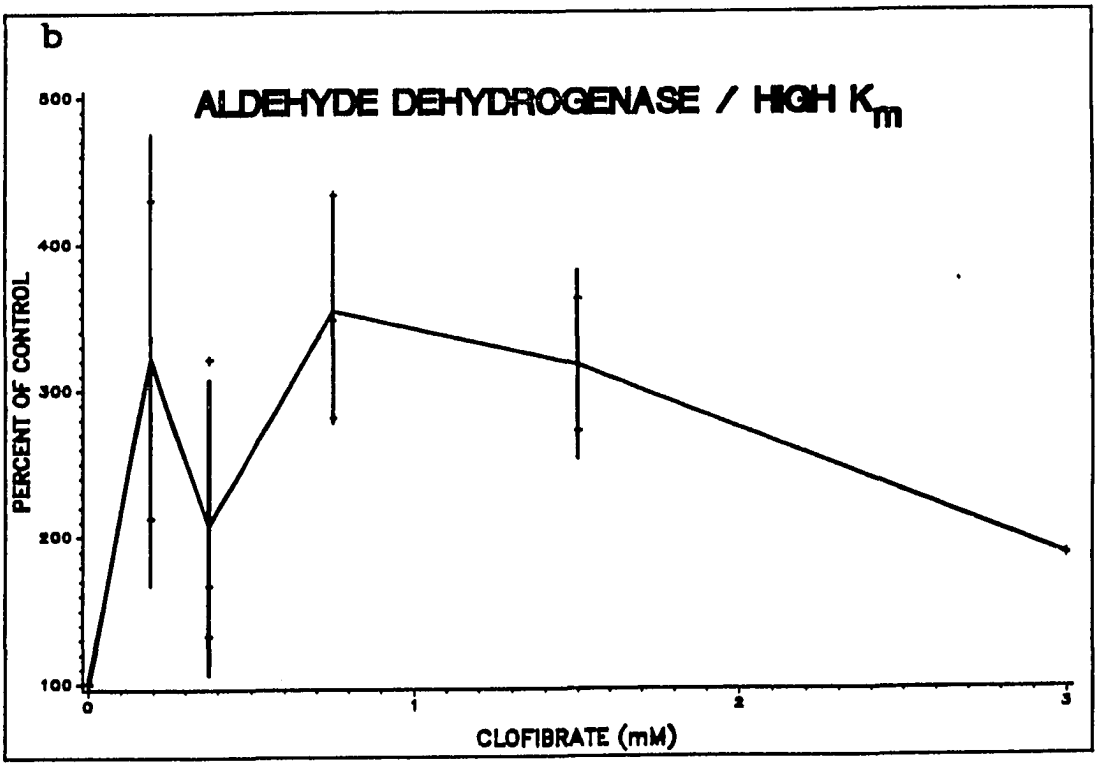
homogenates of the cultured cells, however, which is depicted in Figure 4.

Figure 4 presents the response of SOD activity to treatment with clofibric acid. A significantly (P>.05) quadratic response occurred with maximal activity approaching 150% of that of the controls. We have not, as yet, ascertained which SOD isozyme is being induced as we were unable to measure SOD activity in the organelles isolated by isopynic sucrose ultracentrifugation.

Aldehyde dehydrogenase activity is depicted in Figures 5a and b. Significant induction (P>.05) occurred for both the high K_m and low K_m variants. The response of the low K_m isozyme shows a threshold value at 0.37 mM clofibric acid. The response was linear from 0.37 to 1.5 mM. A decrease in activity occurred at the highest drug dose. The cells may be expressing a biochemical lesion indicating a toxicity at this level. A similar response can be seen for the high K_m isozyme (Fig. 5b). A significant quadratic response occurred (P>.04), although no threshold was observed. Response to the drug was linear to 0.37 mM clofibric acid. The decrease in activity at the 3.0 mM dose level is probably an expression of cell toxicity.

