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ENZYME ACTIVITIES OF HEAPTIC PEROXISOMES OF LEAN AND OBESE MICE

By

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ABSTRACT

ENZYME ACTIVITIES OF HEPATIC PEROXISOMES
OF LEAN AND OBESE MICE

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Hepatic peroxisomes and their enzyme activities have been examined in lean and obese C57BL/6J mice and in HA(ICR) mice. No difference in the numbers of peroxisomes per unit area was observed between lean and obese mice by electron microscopy.

Clofibrate(p-chloro-phenoxyisobutyrate ethyl ester) feeding at 0.5% of the diet caused no increase in catalase activity in crude homogenates of liver or in peroxisome numbers, but did cause an increase in liver weight in lean and obese C57BL/6J male mice. Catalase activity did increase in HA(ICR) male mice fed the same clofibrate diet.

Hepatic peroxisomes have been isolated on isopycnic sucrose gradients from white HA(ICR) mice and lean and obese C57BL/6J mice. Nearly all the catalase activity was in the peroxisomal fraction. Matrix marker enzyme activities, catalase and urate oxidase of the peroxisomes and glutamate dehydrogenase of the mitochondria, were similar in amounts of activity in lean and obese C57BL/6J male and female mice. Membrane components, NADPH:cytochrome c reductase of the microsomes and β-hydroxybutyrate dehydrogenase of the mitochondria, had lower activity in the obese mice in inverse proportion to the

larger liver size. The white HA(ICR) male mice had marker enzyme activities similar to the lean C57BL/6J male mice. Fed and fasted mice had similar marker enzyme activities on a per g liver basis.

Activity for peroxisomal fatty acid β-oxidation was the same for obese and lean mice, fed and fasted mice or male and female mice per g liver, but peroxisomal β-oxidation was approximately 3 times higher in HA(ICR) male mice than in the C57BL/6J mice. Mitochondrial fatty acid β-oxidation was the same when comparing lean and obese mice or male and female mice but higher for fasted mice than fed mice. Mitochondrial fatty acid β-oxidation was higher in HA(ICR) mice than in lean and obese C57BL/6J mice. The ratio of mitochondrial to peroxisomal fatty acid β-oxidation activities were the same in all groups compared.

Hepatic NAD:glycerol-3-P dehydrogenase was higher in obese male mice compared to lean male mice in both the peroxisomes and in total activity in the liver. Fasting reduced the cytosolic fraction of this enzyme activity in the lean mice but not in the obese mice. The peroxisomal enzyme remained unchanged during a fast in lean and obese mice. Female obese mice had higher cytosolic and peroxisomal NAD:glycerol-3-P dehydrogenase activity than the lean females. Obese females had higher total and cytosolic enzyme activities than obese males. Lean C57BL/6J mice and HA(ICR) mice had similar activities of NAD:glycerol-3-P dehydrogenase.

On a per animal basis, there was more hepatic peroxisomal fatty acid β-oxidation and more peroxisomal NAD:glycerol-3-P dehydrogenase activity in obese mice. Thus, there does not appear to be a lowered

amount of hepatic peroxisomal activity associated with increased weight. The evidence in this research does not support the hypothesis that peroxisomal metabolism wastes energy in animals.

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INTRODUCTION

Obesity has been called the principal nutritional disease of humans living in most industrially developed nations (21). Its cause is unknown (7). The complexities of obesity in man are summarized by Garrow (54).

Experimentally, it is very difficult to make accurate measurements of daily energy balance in man in contrast to the rat. A net caloric imbalance of 1-2% in man over a period of a few years has serious consequences in relation to weight gain or loss. The accuracy of techniques presently available to measure energy balance have a greater error than the imbalance they are supposed to measure. In man, energy regulation does not appear to be as accurate nor as rapid as in the rat. There is little evidence that the intake of energy in man is controlled by energy expenditure in the short or long term. In addition, the physiological signals that are effective in experimental animals are easily overridden in man. Garrow (54) suggests that there is good evidence for a metabolic adaptation in man to an energy imbalance. This is accomplished through a change in the metabolic rate in the same direction as the imbalance to maintain the equilibrium. The nature of this change is unknown but circumstantial evidence suggests that it could be a change in the rate of protein turnover (54).

The concept that obesity may be genetically controlled in man finds favor with the close correlation between obesity and an endomorphic somatotype during adolescence and with evidence from studies involving twins (104). Because genetic tests are very expensive, time-consuming and inappropriate for man, the use of experimental animal models is mandated for studies involving energy regulation.

LITERATURE REVIEW

Obese Mouse

There are many forms of obesity in rodents and they have been extensively reviewed by Bray and York (22), Hunt et al. (71) and Assimaopoulos-Jeannet and Jeanrenaud (6). The obese mouse (ob/ob) used in this study arose from the laboratory stock of the Jackson Memorial Laboratory, Bar Harbor, Maine, in 1950 (72). It has been used in many studies of obesity and extensively reviewed (31, 32, 56, 59-61, 128, 129, 153).

The rationale for using this animal for this research is based on several parameters. Animals with the ob/ob gene can be detected at 10-15 days in age by lowered oxygen consumption (51, 78) or lowered body temperature (76, 147). There is a report that the ob/ob genetype can be detected successfully at even earlier ages (20). In the ob/ob animals, the body fat content, fat cell size, and serum insulin all increase prior to weaning (22, 46, 75, 79). Energy expenditure is decreased while hyperphagia and increased efficiency in food utilization result in excess storage of calories as fat (22). Trayburn et al. (147) have recently proposed that the obesity in these animals is due to a major defect in the energy consuming process. To support this concept, it is known that the obese is hypermetabolic prior to weaning (51, 78, 147); it cannot regulate its body temperature on cold exposure (76, 105, 115, 147, 157); and it remains in positive

energy balance even when pair-fed to the lean littermates (5, 30, 65, 102). Even when the obese animal is placed on a restricted diet to reduce its weight to that of the leans, it still has a higher percentage fat content (5, 23, 40, 160). Therefore, it has been concluded that an excess energy intake enhances but does not necessarily cause the obesity (5, 23, 40, 45, 120, 160).

In weanling mice, the obese animals contained 130% more fat than their lean littermates, but the animals were only 14% heavier (93). In a five week period, these mice converted 3-4 times more food energy to body energy but only consumed 20-40% more energy. In contrast, only 70% of the dietary protein was converted to body protein (93). A difference in protein accumulation in preobese and obese mice has been reported (18), as well as a slower protein mass turnover in the obese animal during a fast (39). A significant difference in energy metabolism occurs at an early age in these animals (93).

Bray et al. (24, 158) have recently proposed that obesity in the ob/ob mouse is caused by an enzymatic defect through loss of the thyroid induced sodium- and potassium-adenosine triphosphatase. The difference in enzymatic activity cannot result from obesity in every animal model because mice made obese by gold-thioglucose treatment did not have a defective thyroid induced Na^+-K^+-ATPase. These results could be used to explain the lower body temperature, the inability to regulate body temperature and the increased food utilization efficiency in the obese mouse.

Both liver and adipose tissue in the obese mouse have been studied extensively (22, 60). Histologically, obese mouse adipose tissue has larger (31, 32, 56) and more numerous fat cells (59). Although there is some controversy as to whether ob/ob mice have more fat cells (60), the number of cells has been reported to be dependent on the location of the fat depot (22).

Hyperlipigenesis in adipose tissue of the obese mouse has been observed by several procedures. There is increased incorporation of acetate into fatty acids and lipids when epididymal adipose tissue is incubated in the absence of glucose (33, 66). Hellman and Westman (62) observed increased esterification of palmitate in epididymal adipose tissue in the absence of glucose. The activity of an adipose glycerol kinase for the reesterification of fatty acids has been reported (94, 128).

Obese mice appear to have an impaired ability to mobilize fat. Lochaya et al. (94) reported lowered lipase activity. There are several reports of reduced fatty acid release from isolated adipose tissue after starvation (62, 101, 154) and after stimulation with epinephrin (90, 101).

In hepatic tissue from the obese animal, there is a 5-10 times increase in vivo in glucose incorporation into liver fat (136) even after fasting (11, 159). Kornaker and Lowenstein (81) reported a greater capacity for fatty acid synthesis in the obese mouse liver due to higher levels of citrate cleavage enzyme.

There have been numerous studies of hepatic and adipose tissue enzymes in lean and obese mice. Bray and York (22) reviewed these through 1971. The hepatic glycolytic, lipogenic, and gluconeogenic enzymes are increased in the obese animal with the exception of PEP carboxykinase. In adipose tissue, the lipolytic enzymes are decreased in the obese. Glycerol kinase activity has been reported to be high in obese adipose tissue, but these data are not supported by *in vivo* data on fatty acid reesterification (62).

In vivo, studies of hepatic lipogenesis showed 6-8 times higher activity in the obese mouse (73). This is confirmed enzymatically by the several fold higher activity of acetyl-CoA carboxylase activity in the obese mouse (29, 98). Maragoudakis *et al*. (98) also found a different response to a fasting-refeeding regime with the obese mouse. With a 48 hour fast followed by a 48 hour refeeding period, acetyl-CoA carboxylase increased 9 times in the lean animal while in the obese animal, the activity only increased two times. Therefore, the total activity in the two animals was equal. During a fast, acetyl-CoA carboxylase decreased by 1/3 in the obese mouse and by 1/5 in the lean animals. The workers concluded that there was a quantitative rather than a catlytic or regulatory difference in the enzyme levels in the two mice.

Volpe and Marasa (152) reported an altered response in the obese animal on fasting-refeeding regime where the rate of degradation of fatty acid synthetase did not increase during fasting as it did in the lean animal. Fatty acid turnover and half-life has been determined

by Mayer and colleagues (12). They concluded that significantly less fat was mobilized from stores in the obese mouse leading to a much slower turnover of fat stores and a longer half life.

These reported findings indicate that the obese mouse has many factors in its enzymatic make-up that would contribute to a positive energy balance even under usually adverse conditions. However, little experimental work has explored differences in hepatic lipid degradation in the obese mouse.

Futile Cycles

The concept of futile or energy wasting cycles have been explored in several studies. Hue and Hers (70) reported futile cycling with the loss of ATP for glucose:glucose-6-P in mammalian liver and fructose-6-P:fructose-di-P in muscle but not liver. Rognstad et al. (131) have provided isotopic evidence for futile cycling between glucose:glucose-6-P and fructose-6-P:fructose-di-P in isolated liver parenchymal cells. Newsholme and Gevers (113) reported that the fructose-6-P:fructose-di-P recycling may benefit the system by making it more sensitive to various stimuli. In the bumble bee, Bombus affinis, the cycling is used to produce heat in the muscles (35). Evidence for futile cycling through pyruvate by hydrolysis of phosphoenolpyruvate has been given in perfused liver (52) and kidney cortex slices (132). The operation of futile cycles in glucose metabolism has been reviewed by Scrutton and Utter (131) and Newsholme and Gevers (113). Clark et al. (36) have suggested that futile cycles may serve a regulatory function to balance ATP

production and utilization. They also suggested that the recycling may be an imperfection in regulation or a leak.

The wasted energy in futile cycles may occur at other sites in metabolism as well. The well-known site of energy wasting in plant peroxisomes through photorespiration suggest that animal peroxisomes could have a similar energy wasting function in animal tissues. Several reviewers have made this suggestion (44, 143, 144), so this aspect has been investigated further in my dissertation project.

Peroxisomes

Microbodies or peroxisomes are established as subcellular respiratory organelles (42, 43, 64, 67, 143). Microbodies have a single bounding membrane, dense matrix and, in general, contain enzymes associated with oxidative degradations involving flavin oxidases and catalase (144).

The term microbody was introduced into the microscopy literature by Rhodin in 1954 (130) to describe a special type of cytoplasmic body found in the convoluted tubule cells of mouse kidney having a single bounding membrane and a fine granular matrix. Rouiller and coworkers (53, 133) identified, in rat liver parenchymal cells, a similar particle which contained a dense core, as well as a single membrane and granular matrix. Microbody distribution as observed in numerous tissues and species, was summarized by Hruban (67).

Microbodies were first recognized and called peroxisomes in rat liver through biochemical studies of the centrifugal behavior of urate oxidase, D-amino oxidase and catalase (14, 16, 41). The

term peroxisome comes from the initial use of an assay by de Duve's group for isolated microbodies through the peroxidic release of CO_2 from formate as catalyzed by their catalase content. There is little evidence, however, for a peroxidative function of peroxisomes *in vivo* (144). The term peroxisome was used also by Tolbert *et al.* (143, 146) to describe microbodies in leaves which fulfilled de Duve's criteria of peroxisomes (42, 43).

Studies with peroxisomes from *Tetrahymena pyriformis* showed the presence of enzymes of the glyoxylate cycle, as well as catalase (110, 111). Breidenbach and Beevers (17, 25, 38) introduced the term glyoxysome to describe microbodies in germinating fatty seeds that contained enzymes of the glyoxylate cycle and fatty acid β-oxidation. Catalase containing particles that were not mitochondria have been isolated from yeast (8). Certain algae have particles containing the glyoxylate enzymes (57).

The peroxisomal system, in general, is made up of the association of catalase with various H_2O_2 producing oxidases. This system catalyzes a two step reduction of molecular oxygen to water. The electron donors appear to vary with the cell type, but usually include glycolate, L-lactate and other L-amino acids (43). De Duve has compared the mitochondrial electron transport chain to the peroxisomal system in several important ways. First, peroxisomal respiration is not coupled to any mechanism for the retrieval of energy and, therefore, catalyzes an essentially wasteful form of respiration. Photorespiration in leaves of plants is the clearest example (143, 146).

Second, the rate of peroxisomal oxidation is almost directly proportional to oxygen tension, while mitochondrial respiration is essentially independent of oxygen tension, except at very low oxygen concentrations. In animals, there is no physiological data on the effect of oxygen on partitioning respiration between peroxisomes and mitochondria. However it has been assumed that mitochondria may have precedence over peroxisomes in utilization of limiting amounts of oxygen. Peroxisomes can respond to increases in oxygen content and, in plants, might have a function in protection from oxygen toxicity, although this has not been investigated in animals. Third, peroxisomes and mitochondria have different specificities to selected substrates. Urate and D-amino acids are metabolized preferentially by peroxisomes. Lazarow (88) and de Duve (44) propose that peroxisomes preferentially oxidize long chain fatty acids while mitochondria use shortened fatty acids.

Peroxisomal Enzymes

Peroxisomal enzyme content varies widely depending on the tissue from which they were isolated. By comparing the enzymes found in microbodies from different sources, an understanding of the origin of the hypothesis for their role in energy wasting therapy can be explained.

