A STUDY OF PSEUDOCHOLINESTERASE IN OBESITY, DIABETES, AND NUTRITION. STUDIES USING EXPERIMENTAL MODELS

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BY

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A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Master of Science

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Memorial University of Newfoundland

July 1985

St. John's Newfoundland

ABSTRACT

Human serum pseudocholinesterase (PChE) has been widely studied for its genetic polymorphism, but studies defining its physiological role have not been successful. The origin of plasma PChE is the liver. Human serum PChE has been reported to be increased in hyperlipoproteinemia and obesity.

A relationship between food assimilation and PChE was proposed on the basis of dietary experiments in mice. Further studies in hyperphagic obese mice showed that both serum and liver PChE activity increased in response to overeating. However, adipose tissue PChE activity is decreased in obese mice.

Whether such alterations are evident in genetically (Zucker) fat (fa/fa) rats is not known. What is the age of obviously altered PChE activity in the liver and adipose tissue of the postweanling genetically obese (ob/ob) mouse? What is the sub-cellular location of PChE in the liver of ob/ob mice? Whether dietary protein, carbohydrate, or fat influences PChE induction in the liver is not clear. What effect does specific inhibition of PChE have on epinephrine-stimulated lipolysis?

Male fa/fa rats and ob/ob mice were used as experimental models of obesity and diabetes. Dietary studies were done in male Swiss-Webster mice. Isolated rat adipocytes were used to study the effect of specific inhibition of PChE

on epinephrine-stimulated lipolysis *in vitro*.

PChE was determined by a colorimetric method. Subcellular changes in liver PChE of ob/ob mice were analyzed by a cytochemical method for electron microscopy. Rate of glycerol release was used as an indicator of rate lipolysis. Glycerol was determined by a colorimetric, enzymatic method.

Serum and liver PChE activity is significantly higher ($P < 0.01$) in fa/fa rats when compared to lean rats. Adipose tissue PChE activity was not significantly different between the two groups. Linear regression analysis showed that serum PChE activity has a good positive correlation with liver PChE activity, serum triglycerides, body weight and food intake.

Liver PChE activity was significantly higher ($P < 0.05$) in ob/ob mice than in lean littermates as early as 23 days of age. Adipose tissue PChE activity was, on the other hand, significantly lower ($P < 0.05$) in ob/ob mice than in lean littermates as early as 23 days of age. By cytochemical electron microscopy, increased staining for PChE was observed in the rough endoplasmic reticulum of ob/ob mice. Albino mice with different diets showed that high protein diets produced the greatest increase in PChE activity in the liver compared to high carbohydrate or high fat diets. Mice fed a normal mouse diet *ad libitum* had significantly higher

($P < 0.05$) liver PChE activity than those fed a restricted diet of 2 g of a normal mouse chow per day. In albino mice, liver PChE activity varied directly with the protein content in the diet.

A significantly higher ($P < 0.05$) release of glycerol from rat adipocytes was observed, as expected, in the presence of epinephrine when compared to basal conditions. Propranolol, a beta-adrenoreceptor blocker, decreased the release of glycerol significantly ($P < 0.05$) and also inhibited PChE. The specific PChE inhibitor tetramono-isopropylpyrophosphoramide (Iso-OMPA) also significantly decreased ($P < 0.05$) the epinephrine-stimulated lipolysis. Purified horse serum PChE showed no lipase activity and Iso-OMPA had no significant effect on the lipase activity of purified triacylglycerol acylhydrolase (lipase) in vitro.

Genetically obese (fa/fa) rats show alterations in PChE activity similar to that observed in obese human beings and mice. Liver and adipose tissue PChE activity are altered in ob/ob mice before frank expression of obesity is evident. Increased synthesis is the apparent cause of increased liver PChE activity in the ob/ob mouse. Further confirmation requires studies using immuno-precipitation techniques. Dietary studies suggest that liver PChE induction is a function of the level of protein in the diet.

Specific inhibition of PChE causes a decrease in epinephrine-stimulated lipolysis. The effects of propranolol and Iso-OMPA suggest a relationship between PChE and the beta-adrenoreceptor in adipose tissue.

ACKNOWLEDGEMENTS

For this research, I was grateful for funds from Dr. K.M. Kutty's grants from the National Health Research Development, Health and Welfare Canada, The Faculty of Medicine at Memorial University of Newfoundland and The Dr. Charles A. Janeway Child Health Centre. I am thankful to Dr. K.M. Kutty for his guidance and help during the course of my programme. I am also grateful to Doctors G. Mathieson and V. Prabhakaran for their advice in the preparation of this thesis. I would also wish to thank Dr. S.N. Huang for all his assistance and support.

To my wife, Janet, for her patience, understanding, help and love, I am very thankful.

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INTRODUCTION

Pseudocholinesterase (acylcholine acylhydrolase, EC 3.1.1.8; PChE) has been known to be present in mammals for more than 40 years. During this time, a number of hypotheses regarding the function of PChE have been proposed, including ones that suggest that PChE is involved in nutrition (Waterlow, 1950; Gerebtzoff, 1959) and lipid metabolism (Clitherow, Mitchard & Harper, 1963; Ballantyne, 1966). An association between PChE, obesity and diabetes has been frequently found not only in man but also in experimental animal models of obesity and diabetes (Kutty, 1980). The genetically obese (fa/fa) rat is another widely used model of experimental obesity (Bray & York, 1979). If similar alterations in serum, liver and adipose tissue PChE activity are observed, it would further demonstrate the reproducibility of these alterations in another model of obesity and thus add additional evidence to the association between PChE, obesity and diabetes. Abnormal liver and adipose tissue PChE activity have been observed in adult, genetically obese (ob/ob) mice (Kutty, Huang & Kean, 1981). However, when these changes in liver and adipose tissue PChE of the ob/ob mouse are evident is not known. They could precede the frank signs of obesity. Earlier observations suggest (Kutty, Huang & Kean, 1981) that a high calorie intake is responsible for quantitative changes

in PChE activity in obesity and diabetes. This may not be the sole dietary influence on PChE; the effect of a specific type of diet should be examined. Elevated liver PChE activity is found in certain animal models of obesity (Kutty, Huang & Kean, 1981). Increased synthesis has been suggested but no evidence to support this as the cause has been forthcoming.