Figure 5. Response of the (a) low K_m and high K_m isozyme of aldehyde dehydrogenase as influenced by clofibric acid treatment. Contents expressed as % pf control value/min/10^6 cells. Mean control value low K_m = 11±12 fmoles/min/10^6 cells. Mean control value high K_m = 29±17 fmoles/min/10^6 cells. Assay methods described in Materials and Methods section.


a
ALDEHYDE DEHYDROGENASE / LOW K_m
PERCENT OF CONTROL
0
100
200
300
400
500
600
0
1
2
3
CLOFIBRATE (mM)


b
ALDEHYDE DEHYDROGENASE / HIGH K_m
PERCENT OF CONTROL
100
200
300
400
500
0
1
2
3
CLOFIBRATE (mM)

DISCUSSION

The extrapolation of rodent-generated data on peroxisome proliferators to man has been questioned for some time. Species variation in peroxisomal metabolism seems likely, supporting evidence is limited, and the scope of the phenomena is incompletely understood. We believe that this is due to a lack of adequate non-rodent models to study peroxisome metabolism. Porcine hepatocyte peroxisomal and mitochondrial β-oxidation is not induced by up to 3.0 mM clofibric acid. Isolated rat cells, treated in a similar fashion respond with a dramatic increase in peroxisomal β-oxidation of up to 2900% (33,34). In rat hepatocyte cell culture, clofibric acid causes an induction of catalase activity, approximately 1-2 fold, which, again, did not occur in the present study. Recently, variation in the kinetics of the response of cyanide-insensitive β-oxidation to a peroxisome proliferator (trichloroacetic acid) has been reported between intact rats and mice, with rats being more sensitive to trichloroacetic acid than mice (35). The difference in kinetics existed in isolated hepatocytes as well. Human hepatocyte peroxisomal β-oxidation, in the same study, was totally insensitive to trichloroacetic acid. One may conclude that the liver tissue of phylogenetically higher animals may be less sensitive to peroxisome proliferators than are rodents.

Specificities in metabolism may occur via a number of different mechanisms. Sensitivity to peroxisome proliferators may arise through receptor-mediated processes. Evidence in rats of a cytosolic protein receptor for nafenopen has been reported (36). Evidence also exists in rats for a clofibrate-induced inhibition of adenylate cyclase (37). These effects may be due to intrinsic differences in the type or number of receptors present in hepatocytes for these agents. Factors affecting bio-availability may be involved as well, although the ability to demonstrate kinetic differences in the response of isolated liver cells to peroxisome proliferators indicates factors at the cellular level are involved (35).

Species specificities in the ability to invoke detoxification mechanisms may exist. These may occur either in the ability to deal with oxidative stress or to clear xenobiotics. The behavior of these enzymes in response to insult with clofibric acid will help indicate how the cells deal with the effects of the drug. If a state of oxidative stress exists, these enzymes may dissipate reactive intermediates, preventing toxicity. Although peroxisomal β-oxidation is not affected, other oxidases in the cell may be. The existence of a peroxisomal SOD has not previously been confirmed. SOD exists in association with the capacity to generate oxygen radicals in other regions of the cell. It seems logical that it would exist in the peroxisome which we have demonstrated.

We have also shown that total cellular SOD seems to respond in an inverse manner to what has been reported for rodents treated with clofibrate (38). It may be significant, then, that decreased SOD activity was associated with enhanced peroxidative risk in the rodent (38).

Aldehyde dehydrogenase in the peroxisome may clear reactive aldehydes from the organelle or provide substrate for β-oxidation by carboxylation of aldehydes. Aldehyde dehydrogenase with a high K_m for aetaldehyde is inducible in rats, which we reproduced with porcine cells (39). However, the low K_m isozyme was not inducible in the rat as it was in the porcine cells. At high drug concentrations, the activity of both ALDH's in the porcine cell decreased significantly, possibly indicating toxicity. This effect was not reported in rodents.

Probably the most significant change seen in the porcine cells was the induction of NADPH:cytochrome c reductase, reflecting proliferation of the endoplasmic reticulum. It is well known that, in rodents, clofibric acid induces proliferation of the smooth endoplasmic reticulum (4). This probably occurred in our porcine cells as well.

Porcine hepatocytes do respond to clofibric acid but in a manner distinctly different from than of rodents. An insensitivity to proliferation of peroxisomal enzymes and indications of toxicity at high dosages are

most obvious. Slight induction may occur at toxic levels, however, as occurred when guinea pigs were dosed with WY171883 which is also a peroxisome proliferator (40).