In C_3 plants, very active photorespiration occurs due to glycolate biosynthesis in the chloroplasts and its metabolism in the peroxisomes and mitochondria (1, 38, 116, 143). Oxygen uptake and CO_2 loss in these plants may be close to 50% of the gross photosynthetic rate and drastically reduces net photosynthesis (114).

Several physiologically irreversible steps that are energy wasting occur in photorespiring plants in the conversion of ribulose-bisphosphate to glycine.

In germinating fatty seeds such as peanut and castor bean, glyoxysomes, a micorbody, contain the enzymes of the glyoxylate cycle and fatty acid β-oxidation that are involved in the conversion of fatty acids to C_4 acids that are then used for sugar synthesis in the germinating seeds (38). There is one energy wasting step in the first dehydrogenation in β-oxidation in these particles where the electrons are transferred to H_2O_2 which is then destroyed by catalase. No β-oxidation, apparently occurs in the mitochondria of these seeds. Glyoxysomes disappear from the germinating seed in about ten days, when the plant starts to photosynthetically produce its own sugars. During this changeover, the glyoxysomes are replaced by peroxisomes in the leaves of the plant.

Until recently, peroxisomes in animals were not known to contain a defined cycle of respiratory or synthetic metabolism as do leaf peroxisomes or seed glyoxysomes. But many of the enzymes known to be contained in animal peroxisomes are associated with lipid degradation. Now the existence of fatty acid β-oxidation in liver peroxisomes establishes a metabolic sequence in the peroxisome.

Catalase

Catalase is found in all animal peroxisomes and is used as a marker for peroxisomes both cytologically (49, 114) and biochemically (145). The function of catalase in peroxisomes has been

suggested de Duve et al. (42) to remove hydrogen peroxide produced by other enzymatic reactions in the peroxisomes, as well as extra-peroxisomally produced hydrogen peroxide. Catalase has been implicated in metabolic flux as measured by oxygen consumption that is peroxisomally dependent. In liver homogenates, Aebi and Suter reported that about 1/3 of the normal oxygen consumption by the liver was through the peroxisome (1). Oshino et al. (116) demonstrated that approximately half the ethanol in liver cells was metabolized by catalase. They also showed that perfusing intact liver with urate and glycolate produced peroxisomal hydrogen peroxide and accounted for nearly half of the total liver respiration.

Urate and glycolate metabolism have been shown to stimulate ethanol utilization by catalase several fold (142). Masters and Holmes (103) interpret this to mean that the peroxisomal system is capable of operating beyond its already high steady state levels. They also suggest that peroxisomal oxidation through catalase may occur without simultaneous increase in the pyridine nucleotide redox state in oxidizing ethanol.

Jones and Masters (74) have compared catalase activities from various tissues and species. Catalase activity is always highest in the liver with substantial amounts in the kidney and blood. A much lower activity is found in other tissues. There is a marked species variation in peroxisomal and supernatant catalase activities as measured in crude tissue homogenates. Rat and mouse livers have most of their catalase activity in the peroxisomal particles, while beef

and guinea pig liver catalase is found principally in the supernatant. Jones and Masters (74) attribute this to the need for protection of the less stable catalase of rat and mouse inside an organelle (peroxisomes). No organelle separation was done, however, so peroxisomal breakage during tissue homogenation cannot be estimated.

In rat liver, catalase accounts for as much as 40% of the peroxisomal protein (42). Catalase is used as a marker to identify peroxisomes for electron microscopy using diaminobenzidine (49, 114).

α-Hydroxy Acid Oxidase

α-Hydroxy acid oxidase has been partially characterized from rat liver and kidney as well as pig liver and kidney peroxisomes (106). This enzyme catalyzes the aerobic oxidation of α-hydroxy acids with flavin mononucleotide as a cofactor to produce hydrogen peroxide (42, 43). The enzyme from rat liver peroxisomes is most active with glycolate as its substrate. Considerable activity is observed with lactate but very little with L-α-hydroxy butyrate. The enzyme catalyzes the oxidation of L-α-hydroxy isocaproate almost as well as glycolate and also has activity with α-hydroxy-caproate and α-hydroxy butyrate. The hepatic enzyme was not able to oxidize α-hydroxy acids of chain length greater than C_8. The reason for these various specificities is not known (106).

α-Hydroxy acid oxidase shows very different substrate specificities between tissues and species. Rat liver α-hydroxy acid oxidase utilized glycolate, lactate and α-hydroxy isocaproate while

the enzyme isolated from rat kidney was inactive towards short chain α-hydroxyacids but was active for α-hydroxy isocaproate and α-hydroxypalmitate. The pig liver and kidney enzymes exhibit a principal specificity for glycolate (106).

α-Hydroxy acid oxidase activity has been used to identify peroxisomes by microscopy by using a coupled reaction of nitro blue tetrazolium and α-hydroxy acid (3, 4, 137). Allen et al. (3) reported positive reaction with D,L-α-hydroxyvalerate, D,L-α-hydroxybutyrate and L-lactate in rat kidney.

D-Amino Acid Oxidase

D-Amino acid oxidase has been identified in rat liver and kidney peroxisomes (15) using the oxidation of D-amino acids to keto acids and hydrogen peroxide production coupled to the peroxidation of formate. The enzyme has little activity towards L-amino acids.

Farber et al. (50) has used D-amino acid oxidase to visualize microbodies for microscopy in rat liver and kidney by using nitro blue tetrazolium coupled with various D-amino acids. They reported good staining with D-methionine, D-leucine, D-ethionine and D-isoleucine. Less intense staining was observed with D-tyrosine and D-phenylalanine.

Glyoxylate Aminotransferase

Glyoxylate aminotransferase is a characteristic enzyme of peroxisomes in which glyoxylate is transaminated to glycine with various amino acid donors (69, 80, 127, 143). In rat liver and kidney, the peroxisomal glyoxylate aminotransferase has been characterized (69)

and found to differ from those of spinach leaves in the specificity for the amino donor. The rat hepatic enzyme is specific for glyoxylate and most active with leucine and phenylalanine and some activity with histidine as the amino donor.

The rat hepatic glyoxylate aminotransferase fluctuates widely *in vivo* (69) as is seen with other peroxisomal enzymes (48, 83). Overnight fasting decreases the activity by 50%. The activity is increased moderately on high protein diets and in the terminal stages of starvation. No activity was detected at birth but increased thereafter, to a plateau at about 40 days of age in the rat. Female rats have been shown to have four times the activity of males. p-Chlorophenoxyisobutyrate (similar to clofibrate) increased the activity of the enzyme 2.5 times along with peroxisome proliferation (69).

NADP:Isocitrate Dehydrogenase

NADP:Isocitrate dehydrogenase has been reported to be associated with rat liver peroxisomes (92).

Urate Oxidase

Urate oxidase is always a peroxisomal enzyme if it is present in the tissues (42). Urate oxidase catalyzes an oxidation with oxygen of urate to allantoin and hydrogen peroxide (42). It is used as a peroxisomal marker enzyme (145). Urate oxidase, xanthine dehydrogenase and allantoinase have been reported in peroxisomes from avian and frog liver and avian kidney by Allen and coworkers (134, 151). Tolbert (144)

reported that active xanthine dehydrogenase is not found in peroxisomes from pig and rat kidney and liver. In rat liver, the core observed in peroxisomes is made up of urate oxidase (13, 68, 149, 150). This is probably true for all species with a peroxisomal core and urate oxidase activity (43). Birds, man and other primates lack cores in their peroxisomes and urate oxidase activity (2, 138).

Carnitine Acyl Transferases

Carnitine acetyl transferase and carnitine octanyl transferase have been identified as part of the peroxisomal enzyme complement in rat and pig liver peroxisomes but not in rat and pig kidney (100). These enzymes are also located in the mitochondria and the microsomes. These enzymes are not detected in microbodies, mitochondria or microsomes of plants. The distribution of carnitine acetyl transferase was reported to be: 52% mitochondrial; 14% peroxisomal; and 34% microsomal. Peroxisomal and microsomal activities of carnitine octanyl transferase were approximately equal to carnitine acetyl transferase. The mitochondrial carnitine octanyl transferase exhibited an activity six times greater than the carnitine acetyl transferase (100).

It has been suggested that the carnitine acetyl transferase in peroxisomes and microsomes acts to keep a reservoir pool of carnitine acetyl residues and/or keep acetyl-CoA levels constant (100). The role of carnitine acyl transferases may be to transport acyl residues out across the peroxisomal membrane after fatty acid β-oxidation (89).

NAD:Glycerol-3-P Dehydrogenase

NAD:Glycerol-3-P dehydrogenase is located in peroxisomes isolated from livers of rat, chicken and dogs and from rat kidney (55). This enzyme is not found in spinach leaf peroxisomes. Conversely, no malate dehydrogenase was found in animal peroxisomes but was very active in plant peroxisomes (156). It has been suggested that peroxisomal NAD:glycerol-3-P dehydrogenase acts in a membrane transport shuttle in a manner similar to the glycerol-3-P shuttle between the mitochondria and cytoplasm or to a malate dehydrogenase shuttle in plant peroxisomes (55). The peroxisomal enzyme is different kinetically and has a different mobility on polyacrylamide gels than the cytoplasmic NAD:glycerol-3-P dehydrogenase or the membrane FAD-linked mitochondrial glycerol-3-P dehydrogenase (R. Gee, J. B. Krahling & N. E. Tolbert, in preparation).

Fatty Acid β-Oxidation

Enzymes capable of β-oxidation of fatty acids have been reported in rat (88, 89) and mouse (112) liver peroxisomes. The first enzyme in the sequence, acyl CoA oxidase, has been isolated by Osumi and Hashimoto (117). This enzyme catalyzes a flavin-linked dehydrogenation of the substrate with oxygen uptake to produce hydrogen peroxide. Chain length specificities have been reported (88, 117). The long chain fatty acids (C_{14}-C_{18}) are oxidized best with little or no activity for short chain (C_4-C_8) acyl CoAs. β-Oxidation

activity in peroxisomes can be measured both by oxygen uptake through its catalase linkage in the first step or by substrate dependent NAD reduction in the third step catalyzed by β-hydroxy acyl CoA dehydrogenase (82, 89). Up to five cycles, but not the theoretical seven, have been observed with palmitoyl CoA oxidation by liver peroxisomes (88). This, in part, supports the chain specificity activity of the peroxisomal enzymes. Recently, Shindo and Hashimoto (139) have reported the activity of a fatty acyl CoA synthetase in rat liver peroxisomes, as well as good activity using palmitic acid as a substrate for β-oxidation. Therefore, the liver peroxisomes can activate and oxidize fatty acids.

Hypolipidemic Drugs and Peroxisomes

Feeding hypolipidemic drugs have been reported in numerous cases to proliferate peroxisomes (124-126, 140) and increase liver weight (19). The drugs have also been shown to change some total hepatic enzyme activities (26, 34, 84, 87, 96, 108, 121-123) in rat liver. The effects of clofibrate, a hypolipidemic drug, is selective in increasing enzyme activities. In the male rat liver, the specific activity of carnitine acetyl transferase and carnitine octanyl transferase increased in isolated peroxisomes, mitochondria and microsomes from treated animals. There was also an increase in the mitochondrial carnitine palmitoyl transferase (77, 99). There was no increase in the specific activity of catalase and urate oxidase in the isolated organelle (47, 63, 99). Slight decreases in peroxisomal D-amino acid oxidase and α-hydroxy acid oxidase have been reported with clofibrate

treatment (91). The microsomal NADPH:cytochrome c reductase did not increase in specific activity (91). The flavin-linked glycerol-3-P dehydrogenase has also been reported to increase with hypolipidemic drugs (27, 28, 84).

The decrease in serum triglycerides and neutral steroids has been a known function for hypolipidemic drugs for some time (141), but their mode of action is as yet unknown. A blockage of cholesterol synthesis at both premevalonate (9) and postmevalonate sites (10) has been suggested. Reports of inhibition of fatty acid synthesis at the level of acetyl-CoA carboxylase (97) and acyl-CoA-α-glycerolphosphate acyltransferase (28, 86) have been made. Coenzyme A and its derivatives have been reported to increase (107) with the administration of clofibrate. The effect of hypolipidemic drugs on the availability of acyl CoAs has also been suggested in regulating the hypolipidemic effect (99). More recently, there have been reports of increased fatty acid oxidation in livers of clofibrate fed rats (34, 58, 87, 96, 155).

Peroxisomal proliferation does not seem to be necessary for hypolipidemic effects to be manifested. In female rats, no proliferation of peroxisomes occurs but there is a hypolipidemic response (85) and an increase in the total hepatic carnitine acetyl transferase (108).

Phthalate esters have been reported to proliferate peroxisomes and increase some peroxisomal enzyme activities (109, 118, 119).

In summary, it is known that animal peroxisomes contain various enzymes linked to flavin oxidases and catalase. No defined cycles of futile respiration have been observed in animal peroxisomes as found in plant peroxisomes. There is much inter- and intra-species variation in peroxisomal enzyme content and their response to various agents.

STATEMENT OF THE RESEARCH PROBLEM

This research was designed to examine the involvement of peroxisomes in a proposed energy wasting scheme in the control of calorie utilization by mammals. The animal model chosen was the obese-hyperglycemic mouse (C57BL/6J) and its lean littermate. They were used to determine possible differences in the activities of peroxisomal enzymes for the obese compared to its lean littermate. This animal was chosen because it becomes obese without using calorically dense diets and can be identified by lowered oxygen consumption from its lean littermates before phenotypic expression of obesity. The protocol for this study was to examine the activities of catalase, urate oxidase, palmitoyl-CoA dependent NAD reduction (β-oxidation) and NAD:glycerol-3-P dehydrogenase of peroxisomes isolated from livers of lean and obese mice by isopycnic sucrose density gradient centrifugation.

The data obtained from these experiments will aid in the determination of whether there is a basis for an energy wasting differential between lean and obese animals. A further understanding of peroxisomal involvement in lipid metabolism as well as a connection with obesity has been achieved.

METHODS AND MATERIALS

Male and female lean (C57BL/6J) mice and their genetically obese and hyperglycemic littermates were from Jackson Memorial Laboratory, Bar Harbor, Maine. For comparison and development of procedures, six week old white [HA(ICR)] male mice from Spartan Research, Haslett, Michigan, were used.

Total Hepatic Catalase Content in Crude Homogenates

Total hepatic catalase content in crude homogenates of liver in these mice was examined initially in this research. The effect of clofibrate (CPIB or p-chloro-phenoxyisobutyrate ethyl ester) on these animals was also explored.