Adipose tissue PChE activity is reduced in ob/ob, diabetic (db/db) and gold thioglucose-induced obese mice. A relationship between hormone-sensitive lipolysis and adipose tissue PChE has been put forth but how they are associated is not known and requires further examination. These aspects have to be investigated to show how PChE, obesity and diabetes, and nutrition are related. Experimental models of obesity and diabetes could be used to analyze these aspects.

Characteristics and Purification of Serum and Liver Pseudocholinesterase

Alles & Hawes (1940) showed that there are several biochemical differences between red cell cholinesterase and serum cholinesterase in man. Mendel & Rudney (1943) subsequently showed that the serum cholinesterase was

capable of not only hydrolyzing choline esters but also non-choline esters such as tributyrin. These authors were the first to distinguish between the two cholinesterases as pseudocholinesterase (acylcholine acylhydrolase, EC 3.1.1.8: PChE) and true cholinesterase (acetylcholine hydrolase, EC 3.1.1.7: acetylcholinesterase, AChE). In the sera of most mammals such as human beings, dogs, and rats, cholinesterase activity is due solely to PChE (Silver, 1974). Moreover, Satler, Predan & Brzin (1974) found that in rat liver, there is five times more PChE activity than that of AChE. PChE can be further distinguished from "simple" esterases and AChE by the selective and irreversible inhibition by tetramonoisopropylpyrophosphoramide (Iso-OMPA) (Aldridge, 1953).

PChE exists as a group of isoenzymes in both serum and tissues (Silver, 1974). The C_4 isoenzyme contributes 90-95% of the total human serum PChE activity. It is a tetramer (Muensch, Goedde & Yoshida, 1976). PChE isoenzymes have been purified from a number of different sources and include human serum (Das & Liddell, 1970; Muensch et al, 1976; Lockridge, Eckerson & La Du, 1979), and horse serum (Lee & Harpst, 1973; Main, Soucie, Buxton & Arinc, 1974; Teng, Lee, Zinn & Carlson, 1976). PChE has also been purified from rabbit liver (Rush, Main, Miller & Kilpatrick, 1980) and porcine parotid gland (Tucci & Seifter, 1969).

From these studies, it appears that PChE is a glycoprotein of molecular weight in the range of 340,000 - 365,000 daltons for the tetramer. The other isoenzymes have been shown to be either monomers or dimers depending on the source of purification (Masson, 1979; Rush et al, 1980).

Serum and Liver PChE

The liver is generally assumed to be the site of production of serum PChE in mammals (Silver, 1974) and in the rat, the liver enzyme is considered to be of the same type as the serum PChE (Sawyer & Everett, 1947). Evidence for this conclusion is based on such observations as low levels of serum PChE in certain liver diseases (Antopol, Schifrin & Tuchman, 1938) and after experimental liver damage (Brauer & Root, 1946). Moreover, the correlation between liver and serum PChE is indicated by the absence of PChE activity in both liver biopsy specimens and serum of a patient which probably was of the "silent" gene type (Doenicke, Gunter, Kreutzberg, Remes, Spiess & Steinbereithner, 1963; Gunter, Kreutzberg & Doenicke, 1963). The correlation between the level of PChE and albumin in human serum has also been taken as evidence for the hepatic origin of PChE. The identity of liver and serum PChEs with respect to enzymatic properties is consistent with the conclusion that

the liver is the source of serum PChE (Silver, 1974). Studies of certain molecular properties of liver PChE have also supported this view (Svensmark, 1963, Svensmark, 1965). Garry, Prince & Notari (1974) have made a careful half-life determination of human serum PChE. They reported a value of 3.4 days.

Human serum PChE activity varies with physiological factors such as age and sex (Siddel & Kamenskis, 1975), and pregnancy (Wetstone, La Motta, Bellucci, Tennant & White, 1960). In the mouse liver, PChE activity increases with increasing age (Barrows & Roeder, 1961). There are reduced levels of serum PChE in pathological states such as malnutrition, liver disease, cancer and acute infectious diseases (Antopol, et al, 1938; Vorhaus & Kark, 1953). The reduced levels were interpreted to reflect a decreased rate of protein formation (Brown, Kalow, Pilz, Whittaker & Woronick, 1981).

Physiological Function of PChE

No physiological function has been unequivocally assigned to PChE. A number of roles have, however, been put forth. Of these proposed roles, the involvement of PChE in lipid metabolism and nutrition are of importance to the substance of this thesis.

Function in Lipid Metabolism

A function for PChE in the removal of intermediate choline esters produced during fatty acid metabolism in the liver has been proposed (Clitherow, et al, 1963; Ballantyne, 1966). This proposal was originally made on the supposition that PChE broke down butyrylcholine that could possibly be formed by butyryl CoA and choline. Butyrylcholine can cause PChE induction in the liver (Alisova, Oolgo-Daburov & Panyukou, 1971). To date, however, no evidence exists for the presence of any water soluble acylcholine in serum of tissues of humans and animals, that is supportive of this theory, to assign such a function for PChE.

Role in Controlling Plasma Choline and Acetylcholine in the Brain

Funnell & Oliver (1965) proposed that PChE is involved in the homeostatic mechanism regulating the levels of choline in the plasma and acetylcholine in the brain. They suggested that under certain experimental conditions, acetylcholine synthesis was dependent on the availability of free choline. PChE was proposed to function in the hydrolysis of choline esters and thus it may control the level of free plasma choline. The majority of choline, however, found in plasma is bound to phospholipids such as phosphatidylcholine; the latter is not a substrate for PChE (Kutty, 1980). How

PChE functions to regulate plasma choline is therefore not clear.