A model more accurately reflecting human responses to peroxisome proliferators is needed so that we may gain a more complete understanding of the peroxisome's contribution to metabolism. The hypothesis that peroxisome proliferators are carcinogenic revolves around the ability of the peroxisomal oxidases to generate reactive species of oxygen. Because the ability to generate oxygen radicals is related to the metabolic activity of the peroxisomal oxidases, the inability to demonstrate induction of peroxisomal β-oxidation in tissues of the pig, human, and others probably suggests that the rodent model is invalid for extrapolation to other species.

Our results support the claim that peroxisomal β-oxidation in higher animals is less sensitive to peroxisome proliferators than are rodents (40). From these data, it is reasonable to suggest that rodents are not appropriate models for the study of peroxisomal metabolism in higher animals. Porcine hepatocyte cell culture offers an alternative for the study of peroxisomal metabolism.

REFERENCES

1. Masters, C. and R. Holmes. 1977. Physiol. Rev. 57(4):816.

2. Tolbert, N.E. 1981. Annu. Rev. Biochem. 50:133.

3. deDuve, C. 1983. Sci. Am. 248(50):74.

4. Cohen, A.J. 1974. Food. Cosmet. Toxicol. 19:585.

5. Kawashima, Y., M. Takigishi, H. Watanuki, H. Katoh, Y. Tachibana, and H. Kozuka. 1985. Toxicol. Appl. Pharmacol. 78:363.

6. Reddy, J.K., D.L. Azarnoff, and C.C. Hignite.1980. Nature 283:397.

7. Vaino, H., K. Linnainmaa, M. Kahonen, J. Nickels, E. Hietanen, J. Marmiemi, and D. Peltonen. 1983. Biochem. Pharmacol. 32(18):2775.

8. Sakurai, T., S. Miyazawa, T. Osumi, S. Furuta, and T. Hashimoto. 1981. Toxicol. Appl. Pharmacol. 59:8.

9. Lazarow, P.B. 1977. Science 197:580.

10. Ganning, A.E. and G. Dallner. 1981. FEBS Lett. 130(1):77.

11. Hori, S. and T. Suga. 1985. Biochem. Pharmacol. 34(9):1357.

12. Autian, J. 1973. Environ. Health Persp. 4:3.

13. Lalwani, N.D., M.K. Reddy, S. Gosh, S.D. Barnard, J.A. Mohello, and J.K. Reddy. 1985. Biochem. Pharmacol. 34(19):3473.