Two month old male mice were separated into four groups: lean animals fed the control diet; obese animals fed the control diet; CPIB-fed lean animals; and CPIB-fed obese animals. The groups were pair-fed to the obese mice fed the CPIB diet. The control diet consisted of ground Wayne Lablox (Allied Mills, Chicago, Illinois). The CPIB diet consisted of ground Wayne Lablox and 0.5% (w/w) clofibrate (Ayerst Laboratories, Inc., New York). An additional two groups of mice, lean and obese, were fed the CPIB diet ad libitum. For comparison, the male HA (ICR) mice of 6-8 weeks of age were used to examine the effect of CPIB feeding. These mice were divided into two groups, control and CPIB-fed, ad libitum. These animals were fed the same

batch preparation as the lean and obese mice. All animals were fed this diet for two weeks prior to sacrifice.

The mice were fasted overnight to reduce liver glycogen. The animals were killed by decapitation, the liver rapidly exposed and perfused with cold homogenizing buffer (8.5% sucrose, 0.01 M phosphate, pH 7.5). Portions of the livers from individual animals were processed for peroxisome visualization by electron microscopy according to the procedure of Fahimi (49) and Novikoff et al. (114) using diaminobenzidine.

The weighed livers were minced individually with scissors and homogenized in a loosely-fitting mechanically driven Potter-Elvehjem homogenizer. The homogenate was filtered through miracloth and the volume measured. This material was taken to determine the catalase activity and protein concentration.

Catalase activity was measured spectrophotometrically at 240 nm by following the rate of loss of a standardized solution of H_2O_2 (145). Protein was determined according to the method of Lowry et al. (95).

Examination of Isolated Organelles from Lean and Obese Mice

Obese and lean littermate control mice, 8-12 weeks of age, and HA (ICR) male mice, 6-7 weeks of age, were used to study enzyme activities in isolated organelles as described by Murphy et al. (112, Appendix A). In addition, a second catalase peak of activity from zonal gradients of lean male mouse liver were examined by electron

microscopy. The fraction was fixed in 12.5% glutaraldehyde, 50% sucrose, 0.5 mM EDTA, pH 7.5, for 30 minutes at 4° C. The fixed solution was diluted 1:1 with washing buffer (12.5% glutaraldehyde, 0.5 mM EDTA, pH 7.5) and centrifuged at 40,000 rpm in a Ti 60 rotor for the Beckman L-2 centrifuge for 30 minutes at 4° C. The pellet was washed and collected in 25% sucrose, 0.5 mM EDTA, pH 7.5. The isolated peak was then postfixed with 1% OsO_4 in 0.10 M cacdylate, pH 7.5, overnight. The fixed fraction was processed for electron microscopy. Ultrathin sectioning was done on a LKB 4801A ultramicrotome. The sections were stained with saturated uranyl acetate for 30 minutes and counter stained with lead citrate for 10 minutes. The sections were examined in a Zeiss 100 or a Philips 200 electron microscope.

Comparison of Peroxisomal and Mitochondrial Fatty Acid β-Oxidation

To compare the distribution and magnitude of fatty acid β-oxidation in lean and obese mice as well as the HA(ICR) mice, similar procedures were used for organelle separation as previously described (112, Appendix A) with the following modifications. (1) For obese mice, one liver was used per experiment and distributed in three gradient centrifuge tubes. For lean C57BL/6J and HA(ICR) mice, because of the smaller liver size, two livers were pooled per experiment. (2) For better estimation of the efficiency of tissue homogenation, a fraction of the total homogenate was saved for further thorough homogenation in a Ten-Broeck homogenizer. The remainder of the homogenate was filtered through Miracloth (Chicopee Mills, Inc.,

Milltown, New Jersey), and the organelle separation was carried out as described (112, Appendix A). The total homogenate was used to determine protein and enzyme activities to compare with the limited homogenation of the organelle preparation. (3) An initial centrigugation at 5,000 rpm for 15 minutes was included in the fractionation procedure followed by the 25,000 rpm centrifugation for 3 hours as previously described (112, Appendix A). (4) After development in the centrifuge, the gradients were collected in 1 ml fractions from the top of the tube using a modified ICR tube gradient pump and a Gilson fraction collector.

Marker enzyme activities were determined as previously described (112, Appendix A). Fatty acid β-oxidation was measured spectrophotometrically by NAD reduction as previously described (112, Appendix A) and poloragraphically by oxygen uptake according to Krahling et al. (82). NAD:Glycerol-3-P dehydrogenase activity was measured spectrophotometrically as used previously (112, Appendix A).

Lean and obese male and female mice were used in this portion of the research. All animals were deprived of food for 18 hours prior to sacrifice (from 3 p.m. the preceding afternoon to 9 a.m. the following morning) in all fasted animals. Fed animals were allowed free access to food up until time of sacrifice.

Statistics

Experimental means were compared using Student's t test except for fatty acid β-oxidation data (Table 9) where factorial analysis of variance was employed.

RESULTS

Total Hepatic Catalase Activity

The liver weights and protein contents of the livers are given in Table 1. Obese animals had obviously larger and more fatty livers by visual inspection, as well as larger liver weights. The obese animals fed the control diet had higher protein content than the lean mice fed the control diet. The mice fed the CPIB diet had increased liver weights compared to their ad libitum fed control mice, but there was no increase in liver protein. Thus, in the C57BL/6J strain of mice, clofibrate did induce the reported increase in liver weight but not in protein concentration. Catalase activity did not increase in pair-fed C57BL/6J mice or these ad libitum fed mice in contrast to data reported for other mice (123, 124). The data is presented in Table 2.

Obese C57BL/6J mice fed the control diet had significantly less catalase activity per g liver but significantly higher specific activity than the lean C57BL/6J mice. Clofibrate feeding did not increase catalase content either on a pair-fed basis or an ad libitum basis. In fact, clofibrate seemed to enhance the small difference in activity observed between lean and obese mice.

When HA(ICR) mice were fed the same diet from the same batch preparation used for the lean and obese C57BL/6J mice, the

Table 1. Liver weight and protein from livers of clofibrate pair-fed C57BL/6J lean and obese male mice

	N	Liver weight (g)	Protein mg/g liver
Control			
Lean	7	$1.14 \pm 0.14^{a,b}$	121 ± 20^{a}
Obese	7	$2.13 \pm 0.28^{a,d}$	174 ± 18^{a}
Clofibrate-fed			
Lean	6	$1.60 \pm 0.19^{b,c}$	132 ± 37^{e}
Obese	7	$2.93 \pm 0.35^{c,d}$	163 ± 17^{e}

Note: Values sharing common superscripts are significantly different at level tested:

$p < .001^{a,c,d}$

$p < .005^{b}$

$p < .05^{e}$.

Table 2. Total hepatic catalase activity in clofibrate-fed C57BL/6J lean and obese male mice and HA(ICR) male mice

Animal	n	Catalase	
		mmol min^{-1} g $liver^{-1}$	mmol min^{-1} mg $protein^{-1}$
Pair-fed			
Control lean	7	6.80 ± 1.53^{b}	79.4 ± 3.6^{b}
Control obese	7	5.23 ± 0.50^{b}	$90.4 \pm 11.6^{b,d}$
CPIB lean†	6	5.35 ± 1.59	71.8 ± 15.1
CPIB obese	7	4.61 ± 0.99	75.7 ± 20.2^{d}
Ad lib--fed CPIB			
Lean	7	2.34 ± 0.37^{a}	21.2 ± 2.8
Obese	6	1.81 ± 0.14^{a}	17.8 ± 1.1
Ad lib--fed HA(ICR)			
Control	7	2.75 ± 0.51^{c}	25.1 ± 5.5^{a}
CPIB	7	4.11 ± 0.98^{c}	45.9 ± 14.8^{a}

†CPIB = clofibrate (chloro-p-phenoxyosobutyrate ethyl ester).

Note: Values sharing common superscripts are significantly different at level tested:

$p < .01^{a,c}$

$p < .05^{b,d}$.

increase in catalase activity was observed as previously reported (123, 124) relative to the control.

It was concluded that clofibrate does not affect peroxisomal catalase in the C57BL/6J male mice as it does in other strains of mice (123, 124). It does, however, increase liver weight in the C57BL/6J mice.

Electron microscope examination of thin sections from control lean and obese C57BL/6J mice showed no gross difference in the numbers of peroxisomes, i.e., no higher or lower number in the obese animal, as shown in Figures 1 and 2. Pictures of livers of clofibrate-fed animals showed no increase in peroxisome numbers compared to the respective controls (Figures 3 and 4). These pictures indicate there was no apparent difference in the numbers of peroxisomes between lean and obese C57BL/6J mice and no increase in peroxisome numbers on clofibrate feeding at 0.5% of the diet as reported for other rodents (84, 108, 121-126, 140).

Isolation of Hepatic Mouse Peroxisomes

Cell organelles in liver homogenates from fasted male HA(ICR) mice (Figure 5) and fasted lean and obese C57BL/6J mice (Figure 6) were separated on isopycnic sucrose density gradients. The peroxisomes, identified by catalase and urate oxidase, were well separated from the mitochondria, identified by glutamate dehydrogenase and β-hydroxybutyrate dehydrogenase. The lysozomal marker, acid phosphate, was primarily located in the mitochondrial peak with a small amount of tailing into the peroxisomal area (data not shown). The microsomes,

Figure 1. Liver tissue from lean C57BL/6J male mouse. Peroxisomes (p) shown by dense staining with diaminobenzidine. Magnification = 38,000X.

Figure 2. Liver tissue from obese C57BL/6J male mouse. Same treatment as Figure 1. Magnification = 18,000X.

Figure 3. Liver tissue from lean CPIB-fed C57BL/6J male mouse. Same treatment as Figure 1. Magnification = 18,000X.

Figure 4. Liver tissue from obese CPIB-fed C57BL/6J male mouse. Same treatment as Figure 1. Magnification = 22,000X.

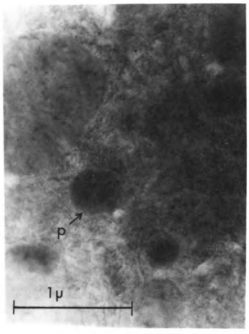


Figure 1

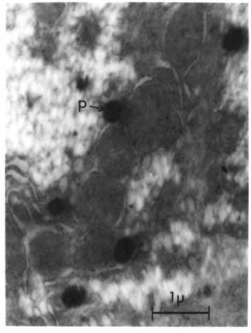


Figure 2

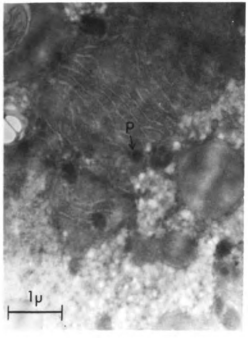


Figure 3

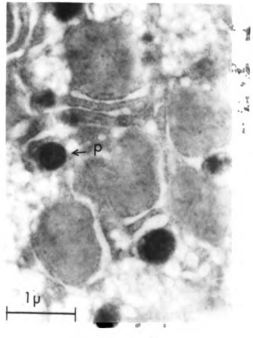


Figure 4

Figure 5. Tube gradient profile for fasted HA(ICR) male mouse containing approximately 1.0 g liver.

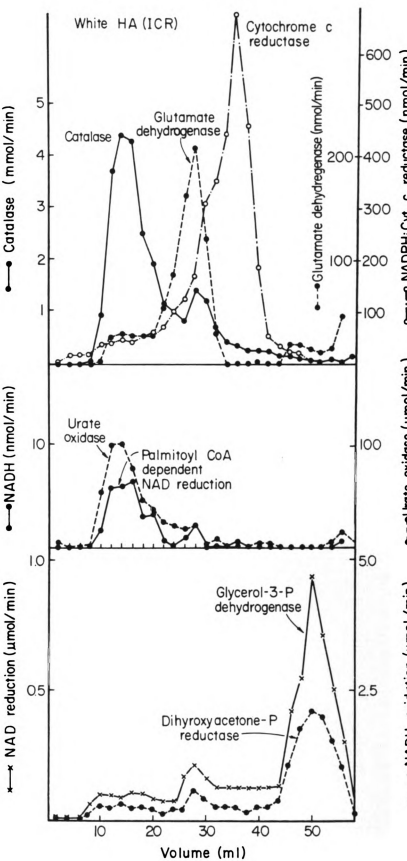
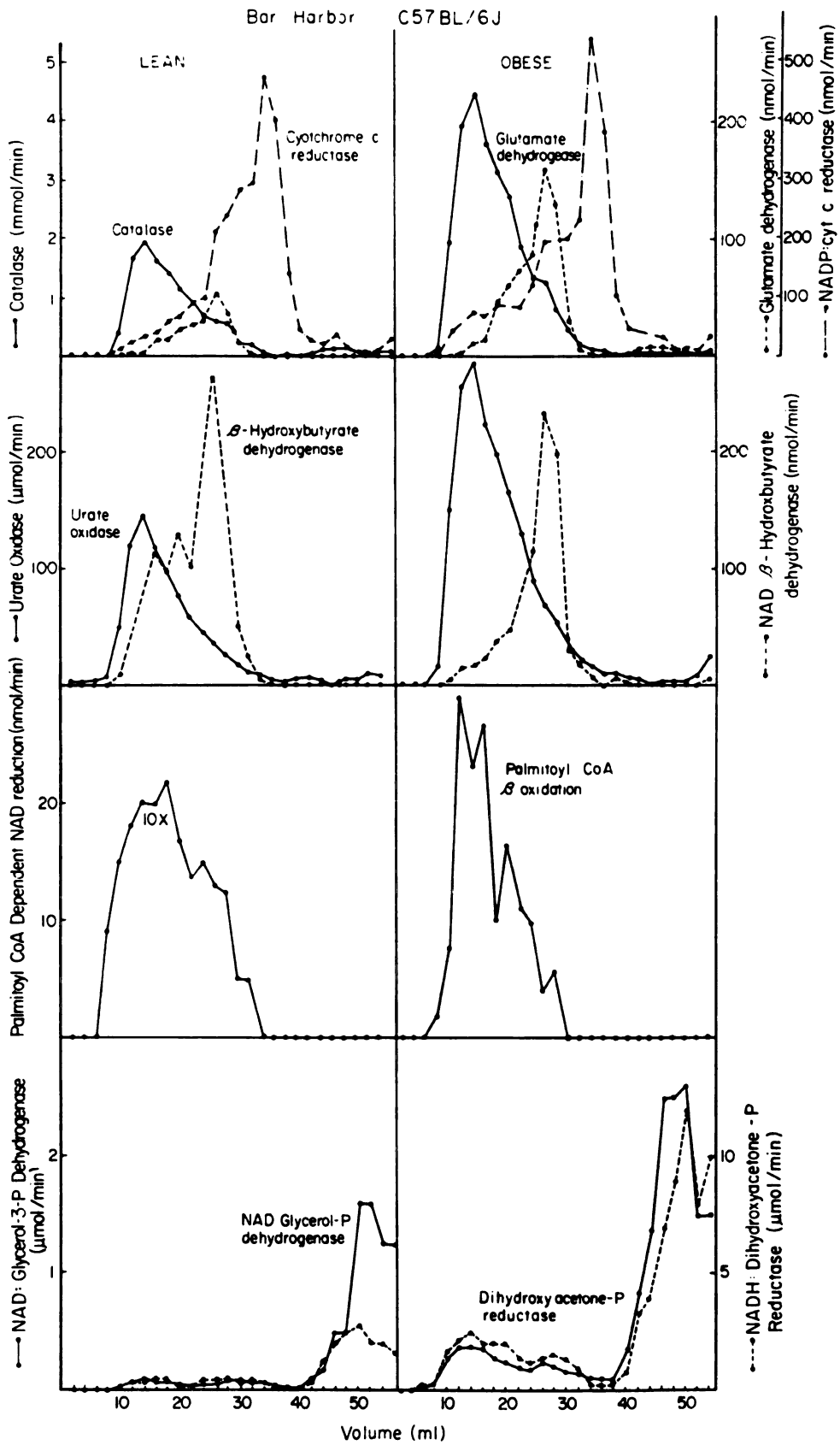

White HA (ICR)
Cytochrome c reductase
Catalase
Glutamate dehydrogenase
Catalase (m mol/min)
Glutamate dehydrogenase (nmol/min)
NADPH-Cyt. c reductase (nmol/min)
Urate oxidase
Palmitoyl CoA dependent NAD reduction
NADH (nmol/min)
Urate oxidase (µmol/min)
Glycerol-3-P dehydrogenase
Dihyroxyacetone-P reductase
NAD reduction (µmol/min)
NADH oxidation (µmol/min)
Volume (ml)

Figure 6. Tube gradient profile for lean and obese C57BL/6J fasted male mice containing approximately 0.7 and 1.5 g of liver, respectively.