PChE and Low Density Lipoproteins

Lawrence & Melnick (1961) suggested that serum low density lipoproteins may form a relatively unstable complex with PChE. They postulated that the complex was formed by a physical interaction. Alternatively, Kutty, Rowden & Cox (1973) hypothesized that the interaction between PChE and low density lipoproteins is an active one similar to the relationship between AChE and acetylcholine. It was proposed that phosphorylcholine, the polar head group of phosphatidylcholine, like acetylcholine, will interact with the esteratic and anionic site of PChE, and therefore block the activity. Two other observations support this proposal that PChE is in an inactive functional state when complexed with low density lipoproteins. First, Kutty, Redheendran & Murphy (1977) observed that rats treated with neostigmine, an anti-cholinesterase, would incorporate lesser amounts of ^{3}H lysine into serum beta-lipoproteins and more into alpha-lipoprotein, compared with the untreated controls. Kutty, Jacob, Hutton, Davis & Peterson (1975) reported similar observations in the serum of a patient who had accidentally ingested Parathion, an organophosphate PChE inhibitor. During the recovery phase, the activity increased in parallel with the increase in beta-lipoproteins

and a concomitant decrease in alpha-lipoproteins occurred.

Only 10% of the total serum PChE activity can be recovered from low density lipoproteins (Chu, Fontaine, Kutty, Murphy & Redheedran, 1978). This would therefore account for only a fraction of the total PChE in the serum and would not assign a role for the remaining unbound PChE in the serum and tissues.

PChE in Obesity and Hyperlipoproteinemia

Berry, Cowin & Davies (1953) were the first to indicate that there was a relationship between PChE and obesity. In a random population study, they showed that plasma PChE activity increases as the proportion of body fat increases. More recently, Bentley, Borel, Vaughan & Gandolfi (1982) showed a positive linear correlation between human serum PChE and body mass index. Serum PChE has been shown to be increased in obese persons (Cuciuanu, Popescu & St. Haragus, 1968). These findings were reaffirmed and extended by Chu, et al, (1978) to demonstrate that a greater number of obese persons with hyperlipoproteinemia had higher PChE activity than obese patients without hyperlipoproteinemia. In a recent study of 200 randomly selected hyperlipoproteinemic patients, over 70% had significantly elevated serum PChE activity compared to normal persons. This was especially

significant in those persons with types IIB and IV hyperlipoproteinemia (Kutty, Jain, Huang & Kean, 1981; Jain, Kutty, Huang & Kean, 1983).

During experimental fattening in pigs, serum PChE activity was observed to increase (Popescu, Fekete, Popescu, Bojthy & Lazlo, 1976). Similarly, Kutty, Hillman, Chandra & Cashin (1979) reported elevated serum and liver PChE activity in genetically obese (ob/ob) mice. These observations were reconfirmed in ob/ob mice and extended to lean mice treated with gold thioglucose to induce obesity (Kutty, Huang & Kean, 1981). In the obese animals, liver and serum PChE activity was twice that of lean animals. The induced changes were attributable to a high calorie diet (Kutty, Huang & Kean, 1981). These observations are supported by a more recent study that associates elevated plasma PChE activity with high calorie intake in ob/ob mice (Kutty, Kean, Jain & Huang, 1983). In addition, obese and lean mice treated with Iso-OMPA showed a lower rate of body weight gain despite food intake similar to the untreated controls (Kutty, Kean & Huang, 1983). The reason for this effect is not known.

PChE in Diabetes

Persons with diabetes mellitus have been shown to have increased levels of serum PChE activity (Antopol, Schifrin,

& Tuchman, 1937; Farber, 1943). Kutty, Huang & Kean (1981) demonstrated that genetically diabetic (db/db) mice have serum and liver PChE activity twice that of lean controls. These observed differences were also concluded to be due to high calorie intake. Further studies in db/db mice showed that plasma PChE activity was significantly higher than lean littermates at an age before obvious signs of obesity such as increased body weight, were evident (Kutty, Jain, Huang & Kean, 1983). When the db/db mice were placed on a restricted diet, no increase in either serum PChE activity or body weight was observed (Kutty, Jain, Huang & Kean, 1983). This was taken as another proof for a relationship between PChE and excessive food intake.

PChE in Nutrition

Harrison & Brown (1951) demonstrated that adult male rats when starved for a period of up to 6 days produced a decrease in liver PChE activity but no change in plasma PChE activity. Henderson, Hamilton & King (1971) re-examined this effect in younger male rats and found that plasma PChE activity decreased within 1 day of starvation. In the liver, Henderson et al (1971) found a 50% decrease within 24 hours of starvation when liver PChE activity was expressed as per 100 g initial body weight. Liver PChE was shown to be depressed in malnourished infants but returns to normal when

their nutrition was improved (Waterlow, 1950). Mice refed bread, after a 48-hour starvation, showed an increase in liver PChE activity after the refeeding and it was concluded that PChE had a role in food assimilation (Gerebtzoff, 1959). In kwashiorkor, a form of protein malnutrition, PChE activity in the serum was decreased; however it increased when treatment was administered (Burch, Arroyave, Schwartz, Padilla, Behar, Viteri & Scrimshaw, 1957). Barrows & Roeder (1961) showed that when rats are depleted of protein, liver PChE activity decreases. Similar results were obtained in the mouse (Leto, Kokkonen & Barrows, 1976). Undernourishment of rat pups caused a decrease in PChE activity of the heart and Eckhert, Barnes & Levitsky (1975) concluded that the nutritional status alters the activity of PChE in rat heart.

Other Proposed Roles for PChE

Koelle, Koelle & Smyrl (1973) and Siddows & Koelle (1977) have suggested that PChE functions as a precursor of AChE in nervous tissue. This proposal was made on the basis of the regeneration rates of ganglionic AChE after 1) irreversible inactivation of both AChE and PChE, 2) selective inhibition of AChE, and 3) inactivation of both AChE and PChE followed by continued suppression of PChE. This theory does not address the role of PChE outside the nervous system.