14. Reddy, J.K., N.D. Lalwani, S.A. Qurishi, M.K. Reddy and C.M. Moehle. 1984. Am. J. Pathol. 114:171.

15. Nederguard, J., S. Alexson, and B. Cannon. 1980. Am. J. Physiol. 239:6208.

16. Neat, C.E., M.S. Thomassen, and H. Osmundsen. 1981. Biochem. J. 196:149.

17. Ishii, H., S. Horie, and T. Suga. 1980. J. Biochem. 87:1855.

18. Turteltaub, K.W., and P.A. Murphy. 1987. Arch. Biochem. Biophys. 255(1):120.

19. Seglen, P.O. 1976. Meth. Cell Biol. 13:29.

20. SAS Institute Inc. 1985. SAS User's Guide: Statistics, Version 5 Edition. Cary, NC: SAS Institute Inc. 956 pp.

21. Tolbert, N.E. 1974. Method Enzymol. 31(pt. A):734.

22. Leighton, F., B. Polle, H. Beafay, H. Baudhuin, J. Coffe, S. Fowler, and C. deDuve. 1968. J. Cell Biol. 37:482.

23. Masters, B.S., C.H. Williams, and H. Kamen. 1976. Meth. Enzymol. 10:565.

24. Wrobleaski, F. and J.S. LaDue. 1955. Proc. Soc. Exp. Biol. Med. 90:210.

25. Beauchamp, C., and I. Fredovich. 1971. Anal. Biochem. 44:276.

26. Koivula, T., and M. Koivusalo. 1975. Biochim. Biophys. Acta 397:9.

27. Krahling, J.B., R. Gee, J.A. Gauger, and N.E. Tolbert. 1979. J. Cell Physiol. 101:375.

28. Battino, R. and H.L. Cliver. 1966. Chem. Rev. 66:395.

29. Lazarow, P.B., and C. deDuve. 1976. Proc. Natl. Acad. Sci. U.S.A. 73:2043.

30. Richardson, N.I., P.J. Snodgrass, C.T. Nuzum, and A.H. Tashjian Jr. 1973. J. Cell Physiol. 83:141.

31. Lowry, O.H., N.S. Rosebough, A.L. Farr, and R.J. Randall. 1951. J. Biol. Chem. 193:265.

32. Picart, L., L. Thayer and M.M. Thaler. 1973. Biochem. Biophys. Res. Commun. 54(2):562.

33. Gray, T.J.B., B.G. Lake, J.A. Beamand, J.R. Foster, and S.D. Gangolli. 1983. Toxicol. Appl. Pharmacol. 67:15.

34. Ozasa, H., S. Miyazawa, S. Furuta, T. Osumi, and T. Hashimoto. 1985. J. Biochem. 97:1273.

35. Elcombe, C.R. 1985. Arch. Toxicol. Suppl. 8:6.

36. Lalwani, N.D., W.E Fahl, and J.K. Reddy. 1983. Biochem. Biophys. Res. Commun. 116:388.

37. Akbar, H., D.T. Witiak, H.A. Newman, R.H. Fertel, and D.R. Feller. 1982. Biochem. Pharmacol. 31(11):2125.

38. Ciriolo, M.R., I.G. Rotilio, V. Borzatta, M. Cristofari, and L. Stanzani. 1982. FEBS Lett. 144(2):264.

39. Antoneov, V.D., S.V. Perozhkov, and L.F. Panchenko. 1985. Eur. J. Biochem. 149:159.

40. Eacho, P.I., P.S. Foxworthy, W.D. Johnson, D.M. Hoover, and S.L. White. 1986. Toxicol. Appl. Pharmacol. 83:430.

SUMMARY

Peroxisomes are ubiquitous in nature. They have been demonstrated in many species and almost all cell types. Yet, after 30 years of study, their contribution to cellular metabolism still remains a mystery. The presence of a β-oxidation pathway, coupled with the capacity to metabolize cholesterol and generate ether glycerolipids, suggests an important role in lipid metabolism. This is further supported by the fact that several congenital anomalies involving alterations in, or complete absence of, peroxisomes are incompatible with life. The organelle must serve a purpose that enhances the survival of the cell. Whether the peroxisome plays a similar role with similar relative importance in all organisms, all tissues, and all cell types remains open to debate. This is because almost the entire spectrum of data on the peroxisome has been collected from relatively few species -- the rat and mouse.

Answering questions of interspecies differences in metabolism is important and quite relevant. Several peroxisome proliferating agents have been shown to be carcinogenic in rodents and it has been proposed that peroxisome proliferators, as a class, are carcinogenic. Does this apply to other species? Does the mechanism proposed to be responsible for the development of carcinomas in rodents after treatment with peroxisome proliferators also occur in other species? No answer is forthcoming.

Numerous examples of species-specific metabolism exist. Peroxisome metabolism may have its own specificities. Differing capabilities of an organism to respond to stimuli such as with sensitivity to peroxisome proliferators may exist such that peroxisome proliferation may occur in some species, be less dramatic in others, or be totally absent. Differences in the response of the peroxisome to peroxisome proliferators may arise through the presence or absence of receptors for the agents in the cells. Various species may differ in the ability to inactivate or detoxify chemicals modulating peroxisome numbers or peroxisomal enzyme activities. Species at different phylogenetic levels may have different subcellular distribution of enzymes associated with detoxification. One may question whether different animals differ in the ability of the cell to cope with the products of peroxisomal metabolism such as hydrogen peroxide, superoxide anions, or oxidized lipid components. Whole animal physiology may be important as well. All species do not possess similar modes of drug absorption, drug distribution, or drug elimination which may affect a species response to peroxisome proliferators. These factors must be dealt with before human risk can be determined for peroxisome proliferators.

A better understanding of interspecies peroxisome metabolism is also relevant from the perspective of understanding metabolism in general.