Bar Harbor C57BL/6J
LEAN
OBESE
Catalase
Cytochrome c reductase
Glutamate dehydrogease
Urate oxidase
β-Hydroxybutyrate dehydrogenase
10X
Palmitoyl CoA β oxidation
NAD Glycerol-P dehydrogenase
Dihydroxy acetone-P reductase
Catalase (mmol/min)
Glutamate dehydrogenase (nmol/min)
NADP:cyt c reductase (nmol/min)
Urate Oxidase (μmol/min)
NAD β-Hydroxybutyrate dehydrogenase (nmol/min)
Palmitoyl CoA Dependent NAD reduction (nmol/min)
NAD: Glycerol-3-P Dehydrogenase (μmol/min)
NADH: Dihydroxyacetone - P Reductase (μmol/min)
Volume (ml)

identified by NADPH:cytochrome c reductase, equilibrated at a lower sucrose density. The observed equilibrium densities of peroxisomes and mitochondria in the gradients from numerous preparations of mouse organelles is presented in Table 3. In the rat, peroxisomes are slightly denser and the mitochondria are slightly lighter (145) than in the mouse so that the two organelle populations from the mouse are not as far apart on a sucrose gradient.

A comparison of the data presented in Figures 5 and 6 indicates that sharper bands of organelles were obtained with the HA(ICR) mice and thus better separation of peroxisomes and mitochondria as compared to the C57BL/6J mice. This was repeatedly observed and no explanation can be offered other than biological variation. Data in Figures 5 and 6 as well as Tables 4 and 5 show that fatty acid β-oxidation activity in the hepatic peroxisomal fraction from the HA(ICR) mice were 3-4 times more active than comparable fractions from the lean C57BL/6J mice. Comparisons were made between the lean and obese and between the lean C57BL/6J mice and the HA(ICR) mice.

Table 3. Equilibrium density of mouse liver organelles at 4° C

Animal	n	Peroxisomes	Mitochondria
Lean	5	1.2348 ± .0038	1.1972 ± .0101
Obese	9	1.2287 ± .0049	1.1918 ± .0105
HA(ICR)	3	1.2293 ± .0098	1.1867 ± .0098

Table 4. Total hepatic enzyme activity of male lean and obese mice (C57BL/6J) and male HA(ICR) mice

Enzyme		n	Per mg protein in peak fraction	Per g liver	Per liver	Per 100 g body weight
Catalase	Lean	5	4.3 ± 1.2	51 ± 20	78 ± 28[a,b]	290 ± 96[b]
(mmol/min)	Obese	5	5.9 ± 1.7	51 ± 14[b]	224 ± 80[a,d]	421 ± 146
	HA(ICR)	3	3.1 ± 3.2	74 ± 16[b]	119 ± 11[b,d]	452 ± 20[b]
Glutamate dehydrogenase	Lean	4	.038 ± .007	1.1 ± 0.7	1.7 ± 0.9[a]	6.4 ± 3.5
(μmol/min)	Obese	4	.047 ± .010	1.3 ± 0.4	5.6 ± 1.5[a,b]	10.4 ± 1.9
	HA(ICR)	3	.034 ± .005	1.7 ± 0.3	2.7 ± 0.5[b]	10.3 ± 2.5
Urate oxidase	Lean	4	.28 ± .08	3.5 ± 1.2	5.2 ± 1.7[a]	19.5 ± 7.5
(μmol/min)	Obese	4	.34 ± .11	3.2 ± 1.4	13.0 ± 4.7[a]	24.7 ± 8.2
Palmitoyl CoA oxidation	Lean	5	9 ± 3[a]	263 ± 143[a,b]	309 ± 199[a,b]	1536 ± 851[a,b]
(nmol/min)	Obese	4	28 ± 12[a,b]	468 ± 59[b,c]	2027 ± 461[a]	3800 ± 734[b]
	HA(ICR)	3	6 ± .2[b]	1210 ± 260[a,c]	1800 ± 730[b]	7190 ± 3120[a]

Note: Values sharing common superscripts are significantly different at level tested:
$p < .01^{a,c}$
$p < .05^{b,d}$.

Table 5. Liver and body weights of 2-3 month old mice

Animal	n	Liver (g)	Body (g)
Male			
Lean	5	$1.56 \pm .15^{a,c}$	26.56 ± 2.32^{b}
Obese	5	$4.36 \pm .64^{b,c}$	53.19 ± 5.20
HA(ICR)	3	$1.54 \pm .25$	26.25 ± 1.85
Female			
Lean	5	$1.23 \pm .34^{a,d}$	21.30 ± 1.48^{b}
Obese	3	$2.73 \pm .50^{b,d}$	49.13 ± 2.31

Note: Values sharing common superscripts are significantly different at level tested:

$p < .05^{a}$

$p < .01^{b,c,d}$.

With both the HA(ICR) and the C57BL/6J mice, less than 5% of the catalase or urate oxidase activity was present in the supernatant fraction. Almost all of the activity was in or tailed into the peroxisomal peak indicating that there may have been little peroxisomal breakage during homogenation. These results differ from those of the rat where about 50-75% of the catalase is in the supernatant and is attributed to peroxisomal fragility or to two subcellular locations of catalase (74). Inclusion of 0.01% ethanol in the gradient, which has been reported to prevent its inactivation by the formation of an inactive peroxide complex (37), did not increase the catalase activity. Data from mouse gradients, particularly the lean mice, indicate some of the peroxisomal material tailed into the mitochondrial area with

a minor peak slightly less dense than the mitochondria (Figure 6). The identity of this material has been examined by electron microscopy (Figure 7). In gradients from both the rat and the mouse liver, the presence of some of the peroxisomal marker enzyme activity in less dense sucrose than the main peroxisomal band appears to be due to an area containing a mixture of microsomes and peroxisomes.

The profile of the mitochondrial matrix enzyme, glutamate dehydrogenase, and the mitochondrial membrane enzyme, β-hydroxybutryrate dehydrogenase, coincide. This indicated that no significant amount of single membrane mitochondrial fragments were present.

Differences in Hepatic Peroxisomal Marker Enzymes

The obese C57BL/6J mice at 3 months of age averaged 53 g body weight and 4.4 g liver weight. On the average, they were two times heavier than their lean littermates and their livers were about three times larger. The HA(ICR) and the lean C57BL/6J mice had the same liver weight and body weight on the average. Under these circumstances, the enzymatic data from the HA(ICR) mice and the C57BL/6J lean and obese mice have been reported as specific activity in the isolated subcellular organelle from the sucrose gradient and as total activity per g liver, per liver and 100 g body weight.

The specific activity of the marker enzymes in the isolated peroxisomes (catalase and urate oxidase) and the mitochondria (glutamate dehydrogenase) and the total activity per g liver were the same in the obese and lean C57BL/6J and the HA(ICR) mice (Table 4). These

Figure 7. Second catalase activity peak from lean C57BL/6J male mouse gradient showing microsomes and peroxisomes. Magnification = 12,000X.

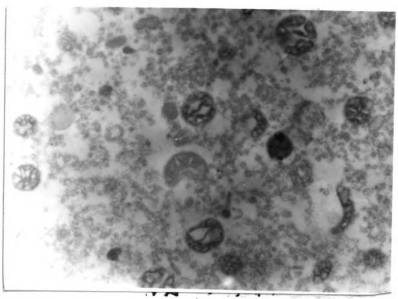


Figure 7

similarities were true even though the obese mice had larger and more fatty livers at three months of age. Consequently, the total peroxisomal and mitochondrial activity increased per liver from the obese mice in proportion to the liver size compared with the lean C57BL/6J but not the HA(ICR). Approximately the same fraction of the liver, about one-third, was used for each gradient so the obvious increase in Figure 6 in the total activity in the obese liver was due to the increased liver size.

An interesting comparison was made between the lean C57BL/6J mice and the HA(ICR) which showed a higher total catalase activity per liver in the HA(ICR) mice even though the liver size is the same in the two animals (Table 5). This increased activity was even more apparent when expressed on a per 100 g body weight basis. The obese C57BL/6J mice still had higher activity per liver for catalase and glutamate dehydrogenase than the HA(ICR) mice but on a per g liver basis, the reverse was found for catalase activity. This indicates that the HA(ICR) mice have a different enzymatic makeup than the lean C57BL/6J even though both animals are lean.

Total hepatic marker enzyme activities for male and female C57BL/6J lean and obese mice are compared in Table 6. No differences were found between the male and female animals with the marker enzyme activities on a per g liver basis. The differences observed between lean and obese male mice are seen again in a comparison of the lean and obese female mice. This is believed to be due to the large size of the liver of the obese animal.

Table 6. Total hepatic marker enzyme activity of fasted male versus female C57BL/6J mice

Enzyme	Animal	n	Per g liver	Per liver	Per 100 g body weight
Catalase (mmol/min)	Lean, male	5	51 ± 20	78 ± 29	290 ± 96
	Lean, female	3	47 ± 13	59 ± 28	272 ± 107
	Obese, male	5	51 ± 14	224 ± 80*	421 ± 146
	Obese, female	3	45 ± 5	128 ± 25	261 ± 44
Urate oxidase (μmol/min)	Lean, male	4	3.5 ± 1.2	5.2 ± 1.7*	19.5 ± 7.5
	Lean, female	3	2.5 ± 0.9	2.9 ± 0.4	13.4 ± 1.8
	Obese, male	4	3.2 ± 1.4	13.0 ± 4.7	24.7 ± 8.2
	Obese, female	3	2.8 ± 1.1	7.2 ± 1.8	14.7 ± 4.1
Glutamate dehydrogenase (μmol/min)	Lean, male	4	1.1 ± 0.7	1.7 ± 0.9	6.4 ± 3.5
	Lean, female	3	0.7 ± 0.3	0.8 ± 0.4	3.7 ± 1.7
	Obese, male	4	1.3 ± 0.4	5.6 ± 1.5**	10.4 ± 1.9**
	Obese, female	3	1.1 ± 0.1	3.0 ± 0.5	6.1 ± 1.0

*$p < .05$.

**$p < .01$.

In comparing the data between male and female mice, it is important to consider the differences in body weight and liver weight between the male and female mice. Lean female mice have a smaller liver size then the lean male (Table 5). The obese female also has a smaller liver size than the obese male. On a body weight basis the lean female had a smaller body size than the lean male. The obese female, however, had a similar body size than the obese male mouse.

Enzyme activities for the peroxisomal marker enzymes, catalase and urate oxidase, changed differently between lean male and female C57BL/6J mice. Catalase, in the lean animals showed no difference on the basis of sex. In the obese animals, catalase increased in the male animal due to the larger liver size, however, no difference was measured on a per 100 g body weight basis.

Urate oxidase activity was different between the male and female lean mice on a per liver basis but not between the obese male and female mice. In neither comparison was there a difference on a per 100 g body weight basis.

The activity of glutamate dehydrogenase, the mitochondrial marker enzyme, changed similarly to catalase. There was no apparent difference in activity between the lean male and female mice. On a per liver basis, glutamate dehydrogenase was higher in the male obese animals than in the obese female mice. This difference was large enough to effect a significant difference to the body weight comparison.

The male and female mice have a similar marker enzyme distribution on a per g liver basis. The lean and obese female mice differ

in the same way as the lean and obese male mice. The differences in body and liver size seem to cause some, but not all, of the differences observed between the male and female mice on a per liver basis and on a per 100 g liver basis.

The marker enzyme activities between fed and fasted male mice are shown by the data in Table 7. No differences were observed in the activity levels due to fed versus the fasted state. The same differences in activity based on a per liver basis were seen in comparing the fed lean and obese males as seen in comparing the fasted lean and obese males.

Comparison of the Matrix and Membrane Associated Enzymes in the Lean and Obese C57BL/6J Mice

Catalase and urate oxidase of the peroxisomes and glutamate dehydrogenase of the mitochondria are located in the matrix of these organelles and were not different in specific activity between the lean and obese animals. In contrast, the membranous enzymes, NADPH:cytochrome c reductase of the microsomes and β-hydroxybutyrate dehydrogenase of the mitochondria, decreased two to threefold per g liver in the obese mice. For these membranous enzymes, there was no difference per liver as shown by the data in Figure 6, even though the livers from the obese mice were about three times larger. Thus, there appears to be a difference of hepatic matrix enzymes of the organelles but no difference in the membrane components with obesity.

Table 7. Total hepatic marker enzyme activity of fed versus fasted C57BL/6J male mice

Enzyme	Animal	n	Per g liver	Per liver	Per 100 g body weight
Catalase (mmol/min)	Lean, fed	3	56 ± 2	95 ± 18[a]	353 ± 84
	Lean, fasted	5	51 ± 20	78 ± 29	290 ± 96
	Obese, fed	3	52 ± 1	204 ± 103[a]	116 ± 122
	Obese, fasted	5	51 ± 14	224 ± 80	421 ± 146
Glutamate dehydrogenase (μmol/min)	Lean, fed	3	0.7 ± 0.2	1.2 ± 0.3[a]	4.6 ± 0.9
	Lean, fasted	4	1.1 ± 0.7	1.7 ± 0.9	6.4 ± 3.5
	Obese, fed	3	1.1 ± 0.3	3.8 ± 2.9[a]	8.7 ± 0.9
	Obese, fasted	4	1.3 ± 0.4	5.6 ± 1.5	10.4 ± 1.9
Urate oxidase (μmol/min)	Lean, fed	2	2.5 ± 0.9	4.4 ± 1.1[b]	16.8 ± 4.1
	Lean, fasted	4	3.5 ± 1.2	5.2 ± 1.7	19.5 ± 7.5
	Obese, fed	2	2.3 ± 0.5	6.7 ± 0.9[b]	16.2 ± 0.8
	Obese, fasted	4	3.2 ± 1.4	13.0 ± 4.7	24.7 ± 8.2

Note: Values sharing common superscripts are significantly different at level tested:

$p < .10^{a}$

$p < .05^{b}$.