Earl & Thompson (1952) proposed that PChE has a role in the maintenance of the myelin sheath. They suggested this on the observation that some organophosphorus anti-cholinesterases caused demyelination at sufficient doses to inhibit PChE. However, not all anti-cholinesterases have been demonstrated to cause this effect (Davies, 1963).

PChE, in neurons, has been suggested to act as a safety mechanism by hydrolyzing acetylcholine should AChE become inhibited by excess transmitter (Shute & Lewis, 1963). How this relates to PChE outside the nervous system is not clear.

PChE has been found to be associated with many membranes across which transport of water and ions is taking place. Augustinsson (1948) showed biochemically that PChE is located in the acinar cells, ducts, or both of salivary glands in the rat. Koblick, Goldman & Pace (1962) observed that PChE is present in frog skin and suggested that PChE is involved in active sodium transport. Fourman (1966) demonstrated that PChE is located in the thick portion of the loop of Henle of rat nephrons. Fourman (1969) also proposed that PChE found in the salt-gland of duck was associated with sodium transport at this site. Because of this association with many different tissues, the hypothesis that PChE has a function in membrane permeability has been put forward (Koblick et al, 1962; Fourman, 1969).

However, these claims for such a role are based (1) on the effects of drugs which were best known for their anti-cholinesterase properties and (2) the coincident presence of PChE at sites where water and ions are represented. These types of evidence are not conclusive.

Adipose Tissue PChE

Localization of PChE in Adipose Tissue

Salvador & Kuntzman (1965) showed the presence of PChE in adipose tissue. They demonstrated the intracellular localization of PChE. However, they considered the presence of such activity to be consistent with a cholinergic innervation of the tissue. In another study, Ballantyne (1968) showed that the PChE activity was present in the fat cells, and only a small amount in nerves. He concluded that these nerves were probably sympathetic in type. Their conclusions were based on histochemical and biochemical methods using differential substrates and specific inhibitors for PChE. The histochemical evidence showed, by light microscopy, that the activity was most intense towards the periphery of the cytoplasm (Ballantyne, 1968).

PChE Activity in Adipose Tissue of Obese Animals

Obese mice, in contrast to lean mice, have lower PChE activity in the adipose tissue. Kutty, Huang & Kean (1981) showed that ob/ob and db/db adult mice have adipose tissue PChE activity that is 50% of lean controls. These authors reported similar observations in lean mice treated with gold thioglucose to induce obesity. The induced changes were considered to be due to high calorie intake.

Possible Lipolytic Function of PChE in Adipose Tissue

The possibility that PChE might have some relationship to lipolytic function was suggested by studies using anti-cholinesterases (Szendzikowki, Patelski & Pearse, 1961/62; Colville, Salvador, Lindsay & Burnes, 1964). These studies showed that both nonspecific (eserine and neostigmine) and specific (Iso-OMPA) inhibitors of PChE, at concentrations defined to inhibit cholinesterases or only PChE, decreased the amount of nonesterified fatty acids in either rat aorta (Szendzikowki et al, 1961/62) or dog plasma (Colville et al, 1964). In the latter study, the nonspecific PChE inhibitor neostigmine inhibited in vivo and in vitro epinephrine-stimulated lipolysis in dogs. The effects were not attributable to nervous stimulation as no parasympathetic stimulation was observed. However, the use of inhibitors

also led to the suggestion that these drugs might also inhibit lipases (Dixon & Webb, 1962). The possibility that PChE has some relationship to hormone-sensitive lipolysis requires further demonstration.

Specific Objectives of this Study

The present study was devised to determine (1) whether or not the serum, liver and adipose tissue PChE show differences in Zucker fat rats as were observed in obese humans and mice when compared to normal lean controls; (2) the age of onset of PChE changes in the liver and adipose tissue of the postweanling ob/ob mice; (3) the subcellular changes of PChE in the liver of ob/ob mice; (4) the influence of dietary protein, carbohydrates, and fat on PChE induction in the liver; and (5) the effect of a specific inhibitor of PChE on epinephrine-stimulated lipolysis in isolated rat adipocytes.

MATERIALS AND METHODS

Experimental Animals

All mice and rats used were males.

Mice

Obese (ob/ob) and lean (ob/+ or +/+) littermate controls of the strain C57GJ/BL were purchased from Jackson Laboratories, Bar Harbor, Maine, U.S.A. The mice were received either 1 or 2 days after shipment from the supplier.

CD-1 albino mice (Swiss-Webster strain) were purchased from Charles River Canada, Montreal, Quebec, Canada.

Rats

Five-month old Zucker fat (fa/fa) and lean (Fa/Fa) rats were obtained from Vassar College, Poughkeepsie, New York, U.S.A.

Sprague-Dawley rats weighing between 200-300 g were obtained from the Animal Care Unit at the Health Sciences Complex, Memorial University of Newfoundland, St. John's Newfoundland.

All animals were maintained at 25°C and light 12 hours per day in the Animal Care Unit. The animals were kept in individual cages during the course of the experiments except for the Sprague-Dawley rats which were housed 3 rats per cage. Animals were either kept in metal cages that had wire

mesh floors or plastic cages which contained heat-treated wood chips as bedding. Mice had free access to Purina Mouse Chow (mouse feed #5015) except where specified. Rats were fed ad libitum Purina Rat Chow #5012. All animals had free access to water.

Sacrifice of Animals and Collection of Tissues

Materials

"Ethrane" (2-chloro-1,1,2-trifluoroethyl difluoromethyl ether), Ohio Medical Anaesthetics; 1L or 4L beaker; gauze; 1 ml syringe with a 26 gauge, 9.5 mm needles, 5 ml syringe with 22 gauge, 25 mm needle, Becton-Dickinson Can. Inc.; Plastic dishes, #1 Whatman filter paper, Canlab.