What does the peroxisome contribute to the cell? How does it improve an organism's ability to survive? These are some of the questions we began to deal with in this work. Our purpose was to help elucidate the metabolism of the peroxisome in a species phylogentically removed from rodents. The two parts of this dissertation have described a non-rodent model for the study of peroxisome metabolism.

In Part I, a peroxisome purification scheme was introduced which allowed for the demonstration of a porcine hepatocyte peroxisomal β-oxidation pathway which has not previously been demonstrated. Through separation of the major organelles, we were able to demonstrate the presence of a peroxisomal β-oxidation pathway and estimate that the relative contribution of the peroxisome to whole cell β-oxidation was 50%. This finding differs from the 30% estimated for rodents. We were also able to show that, as in rats, the porcine hepatocyte peroxisome possesses an ALDH with a high K_m for acetaldehyde. The relative distribution of the ALDH isozymes were similar to that reported for rats. A peroxisomal SOD was identified as well. ALDH and SOD may exist in the peroxisome to scavenge aldehydes and reactive forms of oxygen produced via the peroxisomal acyl-CoA oxidase. Superoxide dismutase has not been reported in the rodent peroxisome.

In Part II of this dissertation, it was demonstrated that a peroxisomal SOD can be found in porcine liver. A protocol to isolate and grow porcine hepatocytes in culture was also described. This technique removes the need to maintain an animal herd and eliminates the disadvantage of using species larger than rodents as research tools. It offers the extra advantage of precise control of environment and the ability to manipulate the liver cells in a specific manner. Through this technique, we demonstrated that ALDH, NADPH:cytochrome c reductase, and cellular protein content are inducible by clofibric acid concentrations in the millimolar range. Our most significant finding was that porcine hepatocyte β-oxidation, both mitochondrial and peroxisomal, is not inducible by clofibric acid. A 4- to 20-fold increase in peroxisomal β-oxidation can be expected in the drug concentrations used in this study when administered to rodents.

These results demonstrate that a dramatic species-specific effect exists in the sensitivity of liver cells to clofibric acid. Our data show that porcine hepatocytes do respond to clofibric acid, but in a manner distinctly different from that of rodents. Porcine hepatocyte peroxisomal β-oxidation may be much less sensitive to this drug than is rodent liver. Our results are in agreement with the findings of others, who show a diminished or non-existent sensitivity to peroxisome proliferators in human liver cells

and primates. We suggest that rodents are more sensitive to peroxisome proliferators than are phylogenetically higher animals and as such are a poor model for the study of the human peroxisome. Future studies in peroxisomal metabolism should focus on the elucidation of the phenomena of peroxisome proliferation and unraveling the metabolic role of the organelle in the cell.

Porcine hepatocyte cell culture could be used to elucidate the nature of the increased sensitivity of rodents to these peroxisome proliferators and thus provide insight into the mechanism of peroxisome proliferation. Radiolabeled clofibric acid could be used to determine the presence and relative concentration of receptors for the drug in various cell types. Correlations should be made to determine if the presence of receptors is related to the ability to induce peroxisome proliferation. Kinetics of the binding of the drug to the receptor could also be determined and correlated to the ability to induce peroxisome proliferation. The receptors could be isolated and purified to help understand the nature of the receptor. The distribution and fate of clofibric acid in the cell should be studied also. Determination of the disposition of clofibric acid in the cell (i.e., is the drug internalized and what happens to it after binding?) is important to understanding the mechanism of proliferation.

The availability of a cell culture system suitable for the study of the peroxisome allows for the use of specific metabolic inhibitors which can shunt energy substrates away from specific pathways without the death of the animal. These inhibitors could be used to see how the porcine hepatocytes deal with the loss of metabolic pathways as a means of understanding the peroxisome's contribution to metabolism. Metabolic intermediates can be quantified via freeze-clamp techniques which can be used to determine kinetic parameters of peroxisomal enzymes *in situ.* Radiolabeled substrates for energy metabolism can be administered to the cells to determine the fate of the intermediates as related to the peroxisome. These studies would add substantially to our understanding of interspecies peroxisomal metabolism and through it an understanding of human peroxisomal metabolism.

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