Comparison of β-Oxidation in the Lean and Obese C57BL/6J and HA(ICR) Mice

The assay procedure for peroxisomal palmitoyl-CoA dependent reduction of NAD did not measure the mitochondrial β-oxidation (82, 88, 89). The palmitoyl-CoA dependent NAD reduction shown by the data in Figures 5 and 6 coincided with the distribution of the peroxisomal catalase. These results with the mouse extend the observation made by Lazarow and de Duve (89) in the rat that liver peroxisomes catalyze β-oxidation. The peroxisomal β-oxidation was 5-6 times more active per g liver, per liver and per 100 g body weight in the HA(ICR) mice compared to the lean C57BL/6J mice (Figures 5 and 6, Table 4). The specific activity did not exhibit a significant difference. Total fatty acid β-oxidation in the peroxisomes for the obese mouse livers was about twice as much per g of liver as in the lean mice. This resulted in a fivefold difference in total β-oxidation on a per liver basis. This increase was pronounced in specific activity of the isolated peroxisomes (Table 4). These results indicated a higher activity for β-oxidation in liver peroxisomes of the obese mice even though the other peroxisomal enzymes were not higher per g liver or per mg protein.

Comparison of the HA(ICR) mice to the obese C57BL/6J mice indicated a higher specific activity of fatty acid β-oxidation in peroxisomes from obese mice, but threefold higher total activity per g liver in the HA(ICR) mice. On a per animal basis, expressed as either per liver or per 100 g body weight, the HA(ICR) mice and the obese C57BL/6J mice have an equal capacity to oxidize fatty acids in the peroxisomes.

Comparison of the Organelle Distribution of Fatty Acid β-Oxidation in the Mouse

The gradient profiles for fed male mice and fasted female mice are shown in Figures 8 and 9. The marker enzymes, catalase and urate oxidase for the peroxisomes and glutamate dehydrogenase for the mitochondria, are fairly well separated for each other. There was significant mitochondrial contamination of the peroxisomal peak in the lean male and female mice. In comparing mitochondrial and peroxisomal β-oxidation, the assays used measured only the activity of the enzyme from the organelle indicated (82).

In the mouse, there is approximately an equal distribution of β-oxidation between the two organelles. This is apparent in Figures 8 and 9 and in Table 8.

NAD Reduction Assay for Fatty Acid β-Oxidation

When β-oxidation was measured in the peroxisomal fraction by the rate of NAD reduction, the only significant differences observed were between the HA(ICR) mice and the lean and obese C57BL/6J mice. In both cases, the HA(ICR) mice had higher activity than the lean or obese. There was no significant difference in the activity between the lean and obese mice whether fed or fasted in this experiment. This is in contrast to data already reported. When the data from both experiments is combined, no significant difference was found due to the large variance in the data. No apparent difference was observed in peroxisomal activity between male and female mice.

Figure 8. Tube gradient profile for lean and obese C57BL/6J fed male mice containing 0.82 and 0.94 g liver, respectively.

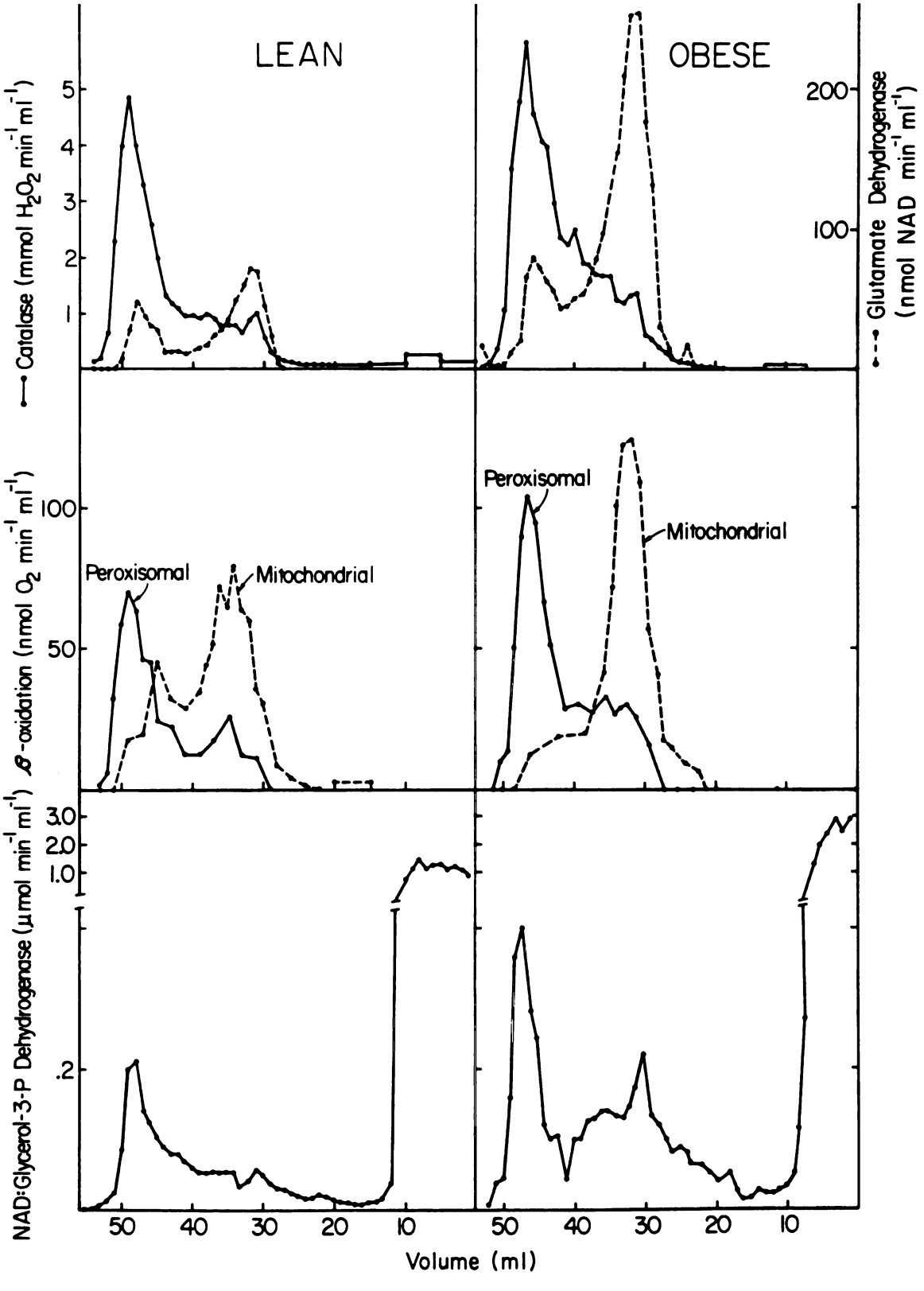

LEAN
OBESE
Catalase (mmol H_2O_2 min^{-1} ml^{-1})
Glutamate Dehydrogenase (nmol NAD min^{-1} ml^{-1})
β-oxidation (nmol O_2 min^{-1} ml^{-1})
Peroxisomal
Mitochondrial
NAD:Glycerol-3-P Dehydrogenase (μmol min^{-1} ml^{-1})
Volume (ml)

Figure 9. Tube gradient profile for lean and obese C57BL/6J fasted female mice containing 0.35 and 0.69 g liver, respectively.

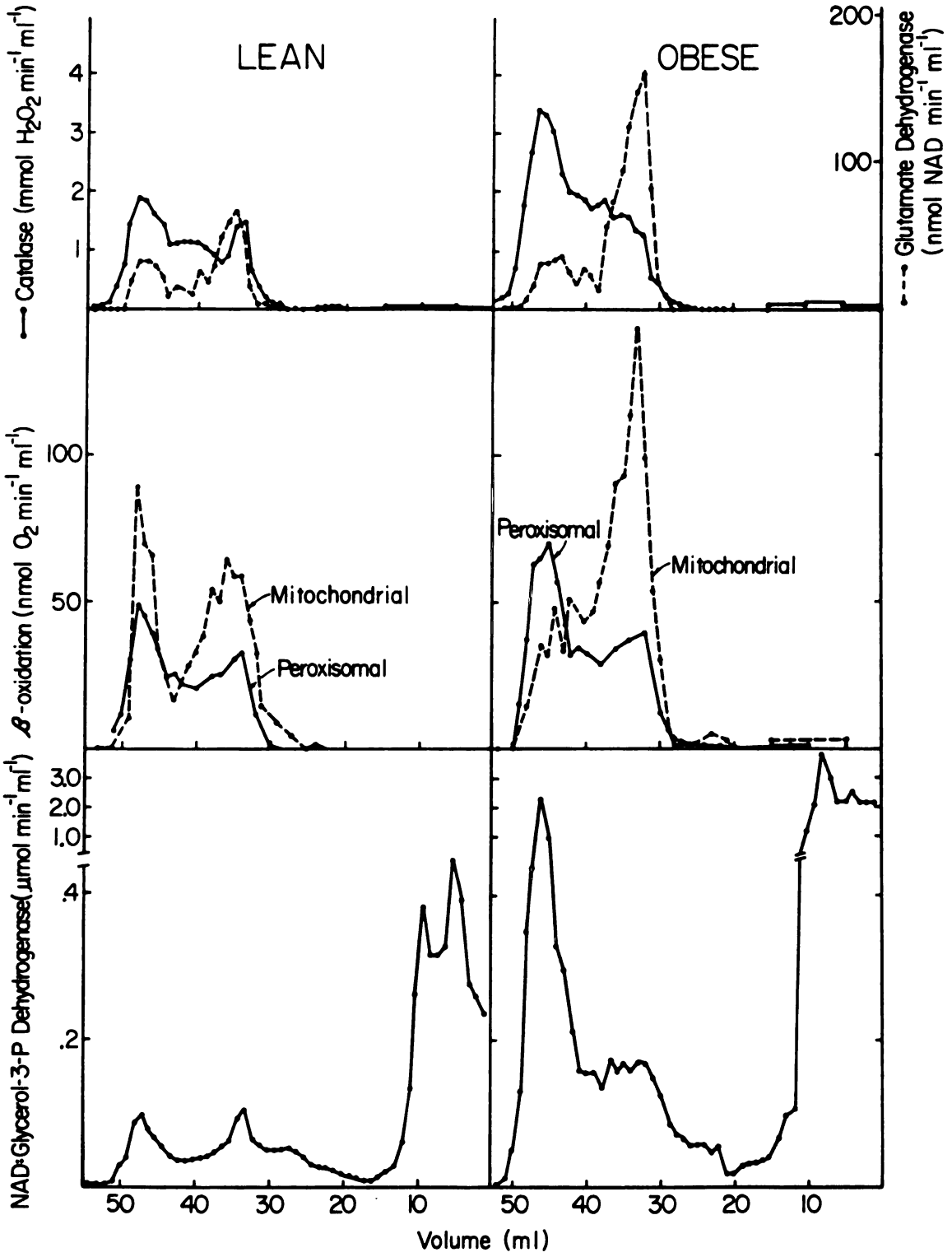

LEAN
OBESE
Catalase (mmol H_2O_2 min^{-1} ml^{-1})
Glutamate Dehydrogenase (nmol NAD min^{-1} ml^{-1})
β-oxidation (nmol O_2 min^{-1} ml^{-1})
Mitochondrial
Peroxisomal
NAD-Glycerol-3-P Dehydrogenase (μmol min^{-1} ml^{-1})
Volume (ml)

Table 8. Hepatic β-oxidation: Palmitoyl CoA dependent

	μmol min^{-1} g $liver^{-1}$				
	Peroxisome		Mitochondria	Mitochondria + peroxisome	Mitochondria / peroxisome
Animal	NAD	O_2	$O_2/2$	O_2	O_2
Male					
Fasted, lean	$0.35 \pm .14^{a}$	$0.98 \pm .30^{b}$	$1.26 \pm .38^{d,f}$	$2.34 \pm .33^{b,d}$	$1.43 \pm .84$
Fasted, obese	$0.54 \pm .23^{c}$	$1.01 \pm .18^{d}$	$1.01 \pm .27^{b,e}$	$2.02 \pm .25^{e,f}$	$1.05 \pm .40$
Fed, lean	$0.48 \pm .10$	$0.98 \pm .41$	$0.65 \pm .10^{f}$	$1.63 \pm .45^{b}$	$0.72 \pm .26$
Fed, obese	$0.33 \pm .09$	$0.92 \pm .24$	$0.71 \pm .19^{b}$	$1.63 \pm .20^{f}$	$0.85 \pm .45$
Fasted, HA(ICR)	$1.21 \pm .26^{a,c}$	$2.07 \pm .98^{b,d}$	$3.17 \pm 1.85^{d,e}$	$4.91 \pm 2.77^{d,e}$	$1.44 \pm .34$
Female					
Fasted, lean	$0.49 \pm .16$	$1.20 \pm .50$	$0.92 \pm .43$	$2.12 \pm .93$	$0.77 \pm .04$
Fasted, obese	$0.82 \pm .26$	$1.34 \pm .42$	$0.85 \pm .02$	$2.19 \pm .45$	$0.67 \pm .19$

n = 3 except for females n = 2.

Note: Values sharing common superscripts are significantly different at level tested in factorial analysis of variance:

$p < .01^{a,c}$

$p < .02^{b,d,e,f}$

Peroxisomal Oxygen Uptake Assay for Fatty Acid β-Oxidation

The activity of the fatty acid oxidation as measured by oxygen uptake demonstrated no significant difference between the lean and obese or the male and female C57BL/6J mice. The fasted HA(ICR) mice had higher activity than either the fasted lean or obese mice.

There was always a consistently higher fatty acid β-oxidation activity as determined by the oxygen uptake assay than by the NAD reduction assay. No NADH oxidase activity, either palmitoyl CoA independent or dependent, was measured in the isolated peroxisomal fraction.