Method

Gauze pads were placed in a beaker and moistened with sufficient "Ethrane". A cover was then placed over the mouth of the beaker and the vapour allowed to build up inside. The animal was then placed in the beaker and the mouth closed with the cover. The stoppage of breathing was used as the indicator of death. Generally, one to three minutes was needed to obtain euthanasia.

A mid-line incision was made to expose the required organs. The diaphragm was cut and the heart exposed. Blood samples were collected by heart puncture using either a 1 ml syringe with 26 gauge, 9.5 mm needle as in the case for the mouse or a 5 ml syringe with a 22 gauge, 25 mm needle as in the case for the rat. Livers were first perfused with cold 0.145M (0.85%) sodium chloride and blotted dry between two or four layers of 12-ply gauze. Epididymal adipose tissue was dissected out very carefully avoiding blood vessels and adjacent tissues. The adipose tissue, except for that used for adipocyte isolation, was rinsed in cold 0.145M sodium chloride and then blotted dry between two or four layers of 12-ply gauze. Sera and tissues were stored at -20°C until ready for use. When adipose tissue was to be used for adipocyte isolation, the epididymal fat pads were dissected out, carefully removing extraneous blood vessels and tissues, rinsed in warm 0.145M sodium chloride solution and blotted dry between two layers of #1 Whatman filter paper.

Homogenization of Tissues and Isolated Adipocytes

Materials

Potassium Chloride, J.T. Baker chemicals; Triton X100, Sigma Inc; distilled water; Ten-Broeck tissue grinders, Wheaton Scientific; Beckman TJ-6 centrifuge, Beckman Inc.;

Eppendorf microfuge, Brinkman; Vortex mixer, Lab-line Instruments; Thermostatically controlled incubator, National Inc.

Method

Livers were homogenized with 1% Triton X100 in 0.155M potassium chloride using the Ten-Broeck tissue grinders. A ratio of 1 gram of tissue to 4 ml of the medium was used. The crude homogenate was centrifuged at 3000 x g for 15 minutes at 4°C in a Beckman TJ-6 centrifuge. The supernatant was retained and then ultracentrifuged in a Beckman "Airfuge" at 110,000 x g for 5 minutes at room temperature. The supernatant from this step was used for the assay of PChE activity. Epididymal adipose tissue and isolated adipocytes were homogenized with cold distilled water. Adipose tissue was ground in a ratio of 1 gram of tissue to 2 ml of cold water using a Ten-Broeck tissue grinder. The isolated adipocytes were homogenized by vortexing the cells with 0.3 ml of cold distilled water for 2-4 seconds on a Lab-line vortex mixer. The crude homogenates were then centrifuged at 12,000 x g for 5 minutes at room temperature (22°C) in an Eppendorf 5412 microfuge. The clear infranatant phase was removed and used to assay for PChE and determination of protein.

Preparation of Liver Tissue From ob/ob and Lean Mice for Cytochemical Studies

Materials

Monobasic and dibasic sodium phosphate, paraformaldehyde, glutaraldehyde, cupic sulphate, sodium citrate and sodium acetate, were all obtained from Fisher Scientific; Propionylthiocholine iodide, Iso-OMPA, osmium tetraoxide, Sigma; Acetic acid, potassium ferricyanide, J.T. Baker Chemicals.

Method

Fresh frozen liver tissue was sectioned (40 micrometers thick) and placed in 0.1M phosphate buffer (pH 7.4) containing 1% paraformaldehyde for 30 minutes. After a 10-15 minute rinse with the 0.1M phosphate buffer, the sections were stained for PChE according to the method of Karnovsky & Roots (1964). A control set of sections were treated first with the PChE inhibitor Iso-OMPA at a concentration of 10^{-5}M for 30 minutes. These sections were then treated with the PChE staining mixture that contained Iso-OMPA at the same concentration. The staining medium was made as follows: 5 mg of propionylthiocholine iodide was dissolved in 6.5 ml of 0.1M acetate buffer, pH 6.0. The following were added in sequence and with stirring between each addition: 0.5 ml 0.1M sodium citrate, 1 ml 0.03M cupic sulphate, 1 ml water,

and 1 ml 0.005M potassium ferricyanide. Iso-OMPA was added in place of the water to give the final concentration of 10^{-5}M. The final staining mixture was clear, greenish in color, and was stable for hours. The sections were incubated at 4°C for 16 hours in the staining medium. After staining, the sections were placed in 2.5% glutaraldehyde in 0.1M phosphate buffer and treated for 1 hour with 4% osmium tetraoxide in phosphate buffer. The sections were finally dehydrated in decreasing concentrations of acetone and embedded in Epon. The ultra thin sections were examined with an electron microscope.

Composition and Preparation of Special Diets

Materials

Casein, technical from bovine milk, Sigma; granulated sugar, lard (Maple Leaf Tenderflake); American Institute of Nutrition mineral 76 and vitamin 76 mixtures, ICN Nutritional Biochemicals or U.S. Biochemicals; high carbohydrate diet, ICN Nutritional Biochemicals; high protein diet, U.S. Biochemicals; Purina Mouse Chow #5015, Purina Ralston; Acacia, BDH Chemicals.

Methods

Purina mouse chow #5015 consisted of 17.5% protein, 11.0% fat, and 53.9% carbohydrate. It was 18.0 KJ/g and 16.7% of the energy content was from protein.

The high protein diet was 64.0% casein, 22.0% sucrose and 8.0% vegetable oil. To 10 g of the powdered diet was added 0.2 g of tap water. To make this into a pellet, portions of the diet were placed in a 50 ml syringe which had one end cut away, and manually pressed with the plunger of the syringe. The open end of the syringe was closed off with a plastic disc to prevent loss of diet. The pellet was then dried for 24 hours at room temperature. It was 17.9 KJ/g and 60.9% of the energy content was from protein.

The high carbohydrate consisted of 68.0% sucrose, 18.0% casein, and 8:0% vegetable oil. It was 17.9 KJ/g and 17.3% of the food energy was from protein.

The high fat diet was prepared by modifying Purina mouse chow #5015 with lard to give 64.2% fat, 6.4% protein and 25.8% carbohydrate. The diet was made into pellets by the same method used to pellet the high protein diet. It was 26.3 KJ/g and 4.2% of the energy content was from protein.

The diets with varying proportions of protein were prepared by reducing the amount of protein with an equal proportion of carbohydrate so that the casein in each diet

was 0,20,40 and 50%. The percent energy content of each diet with 0,20,40 and 50% casein was 0,22.7,45.4 and 56.8% respectively. Fat, as lard, was constant at 4%. The mineral and vitamin content were the same in each diet, 4 and 1% respectively. Acacia, 1 g, was added as a binding agent. For every 100 g of diet, 10 ml of water was added. This made it sufficiently moist for pelleting. The diet was mixed manually and made into pellets as described for the protein diet.

Incubation of Isolated Rat Adipocytes for the Study of Epinephrine-Stimulated Lipolysis

Materials

Sodium chloride, bovine serum albumin (essentially fatty acid free (less than 0.005%)), collangenase from Clostridium histolyticum (lot #8/F-6827), tris (hydroxmethyl) aminomethane (Tris), 4-aminoantipyrine, adenosine triphosphate (disodium salt), peroxidase from horse radish (195 Purpurogallin U/mg solid (Rz = 133), epinephrine bitartrate, propranolol HCl(d,l), tetramonoisopropylpyrophosphoramide (Iso-OMPA), glycerokinase (90 U/mg of protein), Sigma; Magnesium sulphate-7-hydrate, calcium chloride, sodium bicarbonate, potassium phosphate, potassium chloride, disodium ethylene diamine tetraacetate (EDTA), glycerol

(analytical grade), hydrochloric acid, "Sealease" (capillary tube sealing compound), capillary tubes, forceps, Fisher Scientific or J.T. Baker Chemicals; 4-hydroxy-3, 5-dichlorobenzene sulfonate, (sodium salt), Research Organics; 1-glycerophosphate oxidase (40 U/mg of protein), small plastic rods, Boehringer-Mannheim; Digital pH meter, Corning Instruments; Capillary tube centrifuge, Clay-Adams Inc.; 15 ml plastic (polystyrene) conical tubes, Corning; 1.5 ml plastic (polyethylene) tubes, IEC centrifuge (Model CU-5000), Canlab; National thermostatically controlled incubator; Pye-Unicam SP 1800 spectrophotometer; Pharmacia mixer.

Method

This study was done in 4 experiments using the same volumes and concentrations as outlined in Table 1.

Epididymal fat pads from 1-2, male Sprague-Dawley rats (200-300 grams) were immediately dissected out and rinsed in warm (37°C) 0.145M sodium chloride. After removing extraneous blood vessels and tissues, and weighing the fat pads, isolated adipocytes were prepared according to the method of Rodbell (1964) but with the modifications as outlined by Dax, Partilla & Gregerman (1981). The fresh adipose tissue was minced with scissors and placed in 15 ml plastic conical tubes which contained Krebs-Henseleit original Ringer (KHR) bicarbonate

buffer (Krebs & Henseleit, 1932) as modified by Gruen & Greenwood (1981) to contain 4% bovine serum albumin (essentially fatty acid free) and one-third the concentration of calcium. The albumin KHR bicarbonate buffer was gassed with 95% O_2/5% CO_2 prior to use until the pH was 7.4 and 3 ml of this buffer per gram wet weight of fat was used. A maximum of 2.000 gram of fat per tube was used and 5 mg of collangenase per gram wet weight of adipose tissue was used to liberate the fat cells. A single batch of collangenase was used in all experiments. The tubes were incubated for one hour at 37°C with gentle rocking (about 60 cycles per minute).

The tissue was dispersed into small fragments within one hour of incubation with the collangenase. Liberation of adipocytes from the tissue fragments was achieved by gently stirring the tissue with a small plastic rod. Increased turbidity in the medium indicated liberation of the cells. Fragments of tissue still remaining after this treatment were removed with a plastic spatula and forceps. The suspension of cells was centrifuged in the plastic tubes for 1 minute at about 400 x g. The fat cells floated to the surface, and the stromal-vascular cells were sedimented. The stromal-vascular cells and buffer were removed with a Pasteur pipet, and the adipocytes were washed by suspending them in 10 ml

of warm (37°C) KHR bicarbonate buffer containing albumin. The suspended cells were then centrifuged for 1 minute at about 400 x g. This procedure was repeated once more. Fat droplets which floated more rapidly to the surface were aspirated from the surface, after gentle mixing the cell suspension.

Packed fat cells were diluted 1 in 4 in KHR bicarbonate buffer containing albumin and resuspended. Into four separate plastic conical test tubes was pipetted the volumes (in ml) of each of the reagents given in Table 1.

Table 1: Volumes used in incubation mixtures to examine the effect of specific inhibition of PDhE on epinephrine-stimulated lipolysis.

Tube #	1	2	3	4
KHR Bicarbonate buffer containing albumin	0.65	0.64	0.63	0.63
Cell Suspension	0.35	0.35	0.35	0.35
Epinephrine Bitartrate, 5×10^{-3}M	-	0.01	0.01	0.01
Iso-OMPA 1×10^{-3}M	-	-	0.01	-
Propranolol HCl (S-I) 1×10^{-3}M	-	-	-	0.01

All stock solutions were made fresh daily. The final concentration for epinephrine bitartrate was 5×10^{-5}M, for Iso-OMPA 1×10^{-5}M and for propranolol HCl 1×10^{-5}M. The packed cell percent volumes for the incubation mixtures were determined by taking up an aliquot of suspended cells into a capillary tube, plugging one end of the tube with sealing compound and then centrifuging the tube in a Clay-Adams haematocrit centrifuge for 4 minutes. The packed cell percent volume was read from a calibrated scale by alligning the meniscus with 100% of scale and bottom of the solution at the plugged end with 0% of scale. After incubation at 37°C with gentle mixing (about 60 cycles per minute) for 1 hour, the tubes were placed on ice. The tubes were then centrifuged for 1 minute at about 400 x g at a temperature of 0°C in a Beckman TJ-6 centrifuge. An aliquot of the infranatant was removed and saved for glycerol determination. The remaining layer of cells was washed with KHR bicarbonate buffer to remove the albumin that was contained in the incubation mixture and saved to assay for PChE activity and protein determination. The cells and infranatant were stored at -20°C until assayed.