Mitochondrial Oxygen Uptake Assay for Fatty Acid β-Oxidation

There was a significant difference between lean fed and fasted mice and between fed and fasted obese male mice in mitochondrial fatty acid β-oxidation activity. The increase due to fasting indicates that it is the mitochondrial enzyme that changes due to this diet manipulation. There was no difference in the mitochondrial fatty acid β-oxidation activity between the lean and obese or male and female animals. Again the fasted HA(ICR) mice had higher fatty acid oxidation activity in the mitochondria than the lean or the obese C57BL/6J mice.

When the peroxisomal and mitochondrial oxygen uptake data were combined for a total activity figure, only the activity for the fed versus the fasted animals was significantly higher between lean and

obese animals compared. The fasted HA(ICR) mice had higher total β-oxidation capacity when compared with the fasted lean or obese animals.

Mitochondrial/Peroxisomal Ratio of Fatty Acid β-Oxidation Activity

In the mouse, the ratio of mitochondrial to peroxisomal β-oxidation was approximately one. There was no significant difference in the ratio of mitochondrial to peroxisomal activity in comparisons between any group of mice. These data indicate that there was no difference between any compared group in the site of fatty acid oxidation. Thus, there appears to be no apparent difference in the distribution of fatty acid β-oxidation capacity between the lean and obese mice, fasted and fed mice, lean C57BL/6J and HA(ICR) mice or male and female mice.

Comparison of NAD:Glycerol-3-P Dehydrogenase from Lean and Obese Mice

The profile of NAD:glycerol-3-P dehydrogenase activity across the gradients are shown in Figures 5, 6, 8 and 9. The activity of NAD:glycerol-3-P dehydrogenase is summarized in Table 9 and expressed on a per g of liver basis. The data have been presented as total activity distributed between cytosolic enzyme and peroxisomal enzyme. Dr. Robert Gee in our laboratory has been investigating these two activities. He reports that the cytosolic and peroxisomal forms are isoenzymic. They have similar mobilities during thin layer isoelectric focusing but different mobilities of 7% polyacrylamide gels. The

Table 9. Hepatic NAD:Glycerol-3-P dehydrogenase

Animal	n	μmol NAD mm^{-1} g $liver^{-1}$ Total	Supernatant	Peroxisome	% Peroxisome
Male					
Lean, fed	3	$21.8 \pm 6.1^{b,d}$	18.8 ± 4.8^{b}	3.0 ± 1.3^{f}	13.3 ± 1.8^{b}
Lean, fasted	5	$12.9 \pm 5.0^{b,e}$	10.0 ± 5.0^{b}	2.5 ± 0.9^{b}	21.8 ± 4.6^{b}
Obese, fed	2	32.0 ± 6.7^{d}	25.7 ± 8.6	6.2 ± 1.9^{f}	20.2 ± 9.7
Obese, fasted	5	$22.3 \pm 6.8^{a,e}$	18.0 ± 12.0^{e}	5.2 ± 2.0^{b}	20.3 ± 6.3
Female					
Fasted, lean	3	12.2 ± 6.5^{c}	9.6 ± 5.2^{a}	3.0 ± 1.4^{d}	25.0 ± 8.5
Fasted, obese	3	$41.2 \pm 9.3^{c,a}$	$34.8 \pm 6.7^{a,e}$	6.4 ± 2.8^{d}	14.9 ± 3.7
Male					
HA(ICR), fasted	2	10.8 ± 0.5	8.1 ± 0.3	2.7 ± 0.5	24.5 ± 3.2

Note: Values sharing common superscripts are significantly different at level tested:

$p < .01^{a,c}$

$p < .05^{b,e}$

$p < .10^{d,f}$.

activities of the two compartments differ kinetically also (R. Gee, unpublished).

Comparison of NAD:Glycerol-3-P Dehydrogenase of Fasted Male Obese Versus Lean Mice

The total activity of the enzyme is significantly higher in the obese animal. The peroxisomal form is two times higher in the obese male mice. The amount of the cytosolic enzyme was the same in the obese and lean male mice. The percentage of the activity that was peroxisomal was not different between the two animals. The increase in peroxisomal enzyme activity is due to an increase in the amount of enzyme and not a difference in the distribution.

Comparison of NAD:Glycerol-3-P Dehydrogenase of Fed Male Obese Versus Lean Mice

The total and peroxisomal activity are both significantly higher in the obese mice. The cytosolic enzyme activity was the same in the two animals on a per g liver basis. The percentage of peroxisomal enzyme was also the same between the two animals indicating that the amount of peroxisomal enzyme was higher in the obese and not just a redistribution of the activity.

Comparison of NAD:Glycerol-3-P Dehydrogenase of Lean Male Fed Versus Fasted Mice

An overnight fast significantly decreased the total amount of the enzyme activity. The difference appears to be the result of a significant decrease in the cytosolic enzyme and not the peroxisomal form where there was no difference in an overnight fast. A significant

difference was observed in the percentage distribution of the peroxisomal enzyme. This may be due to the better separation achieved with the fed mice (see Figures 5 and 8).

Comparison of NAD:Glycerol-3-P Dehydrogenase of Obese Male Fed Versus Fasted Mice

In the obese mice, an overnight fast did not decrease either enzyme as it did in the lean animals.

Comparison of NAD:Glycerol-3-P Dehydrogenase of Fasted Lean Male Versus Female Mice

For lean male and female mice, there was no difference in enzyme activity.

Comparison of NAD:Glycerol-3-P Dehydrogenase of Fasted Obese Male Versus Female Mice

Fasted female obese mice had two times higher total NAD: glycerol-3-P dehydrogenase activity than the males. This was due to the two times higher activity of the cytosolic enzyme in the female obese mice. No difference was observed in the activity of the peroxisomal enzyme.

Comparison of NAD:Glycerol-3-P Dehydrogenase of Fasted Female Obese Versus Lean Mice

The obese female mice had significantly higher total activity. This is reflected in the higher activity of both the cytosolic and peroxisomal enzymes. The percentage distribution of the peroxisomal enzyme was not different between the lean and obese mice. This is similar to the comparison between lean and obese male mice. The total

enzyme activity of the obese female was much higher than the lean being on the order of four times, whereas the male obese mice had activities that were three times higher than their lean counterpart.

Comparison of NAD:Glycerol-3-P Dehydrogenase of Fasted Male HA(ICR) and C57BL/6J Mice

There was no difference in the activities of the two enzymes between these animals.

DISCUSSION

The mouse has been found to be a particularly good animal for studying liver peroxisomes. The peroxisomal enzymes are very active per g of liver and the peroxisomes appear to be much less fragile than those of the rat liver as judged by the little catalase activity in the supernatant fraction. Better peroxisomal separation was achieved with the HA(ICR) mouse than with the C57BL/6J mouse. In addition, the HA(ICR) mouse provided several fold higher specific activity.

In crude homogenates of liver, the lean C57BL/6J mice appear to have slightly more catalase than the obese. Clofibrate treatment of these animals did not induce the increase in catalase activity or protein content that it does in other rodents (26, 84, 108, 121-126, 140), although liver size did increase. The same clofibrate diet increased catalase activity and liver weights in the HA(ICR) mice. These data suggest that the two different strains of mice respond differently to clofibrate when fed at the 0.5% of the diet.

Electron microscopic photographs of peroxisomes of lean and obese C57BL/6J mice showed no apparent difference in the numbers of peroxisomes. The feeding of clofibrate at 0.5% of the diet did not cause an increase in the numbers of peroxisomes that was observable in thin sections of liver by the electron microscope. Other rodents, even the acatalasemic mouse, do have an enormous increase in peroxisome number on clofibrate treatment at this level (26, 84, 108, 121-126,

140). Therefore, it seemed inappropriate to continue using clofibrate or other hypolipidemic drugs to increase peroxisomes or peroxisomal enzymes in the C57BL/6J strain of mouse.

In isolated hepatic organelles, three comparisons can be made between the obese and lean C57BL/6J male mice for enzyme activities. (1) The activity of the matrix enzymes, catalase and urate oxidase of the peroxisomes and glutamate dehydrogenase of the mitochondria, remained about constant per g of liver from either the lean or the obese animal. Since the obese male mice had larger livers, the total activity per liver increased proportionally. (2) The two membrane enzymes that were examined, β-hydroxybutyrate dehydrogenase of the mitochondria and NADPH:cytochrome c reductase of the microsomes, were lower in the obese male mice compared to the lean in inverse proportion to the large liver size. Therefore, the amount of these two membranous enzymes was the same per liver in the lean and obese male mice. (3) A higher specific activity and total activity of peroxisomal NAD:glycerol-3-P dehydrogenase was observed in the obese male mice.

The HA(ICR) male mice and the lean C57BL/6J male mice had different amounts of peroxisomal enzymes even though there was no difference in their body weights of their liver weights. Fatty acid β-oxidation as measured by NAD reduction was four times higher in the HA(ICR) mice per g of liver. Catalase activity was approximately 50% higher per liver in the HA(ICR) mice as compared to the lean and two times higher per 100 g of body weight. This demonstrates that while both animals were lean, the amounts of peroxisomal enzymes was not

the same. This raises the question of what is the proper control animal for testing the theory that hepatic peroxisomes are energy wasting. This question will be considered further in the discussion of fatty acid β-oxidation activity.

In contrast to the comparisons between the lean and obese C57BL/6J mice, the comparisons between the lean HA(ICR) mice and the obese C57BL/6J mice present a different interpretation. Catalase activity for the peroxisomes per g of liver was higher in the HA(ICR) mice than the obese C57BL/6J mice. Marker enzymes for the other organelles were the same in these two animals. As will be discussed later, the other peroxisomal enzymes were also different in the HA(ICR) mice as compared to the obese mice.

A comparison of activities for the organelle marker enzymes between male and female C57BL/6J mice showed no difference in any of the activities per g of liver. The significant differences on a per liver basis of the marker enzyme activities observed between lean males and females is probably due to the large liver size of the male animals.

The same differences in marker enzyme activities observed between lean and obese male mice were observed between lean and obese female mice. Therefore, it can be concluded that the sex of these mice does not change the kinds of differences observed for marker enzyme activities between lean and obese C57BL/6J mice.

An 18 hour fast prior to killing the mice did not change the amount of organelle marker enzyme activities.

The obese male mice had been reported to have approximately three times the peroxisomal fatty acid β-oxidation activity as its lean littermate (112). Therefore, a comparison of the organelle distribution for fatty acid β-oxidation was extended to explore for any differences that might be associated with weight. The first step in peroxisomal β-oxidation is proposed to be energy wasting. It is possible to calculate the loss of energy at this step, assuming the rest of the energy from peroxisomal oxidation of the fatty acid is not lost in comparison to the energy that would be obtained by complete mitochondrial oxidation of the fatty acid. If one mole of palmitate is completely oxidized in the mitochondria, 130 moles of ATP are theoretically produced. In contrast, if the peroxisomes are used to completely oxidize one mole of palmitate, 14 moles of ATP would be lost in the first dehydrogenation step. This would result in a 10.8% loss of the potential energy available from palmitate had it been oxidized in the mitochondria. It has been proposed that the peroxisomes and mitochondria have chain length specificities for fatty acid oxidation (88, 117), therefore, the organelle distribution of fatty acid oxidation is probably more complex. The peroxisomes have been proposed to oxidize long chain fatty acids to acetyl CoA and medium chain length fatty acids, after which they can be transported to the mitochondria for complete oxidation.

The ratio of β-oxidation in the mitochondria to the peroxisomes of the adult C57BL/6J mice and the HA(ICR) mice was approximately one. For the adult male rat, this ratio was reported as 3.2 (82). The ratio in the rat changes with the age of the animal (83).

Peroxisomal β-oxidation, as measured by the NAD reduction assay or the oxygen uptake assay, was the same in the lean and obese C57BL/6J mice, whether or not the animals were fasted. The NAD reduction assay did not show a higher activity for the obese mice as reported earlier (112). Although the magnitude of the values was similar, the variance of the numbers was greater and, therefore, the significance of the difference was not apparent in this study. The fact that the oxygen uptake data also indicated no change in the peroxisomal β-oxidation activity, would tend to indicate that the peroxisomal enzyme activities were not different between the lean and obese fasted male mice. The peroxisomal β-oxidation activities did not differ over a one night fast nor was there a sex difference in peroxisomal β-oxidation activity.

Difficulty in obtaining the same fatty acid β-oxidation rates by the NAD reduction assay and the oxygen uptake assay was not caused by NADH oxidase activity in the peroxisomal peak, as no oxidation of added NADH was detected. One reason for the lack of agreement in the stoichiometry of the two assays may be because the oxygen uptake assay measures the first step in the sequence and the NAD reduction assay measures the third step. During NAD reduction, the amount of β-hydroxy acyl CoA available to the enzyme may not be at saturating amounts when assayed in this manner.

Mitochondrial β-oxidation was the same in the obese and lean mice. In an overnight fast, the activity of the mitochondrial fatty acid β-oxidation increased in both the lean and the obese animals.

It was only the mitochondrial system that responds to fasting and not the peroxisomal fatty acid β-oxidation.

The ratio of mitochondrial to peroxisomal activity in the mice is the same in any comparison. If there was a difference in the ratios between the lean and obese mice, it might have indicated a difference in the distribution of β-oxidation activity between a proposed energy wasting organelle, the peroxisome, and an energy conserving organelle, the mitochondria. In this obese animal model, there is no evidence of energy wasting associated with a difference in weight through hepatic β-oxidation.

In contrast, comparisons made between the lean HA(ICR) mice and the obese C57BL/6J mice in fatty acid β-oxidation activity are much different. While peroxisomal β-oxidation was the same per g liver in the lean and obese C57BL/6J mice, it was two times higher in the HA(ICR) mice than in the obese C57BL/6J mice. Mitochondrial β-oxidation was also higher in the HA(ICR) mice as compared to the C57BL/6J obese mice. Therefore, total β-oxidation capacity was higher in the HA(ICR) mice compared to the C57BL/6J mice but was no different between the lean and obese C57BL/6J mice.

Thus, when the HA(ICR) mice are compared to the obese C57BL/6J mice, the HA(ICR) mice have more peroxisomal β-oxidation than the obese. The HA(ICR) mice also have more energy conserving mitochondrial β-oxidation than the obese. The ratio of mitochondrial to peroxisomal activity was the same although there was a large variance in the data.

Because the mice are from two different strains, it might be erroneous to use the HA(ICR) mice as the lean control for the C57BL/6J obese animal. It is probably proper to use the lean C57BL/6J mice as the control for the obese C57BL/6J mice. But it is interesting to note that while both the lean C57BL/6J mice and the HA(ICR) mice are quite similar in body size and marker enzyme activities, the key enzymes of peroxisomal and mitochondrial fatty acid β-oxidation are different in the two animals.