Measurement of Lipolysis

The rate of lipolysis was measured as glycerol release. Glycerol was measured by a colorimetric, enzymatic method.

The method involved incubating 0.05 ml of sample or standard with 0.5 ml of reagent mixture at 37°C for 60 minutes. Standard solutions of analytical grade glycerol were prepared in albumin KHR buffer. The reagent mixture consisted of 3.75 ml of 0.15M of Tris-HCl buffer, pH 7.6; 1 ml of 0.175M $MgSO_4$; 1 ml of 0.1M EDTA, disodium; 1 ml of 0.005M ATP, disodium salt; 1 ml of 0.0075M 4-aminoantipyrine; 1 ml of 0.03M 2-hydroxy-3, 5-dichlorobenzene sulfonate, sodium salt; 0.05 ml of 450 U/ml glycerokinase; 1 ml of 40 U/ml glycerophosphate oxidase and 0.1 ml of 27.5 U/ml horse radish peroxidase. Final volume was 10 ml. The resulting color was measured at 510nm in a Pye-Unicam 1800 spectrophotometer with 1 cm pathlength glass cuvettes against a reagent blank of 0.05 ml of albumin KHR buffer and 0.5 ml of reagent mixture. The concentration of glycerol present was determined from the standard curve of glycerol concentration versus optical density at 510nm.

Lipolytic Activity of Purified Horse Serum PChE

Materials

Butyrylcholinesterase (EC 3.1.1.8) from horse serum, 230 U/mg of protein, Sigma; Triacylglycerol acylhydrolase, (lipase EC 3.1.1.3) (from porcine pancreas 483 ± 109 U/L),

lipase reagent, Boehringer Mannheim; Pye-Unicam SP 1800 spectrophotometer.

Method

Determination of lipase activity was by the method described by Ziegenhorn, Neumann, Knitsch & Zwez, 1979.

Reagents

Lipase reagent consisted of the following: triolein, 0.0003M; Tris buffer, 0.026M, pH 9.2; sodium desoxycholate, 0.019M; calcium chloride, 0.0001M; colipase, 3 mg/L. The freeze-dried lipase reagent was reconstituted in 2.5 ml of distilled water.

The standard freeze-dried lipase was reconstituted in 1.0 ml of distilled water by gentle swirling over a period of 30 minutes. This gave an activity of 483 ± 109 U/L.

To determine the lipase activity of a purified horse serum PChE, the standard lipase preparation was used as a reference.

To assay the lipase activity of the purified horse serum PChE, 0.05 ml of 1 U/ml of the purified horse serum PChE was pipetted into a quartz cuvette that contained 2.5 ml of lipase reagent and 0.05 ml of 0.145M sodium chloride. This was mixed and 4 minutes after the sample was added, the

absorbance at 340nm in a Pye-Unicam SP 1800 spectrophotometer was noted (A_1). After another 5 minutes had elapsed, the absorbance was again noted (A_2). The difference between A_1 and A_2 was calculated and used to determine the lipase activity. The assay of the standard lipase was similar except that 0.1 ml of the standard lipase solution was used. All measurements were at 25°C and against air. To calculate the lipase activity of the purified horse serum PChE in U/L, the ratio of the change in absorbance for the PChE to the change in absorbance for the standard was found and multiplied by the activity for the standard.

Incubation of a Specific Inhibitor of PChE with Purified Lipase

Materials

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) (from porcine pancreas, 483 ± 109 U/L), lipase reagent, Boehringer Mannheim; Tetramonoisopropylpyrophosphoramide (Iso-OMPA), Sigma; Pye-Unicam SP 1800 spectrophotometer.

Method

Determination of lipase activity was by the method of Ziegenhorn et al, 1979.

Reagents

Lipase reagent containing: triolein, 0.0003M; Tris buffer, 0.026M, pH 9.2; sodium desoxycholate, 0.019M; calcium chloride, 0.0001M; colipase, 3 mg/L. The freeze-dried reagent was dissolved in 2.5 ml of distilled water.

The freeze-dried lipase was reconstituted in 1.0 ml of distilled water by gentle swirling over 30 minutes. This gave an activity of 483 ± 109 U/L.

The inhibitor used in this experiment, Iso-OMPA, was made up in distilled water. The concentration used was 0.001M.

Pipet 0.100 ml of purified lipase solution and 0.001 ml of 0.001M inhibitor solution into a clean tube. This gave an effective final concentration of the inhibitor of 0.00001M. Each incubation was for 20-30 minutes. A control of 0.100 ml of lipase solution and 0.001 ml of water was treated in the same way.

To assay for the lipase activity, 2.5 ml of lipase reagent was added at the end of the incubation to all the tubes containing the purified lipase and either an inhibitor or water. This was mixed and then transferred to a quartz cuvette taking care that all the mixture was removed from the tube. The spectrophotometer was set to 340nm and zeroed. An initial absorbance, A_1, was recorded 4 minutes after the lipase reagent was added to the solution of lipase

with either inhibitor or water. After another 5 minutes, the absorbance was again noted (A_2). The difference between A_1 and A_2 was determined for the inhibitor treated lipase preparations and compared with the control for calculation of lipase activity present. All measurements were at 25°C and against air. To calculate the lipase activity in U/L, the ratio of the change in absorbance for the inhibitor treated lipase sample to that for the control standard lipase solution was found and multiplied by the activity for the control standard.