The higher total and peroxisomal activity of NAD:glycerol-3-P dehydrogenase observed in C57BL/6J obese fasted male mice compared to the lean mice (112), is found when the animals were not fasted. The total activity decreases in the lean male mice when they are fasted. This is a logical step in responding to the stress induced by the fast in the lean male mice. Lowered activity would be expected as the animal tries to maximize the use of carbon for gluconeogenesis. To cycle glycerol-3-P through NAD:glycerol-3-P dehydrogenase at a branch point for carbohydrate and lipid synthesis would be wasteful while fasting. The obese male mice do not compensate similarly. The obese mice would appear to be wasting more energy at this point. This is not an unusual observation for the obese mouse as it is reported to synthesize triglycerides even under very adverse conditions (11, 136, 159).

In contrast to the cytoplasmic glycerol-3-P dehydrogenase activities, the peroxisomal NAD:glycerol-3-P dehydrogenase does not change in the fasted versus the fed state in the lean and obese animals. This is similar to the activity of the peroxisomal β-oxidation enzymes. These peroxisomal enzymes did not change with an 18 hour fast.

Female mice had similar differences between the lean and obese in total and peroxisomal NAD:glycerol-3-P dehydrogenase activity similar to the male mice. However, the differences between the obese and lean females were greater than that observed for fasted males (112). The obese females have activities that were even greater than that observed for the obese males. This difference was due to the amounts of cytoplasmic enzyme activities rather than in the peroxisomal enzyme activities. The peroxisomal activities were the reason for the observed differences between the lean and obese fasted males (112). The female obese mice had higher enzyme activities in the cytoplasm and the peroxisome than their lean controls. It is not known why the obese female mice have much higher activities than the obese male mice.

The higher activity of NAD:glycerol-3-P dehydrogenase is consistent with the five to tenfold increase in hepatic lipid synthesis observed in the obese animal (136). Since NAD:glycerol-3-P dehydrogenase functions principally as a reductase (55), it could be providing a source of glycerol-3-P for triglyceride and phospholipid synthesis. The peroxisomal enzyme has also been suggested to function as a shuttle to transport reducing equivalents across the peroxisomal membrane (55) in a manner similar to the malate dehydrogenase in plant peroxisomes (156). Because peroxisomal fatty acid β-oxidation activity, the only NADH producing reaction so far described in animal peroxisomes, is the same between lean and obese mice, the higher activity of the NAD:glycerol-3-P dehydrogenase in the obese mice does not necessarily support the shuttle idea.

It is not known if the enzyme activities measured for peroxisomal fatty acid β-oxidation and NAD:glycerol-3-P dehydrogenase, which are V_{max} values, properly reflect the physiological milieu that the enzymes are exposed to in the peroxisomes. These in vivo conditions might be quite different.

The interaction of metabolism between peroxisomes and mitochondria becomes more complex as more data on peroxisomal enzymes become available. It appears that peroxisomes and mitochondria of liver share the ability to oxidize fatty acids dependent on chain length. Peroxisomes handle long chain fatty acids which, after partial oxidation, can be transported to the mitochondria for complete oxidation (38, 143, 146). The fate of peroxisomally produced NADH and acetyl-CoA is unknown. The possible site(s) of control for β-oxidation could be: at the peroxisomal membrane level of transport in and out of the organelle; the rate of synthesis and/or degradation of the rate limiting enzyme(s); and the oxygen concentration available to the peroxisomal enzymes as suggested by de Duve (43). Because the peroxisomal enzymes are affected selectively by hypolipidemic agents, i.e., increases are seen in the lipid metabolizing enzymes (34, 58, 77, 87, 96, 99), the suggestion of peroxisomal participation in lipid metabolism is still attractive (44).

In the obese animal model, there does not appear to be a lowered amount of hepatic peroxisomal β-oxidation in the obese animal that would be expected if the obese mouse was wasting less energy than its lean control. In fact, on a per liver basis, the obese animal has

more fatty acid β-oxidation capacity because of its larger liver. Nor does there appear to be more mitochondrial fatty acid β-oxidation in the obese mouse if it were conserving more energy from β-oxidation. The ratio of mitochondrial to peroxisomal β-oxidation activity suggest no difference in the site of fatty acid β-oxidation in the liver. The obese animal does respond to a fast with increased mitochondrial β-oxidation. No change, however, is seen in either peroxisomal β-oxidation or NAD:glycerol-3-P dehydrogenase. These two observations are true for the lean mouse also. Peroxisomal activity of these two enzymes is unchanged for a variety of comparisons.

There does not appear to be a differential in hepatic peroxisomal enzymes due to a difference in weight as investigated in this study. The exact physiological function of peroxisomes remains unclear but participation in lipid metabolism is now a certainty. The significance of the energy lost in the first step of peroxisomal fatty acid β-oxidation remains to be determined. In this study, there does not appear to be a difference between lean and obese mice in energy lost by hepatic peroxisomal activity.

CONCLUSIONS

1. Peroxisomal numbers were the same in the liver of lean and obese C57BL/6J mice.
2. Clofibrate fed at 0.5% of the diet did not affect peroxisomal catalase in this strain of mouse.
3. Matrix marker enzyme activities for the peroxisomes and mitochondria were similar in male and female lean and obese C57BL/6J mice and HA(ICR) male mice.
4. Membranous enzyme activities were lower in obese males than lean male mice in inverse proportion to the larger liver size.
5. Peroxisomal β-oxidation was similar in lean and obese, male and female and fed and fasted mice. It was higher in the male HA(ICR) mice than in the C57BL/6J mice.
6. Mitochondrial β-oxidation activity changed during fasting but the change was similar in lean and obese or male and female mice. The activity was higher in the male HA(ICR) mice.
7. Peroxisomal NAD:glycerol-3-P dehydrogenase was higher in obese male and female C57BL/6J mice than in the lean C57BL/6J mice. This peroxisomal enzyme did not change during an 18 hour fast.
8. Cytosolic NAD:glycerol-3-P dehydrogenase decreased during fasting, but the activity was not significantly different between lean and obese mice. Female obese mice have higher cytosolic activity than the lean females and obese and lean males.

9. On a per animal basis, there was more hepatic peroxisomal β-oxidation and more peroxisomal NAD:glycerol-3-P dehydrogenase activity in the obese animals. Thus, there did not appear to be a lowered amount of hepatic peroxisomal activity associated with increased weight. Quite the opposite was observed.
10. The evidence in this research does not support the hypothesis that peroxisomal metabolism wastes energy in mammalian liver.

APPENDIX

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Enzyme Activities of Isolated Hepatic Peroxisomes from Genetically Lean and Obese Male Mice[1]

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Hepatic peroxisomes have been isolated on isopycnic sucrose gradients from white mice [HA(ICR)] and lean and obese (C57BL/6J) mice. Nearly all of the catalase activity was in the peroxisomal fraction. The activity for β-oxidation of palmitoyl-CoA was about three-fold higher per milligram of protein in the isolated peroxisomal fraction or per gram of liver from the obese mouse compared to its lean littermates. Glycerol-3-phosphate dehydrogenase activity also was higher in the peroxisomes and cytoplasm of the obese mouse. The matrix enzymes of the organelles, catalase and urate oxidase of the peroxisome and glutamate dehydrogenase of the mitochondria, had similar activities per gram of liver from either lean or obese mice. Membrane components, NADPH: cytochrome *c* reductase of the microsomes and β-hydroxybutyrate dehydrogenase of the mitochondria, had lower activities in the obese mouse in inverse proportion to the larger size of the liver.

One hypothesis for weight maintenance has been a balance between energy-conserving mechanisms versus futile respiratory processes (1, 2) for energy wasting. Peroxisomes in liver (3) and leaves (4) contain metabolic sequences linked to energy-wasting flavin oxidases and conceptually are the antithesis to energy-conserving mitochondrial respiration. For instance, up to 50% of gross photosynthetic carbon reduction can be immediately lost by leaf photorespiration which is associated with glycolate metabolism in leaf peroxisomes (4). A similar physiological manifestation of peroxisomal activity in animals has not been delineated. Consequently, a comparison of the level of peroxisomal activity in the livers of obese and lean mice was initiated to explore for differences which might be associated with body weight. The C57BL/6J mice, lean and obese littermates, were used because obesity occurs without feeding calorically dense diets and can be easily identified by lowered O_2 consumption and phenotypic expression (5).

The recently discovered fatty acid β-oxidation complex (6) and glycerol-3-P dehydrogenase (7) in the peroxisomes were measured in this study because of their association with lipid metabolism and obesity. The use of mice to study peroxisomal β-oxidation has proven to be fortuitous because about equal distribution of total β-oxidation has been found between the mitochondrial and peroxisomal system in mice (unpublished). In the male rat less than 25% of β-oxidation has been reported to occur in the peroxisomes (8). In addition, data from this study indicate that nearly all of the catalase activity in the mouse liver homogenate was retained in the isolated peroxisomes. This is in contrast to less than 50% recovery of catalase in the peroxisomal fraction from rat liver homogenates (7, 11). Such results indicate that mouse liver peroxisomes can be isolated nearly quantitatively.

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MATERIALS AND METHODS

Male lean mice (C57BL/6J) and their genetically obese and hyperglycemic littermates were from the Jackson Laboratory at Bar Harbor, Maine. Male white mice [subspecies HA(ICR)] from Spartan Research (Haslett, Michigan) were also used for comparison. Young mature animals of about 3 months of age were fasted overnight, weighed, and killed by decapitation. The liver was immediately exposed and perfused *in situ* through the hepatic portal vein with an ice-cold medium of 8.5% sucrose and 0.5 mM EDTA at pH 7.5 until it changed to a light tan color. Livers from the obese mice were very fragile compared to those from the lean mice. The difference in the consistency of the livers necessitated taking a portion of each liver which could be thoroughly homogenized for total activity to compare with the limited homogenization of the organelle preparation. About 0.2 g of the liver was used for total enzyme activity and the remaining weighed portion for preparation of subcellular organelles after careful limited homogenization. Total homogenization was performed with 0.2 g of liver in approximately 20 vol of the medium with repeated passes in a chilled Ten Broeck homogenizer. For organelle preparations the other liver portion was minced with scissors and homogenized in approximately 20 vol of the medium by one pass in a mechanically driven loosely fitting Potter–Elvehjem homogenizer. The homogenate for organelles was filtered through miracloth, its volume measured, and centrifuged at low speed to discard whole and broken cells and nuclei. A 10-ml aliquot was fractionated by isopycnic density centrifugation on nonlinear sucrose gradients for 3 h at 25,000 rpm in 60-ml tubes for the Beckman SW25.2 rotor and L-2 ultracentrifuge. Other details concerning this separation technique have been described (10). The sucrose gradients were prepared about 18 h before use to reduce by diffusion the sharp interfaces between sucrose fractions. After development in the centrifuge, the gradients were unloaded from the bottom of the tube in 2-ml fractions and analyzed for enzyme activity on a per milliliter basis as presented in Figs. 1 and 2. The sucrose density and protein profiles have been omitted for simplification. These data were analyzed in Tables I and II by conversion to activity per milligram protein, per gram liver, or per liver.

Enzyme assays (10). Catalase, the peroxisomal marker, was assayed immediately after collection of the gradient fractions. Urate oxidase was measured later spectrophotometrically. NAD:glutamate dehydrogenase, a mitochondrial matrix marker, was assayed (11) in the presence of 55 ng antimycin A and 5 ng rotenone per milliliter to inhibit NADH oxidation. These marker enzyme assays were run with a Gilford enzyme autoanalyzer Model 3500. β-Hydroxybutyrate dehydrogenase, an inner mitochondrial membrane marker, was assayed (12) as a mitochondrial marker. NADPH:cytochrome c reductase was assayed (13) as the microsomal marker. Protein was determined by a modified Lowry procedure (14). Sucrose density was measured by refractometry.

For fatty acid β-oxidation, palmitoyl-CoA (P–L Biochemicals, Inc.) oxidation was measured spectrophotometrically by the substrate-dependent reduction of NAD, as described by Lazarow and De Duve (6). Five nanograms of rotenone/ml of assay volume was used instead of KCN to inhibit mitochondrial oxidation.

NAD:glycerol-3-P dehydrogenase and NADH:dihydroxyacetone-P reductase activities were measured spectrophotometrically in the absence of electron transport inhibitors without prior freeze–thawing of the samples. These changes from the assay procedure used with the rat homogenates (7) were made possible because there was little oxidation of added NADH by the mitochondrial fractions from the mouse. The dehydrogenase reaction was run in 100 mM glycine at pH 9.7, 1.5 mM NAD, 20 mM glycerol-P and enzyme. The reductase was run in 100 mM Tricine at pH 7.8, 0.46 mM dihydroxyacetone-P, enzyme, and 0.13 mM NADH for the peroxisomal fractions or 0.26 mM NADH for the cytosolic fractions.

RESULTS

Peroxisomal Isolation

Cell organelles in liver homogenates from male white HA(ICR) mice (Fig. 1) and male black Bar Harbor (C57BL/6J) lean and obese mice (Fig. 2) were separated on isopycnic sucrose density gradients. The peroxisomes, identified by catalase and urate oxidase, were well separated from the mitochondria, identified by NAD:glutamate dehydrogenase and NAD:β-hydroxybutyrate dehydrogenase. The lysosomal marker enzyme, acid phosphatase, was primarily located in the mitochondrial area with a small amount of activity tailing into the peroxisomes (data not shown). The microsomes, identified by NADPH:cytochrome *c* reductase, equilibrated at a lower sucrose density. The equilibrium density for the mouse peroxisomes was at 1.23 g/cm^3 and for the mitochondria at 1.19 g/cm^3. In the rat the peroxisomes are slightly denser and the mitochondria lighter (10) than from the mouse so that the two organelle populations from the mouse are not as far apart on the sucrose gradient.

A comparison of the data in Figs. 1 and 2 indicates that sharper bands of organelles indicating better separation of peroxisomes and mitochondria were obtained with the

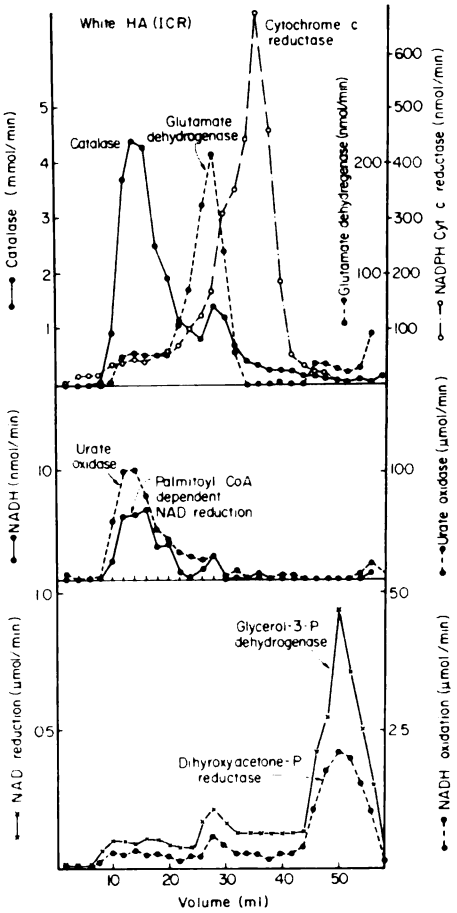

FIG. 1. Isopycnic sucrose density gradient fractionation of the homogenate containing 1.0 g of liver from a white mouse HA(ICR). All the assays were run on the same preparation except for glycerol-3-P dehydrogenase and dihydroxyacetone-P reductase which were from another preparation containing 0.77 g of liver.

white mice as compared with the C57BL/6J mice. This was repeatedly observed and no explanation can be offered for this other than biological variation. Data in Figs. 1 and 2 also show that the β-oxidation activity in the hepatic peroxisomal fraction from the white mouse was two- to threefold more active than in comparable fractions from the lean C57BL/6J mice. In this study, however, the Bar Harbor lean C57BL/6J mice have been used so that comparisons could be made with the obese mutant and its lean littermate.

With both C57BL/6J and the HA(ICR) mice, less than 5% of the catalase or urate oxidase activity was present in the supernatant fraction. Almost all the activity was in or tailed from the peroxisomal peak, as if there had been little breakage of the peroxisomes during homogenization. These results differ from those reported for the rat, where about 50 to 75% of the catalase is in the supernatant and attributed either to peroxisomal fragility or to two subcellular locations of catalase (9). Inclusion of 0.01% ethanol in the gradient, which has been used to prevent the formation of an inactive peroxide complex (15), did not increase the catalase activity. Data from mouse gradients, particularly the lean mouse, indicate some of the peroxisomal catalase tailed into the mitochondrial area (Fig. 2). The identity of this tailing material in the mouse gradients has been examined by electron microscopy. For both the mouse and the rat, the peroxisomal marker enzyme activity above the main band appears to be due to an area containing a mixture of peroxisomes and microsomes.

The profile for the mitochondrial matrix enzyme, glutamate dehydrogenase, and the mitochondrial membrane enzyme, β-hydroxybutyrate dehydrogenase, coincide. This indicates that no significant amount of single membrane mitochondrial fragments were present.

Differences in Hepatic Peroxisomal Enzymes from Lean and Obese Mice

The obese Bar Harbor C57BL/6J mice at 3 months of age averaged 53 g body wt and 4.4 g liver wt. On the average they were twofold heavier than their lean littermates and their livers were about threefold larger. Under these circumstances enzymatic data in Fig. 2 were evaluated and reported in Table I as specific activity in the isolated subcellular organelle from the sucrose gradient and as total activity per gram of liver, per liver, or per 100 grams body weight.

The specific activities of the marker enzymes in the isolated peroxisomes (catalase and urate oxidase) and the mitochondria (glutamate dehydrogenase) and the total activity per gram of liver were the same from

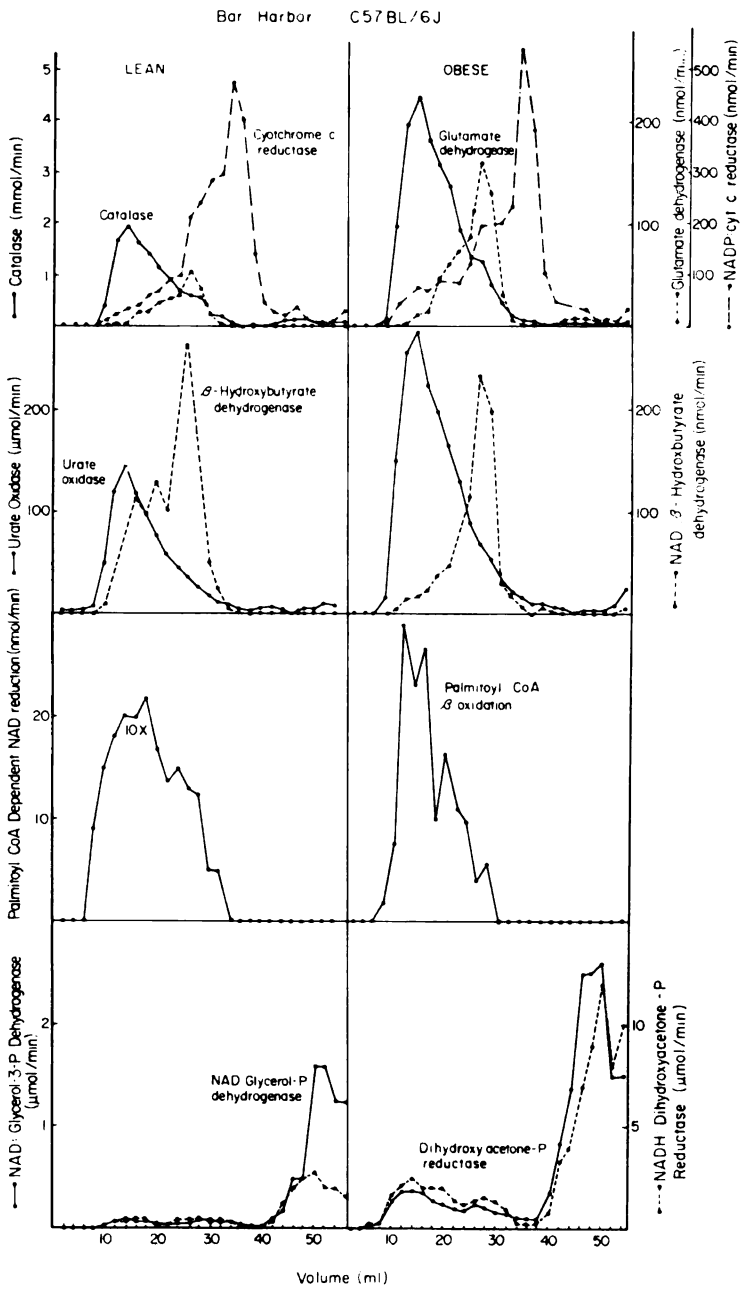

FIG. 2. Isopycnic sucrose density gradient fractionation of the homogenate containing 0.75 and 1.71 g of liver from a lean or obese C57BL/6J mouse, respectively. The β-oxidation activity from the lean mouse has been multiplied by 10 for comparison.

the lean and obese mouse (i.e., ratio of about 1 in Table I). This similarity was true even though the obese mouse at 3 months of age had a larger and more fatty liver. Consequently, the total peroxisomal and mitochondrial activity increases per liver from the obese mouse in proportion to the liver size. In Fig. 2 approximately the same

TABLE I

TOTAL HEPATIC ENZYME ACTIVITY OF LEAN AND OBESE MICE (C57BL/6J)[a]

Enzyme		N	Per mg protein in peak activity fraction		Per g liver		Per liver		Per 100 g body wt	
			Units	Obese/ lean	Units	Obese/ lean	Units	Obese/ lean	Units	Obese/ lean
Catalase (mmol/min)	Lean	5	4.3 ± 1.2	1.4	51 ± 20	1.0	78 ± 29	2.9	290 ± 96	1.5
	Obese	5	5.9 ± 1.7		51 ± 14		224 ± 80*		421 ± 146	
Glutamate dehydrogenase (μmol/min)	Lean	4	0.038 ± 0.007	1.2	1.1 ± 0.7	1.1	1.7 ± 0.9	3.2	6.4 ± 3.5	1.6
	Obese	4	0.047 ± 0.010		1.3 ± 0.4		5.6 ± 1.5*		10.4 ± 1.9	
Urate oxidase (μmol/min)	Lean	4	0.28 ± 0.08	1.2	3.5 ± 1.2	0.9	5.2 ± 1.7	2.5	19.5 ± 7.5	1.2
	Obese	4	0.34 ± 0.11		3.2 ± 1.4		13.0 ± 4.7*		24.7 ± 8.2	
Palmitoyl-CoA oxidation (nmol/min)	Lean	5	9 ± 3	3.1	263 ± 143	1.8	309 ± 199	5.1	1536 ± 851	2.5
	Obese	4	28 ± 12*		468 ± 59**		2027 ± 461*		3800 ± 734**	

[a] The units are cited in column one for each enzyme and the specific activity is calculated on the basis of the protein content of the isolated peak activity fraction, peroxisomes for catalase, urate oxidase, and palmitoyl-CoA oxidation and mitochondria for glutamate dehydrogenase.

* $P < 0.01$.

** $P < 0.05$.

fraction (about ⅓) of the total liver homogenate had been used for each gradient, so the increase in total activity in the obese liver was due to an increase in the liver size. Since the liver in the obese mouse was larger on a body weight basis than in the lean mouse, the change in peroxisomal and mitochondrial marker enzymes increased per 100 gram of body weight.

Comparison of Matrix and Membrane-Associated Enzymes

Catalase and urate oxidase of the peroxisomes and glutamate dehydrogenase of the mitochondria are located in the matrix of these organelles and were not different in specific activity between the lean and obese mouse. In contrast the membrane enzymes, NADPH:cytochrome *c* reductase of the microsomes and β-hydroxybutyrate dehydrogenase of the inner mitochondrial membrane, decreased two- to threefold per gram of liver in the obese mouse. For these membranous enzymes there was no difference per liver, as seen in Fig. 2 even though the liver from the obese mouse was about threefold larger. Thus there seems to be a difference of hepatic matrix enzymes of the organelles but no difference in membrane components with obesity. This difference should be examined for other enzymes and on a larger scale with zonal sucrose gradients.

Comparison of β-Oxidation in the Lean and Obese C57BL/6J Mouse and the White Mouse

The assay procedure for peroxisomal palmitoyl-CoA-dependent reduction of NAD did not measure mitochondrial β-oxidation (6, 8). The palmitoyl-CoA-dependent NAD reduction shown in Figs. 1 and 2 coincided with the distribution of the peroxisomal catalase. The results with the mouse extend the observation of Lazarow and De Duve (6) with the rat that liver peroxisomes catalyze β-oxidation. The peroxisomal β-oxidation was two- to threefold more active per gram of liver from the white mouse (Fig. 1) than from the black Bar Harbor lean mouse (Fig. 2). To compare the distribution of β-oxidation between peroxisomes and mitochondria, it will be necessary to extend these assays using procedures (8) that will quantitatively measure β-oxidation in mitochondria from the sucrose gradient used to separate the two organelles. Preliminary results indicate about equal distribution of β-oxidation between the two particles from the mouse liver.

Total β-oxidation in the peroxisomes from the obese mouse liver was about twofold as much per gram of liver as in the lean mouse, which resulted in a difference of over fivefold per liver. This increase was pronounced in the enzymatic specific activ-

ity with the isolated peroxisomes (Fig. 2 and Table I). The results indicate a higher activity for β-oxidation in the liver peroxisomes of the obese mouse, even though the other peroxisomal enzymes, catalase and urate oxidase, were not higher per gram liver or per milligram protein.

Differences in Hepatic NAD:glycerol-3-phosphate Dehydrogenase in the Obese Mouse

The amount of this enzyme in the peroxisomal fraction of the liver was small, being about one-tenth of that in the cytosol (Figs. 1 and 2, Table II). This limited observation with mice indicates that NAD: glycerol-P dehydrogenase previously reported for rat liver peroxisomes (7) may also be a constituent of mouse peroxisomes. The glycerol-3-P dehydrogenase activities in the peroxisomal and supernatant fractions from the mouse liver migrated similarly during thin layer isoelectric focusing but have different mobilities during electrophoresis on 7% polyacrylamide gels. The activities from the two compartments also differ kinetically (R. Gee, unpublished). The activity of glycerol-3-P dehydrogenase in the peroxisomal fraction from the obese mouse liver was higher than that in the lean mouse when expressed on either a protein or gram of liver basis (Table II). The cytoplasmic pool of glycerol-3-P dehydrogenase was higher in the obese mouse by about the same magnitude as in the peroxisomal fraction.

TABLE II

DISTRIBUTION AND TOTAL ACTIVITIES OF NAD:GLYCEROL-3-PHOSPHATE DEHYDROGENASE IN THE LIVERS OF FIVE LEAN AND OBESE MICE

	Peroxisomal fraction[a]	Supernatant fraction
	Percentage distribution	
Lean	10 ± 3.5	76 ± 6
Obese	14 ± 2.5*	76 ± 2
	μmol/min g liver	
Lean	0.9 ± 0.3	10 ± 5
Obese	2.8 ± 2.1*	18 ± 12
	μmol/min mg protein	
Lean	0.2 ± 0.1	0.5 ± 0.5
Obese	0.8 ± 0.3**	0.9 ± 0.4

[a] Peroxisomal fraction includes volumes 8–24 ml on the gradients; supernatant fraction includes volumes 44–55 ml on the gradient.

* $P < 0.01$.

** $P < 0.05$.

DISCUSSION

The mouse has been found to be a particularly good animal for studying liver peroxisomes. The peroxisomal enzymes are very active per gram of tissue and the peroxisomes appear to be much less fragile than in the rat liver as judged by little catalase activity in the supernatant fraction. Better peroxisomal isolation was experienced with the white HA(ICR) mouse than with the lean C57BL/6J mouse. In addition the white mouse provided several fold higher specific activity for β-oxidation in the isolated peroxisomes.

Three comparisons can be made for the enzyme activities of subcellular particles from the livers of obese with those from the lean littermates of the C57BL/6J mouse at 3 months of age. (A) The matrix enzymes, catalase and urate oxidase of the peroxisomes and glutamate dehydrogenase of the mitochondria, remained about constant per gram of liver from either lean or obese animal. Since the obese animal had a larger liver, the total activity per liver increased proportionately. (B) Two membrane enzymes that were examined, NADPH:cytochrome *c* reductase of the microsomes and β-hydroxybutyrate dehydrogenase of the mitochondria, were lower in the obese mouse in inverse proportion to the larger size of the liver. Therefore, the amount of these two enzymes per liver was not different. (C) A higher specific activity and total activity of peroxisomal fatty acid β-oxidation, as well as some increase in NAD:glycerol-3-P dehydrogenase, was observed in the obese mouse.

Peroxisomal fatty acid β-oxidation in the obese mouse was about threefold higher in

specific activity and total activity per gram liver and over fivefold higher per total liver. This increase in peroxisomal β-oxidation was the opposite from the anticipated result (see introductory statement). Consequently, further interpretation and hypotheses about these data may be equally as erroneous. If the obese trait is associated with overproduction of fatty acids and lipids, the resultant increase in peroxisomal oxidative capacity may be an attempt to dispose of the excess. The higher peroxisomal and cytosolic NAD:glycerol-3-P dehydrogenase in the obese mouse must also indicate a faster turnover of glycerol-3-P. These observations together with hyperglycemia and hyperinsulinemia with lowered response to insulin (16) in the obese mouse are all indicative of altered metabolic relationships.

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