Biochemical Methods

Determination of PChE Activity in Serum, Liver and Adipose Tissue

Materials

Propionylthiocholine iodide, 5,5' dithiobis - (2-nitro benzoic acid) (DTNB), tris (hydroxymethyl) aminomethane (Tris), Butyrylcholinesterase (EC 3.1.1.8) (from horse serum, 230 U/mg of protein), Sigma; volumetric flask, tubes, Fisher Scientific; water bath, Canlab.

PChE was assayed by the method of Dietz, Rubinstein & Lubrano (1973) using propionylthiocholine iodide as substrate

and the color forming reagent DTNB. The enzyme activity was calculated from a standard curve constructed using as reference propionylthiocholine iodide, which had been treated with horse serum butyrylcholinesterase, as a source of free thiocholine.

Reagents

0.05M Tris-HCl-DTNB buffer, pH 7.4. Dissolve 6.055 g of Tris in 900 ml of distilled water. Adjust the pH to 7.4 with about 40 ml of 1M HCl. After the solution has been adjusted to pH 7.4, 0.100 g of DTNB was added and dissolved. The solution was then made up to 1L with water.

To prepare the substrate-buffer solution, 60 ml of the pH 7.4 Tris-HCl-DTNB buffer was mixed with 6 ml of 0.005M propionylthiocholine iodide. Fifteen (15) ml of water was then added and the solution mixed. Into two glass cuvettes was pipetted 2.70 ml of the substrate-buffer solution and water equal of the sample volume to be used. The cuvettes were then placed in the spectrophotometer, a Pye-Unicam SP 1800 set with slit width 0.3 mm and wavelength 410nm, with one cuvette in the reference path and the other in the path for the sample. The spectrophotometer was zeroed and the recorder, a Unicam AR25, adjusted to baseline. Into a clean cuvette containing 2.70 ml of substrate-buffer solution was pipetted the sample and the change in absorbance of the

thiocholine-DTNB complex formed was recorded at 410nm using the recorder to plot the rate of change. In the case of the mouse, 0.02 ml of liver or serum sample was used as the assay volume whereas for the rat, 0.05 ml was used to assay these samples. For assay of PChE activity in adipose tissue or adipocyte samples, 0.100 ml was the sample volume.

To prepare the standard curve for quantitating the activity of PChE present, I used propionylthiocholine as the reference standard. This entailed use of nascent thiocholine liberated by the hydrolysis of propionylthiocholine iodide. To make the stock standard solution of propionylthiocholine iodide, 0.03035 g of propionylthiocholine iodide was made up to 5 ml in a volumetric flask. This gave a concentration of 1 micromole of propionylthiocholine iodide per 0.05 ml of solution. Into a clean 13 x 100 mm test tube was pipetted 0.05 ml of this stock solution and 0.05 ml of a 1U/ml solution of horse serum butyrylcholinesterase. The tube was then mixed by inversion and the mixture incubated for 10 minutes in a 37°C water bath. After incubation was complete, serial dilutions from the enzyme treated mixture was made to give 0.2, 0.1, ..., 0.00625 micromole of nascent thiocholine per 0.05 ml. Standard solutions were then made by adding 0.05 ml of each serial dilution to 2.70 ml of the

Tris-HCl-DTNB buffer. The serial standards were then read at room temperature against a reagent blank of 0.05 ml of water and 2.70 ml of Tris-HCl-DTNB buffer in a Pye-Unicam SP 1800 spectrophotometer with slit width 0.3 mm and wavelength 410nm. A curve of optical density versus concentration of thiocholine in micromole per test volume was plotted.

Determination of Triglycerides in Serum

Materials

Triglycerides fully enzymatic kinetic UV-method kit, Boehringer Mannheim; Centrichem 600 autoanalyzer, Union Carbide.

The biochemical reactions of this test are:

$$\text{Triglycerides} \xrightarrow[\text{esterase}]{\text{lipase}} \text{glycerol + fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{Glycerol kinase}} \text{glycerol-3-phosphate + ADP}$$

$$\text{ADP + phosphoenolpyruvate} \xrightarrow[\text{Kinase}]{\text{Pyruvate}} \text{pyruvate + ATP}$$

$$\text{Pyruvate + NADH + } H^{+} \xrightarrow[\text{Dehydrogenase}]{\text{Lactate}} \text{lactate + } NAD^{+}$$

The oxidation NADH was followed at 340nm. The reaction was performed at 37°C, pH 7.0 and was incubated for 95 seconds (Wahlefeld, 1974).

Reagents (Provided in the kit)

Buffer: 0.05M phosphate, pH 7.0 containing 0.004M $MgSO_4$, 0.00034M sodium dodecylsulphate and 2g/L bovine serum albumin.
Solution 1: NADH/ATP/phosphoenolpyruvate solution of initial concentrations 0.012M, 0.033M and 0.018M, respectively.
Solution 2: Lactate dehydrogenase, 300 U/ml; pyruvate kinase, 50 U/ml; lipase, 5000 U/ml; and esterase, 30 U/ml.
Solution 3: Glycerol kinase, 150 U/ml. A stock working solution was made by mixing in sequence 25 ml of buffer, 0.05 ml of solution 1, 0.05 ml of solution 2 and 0.12 ml of solution 3.

For each series of measurements, a reagent blank with water instead of serum was carried out. The concentration of triglycerides was determined from a standard curve prepared concurrently. For each measurement, 0.010 ml of sample or standard and 0.500 ml of stock working solution were pipetted into a cuvette, incubated for 95 seconds at 37°C and the absorbance read at 340nm. The difference in absorbance between that for the reagent blank and that for the sample or standard was used to calculate the quantity of triglycerides present.

Determination of Cholesterol in Serum

Materials

Cholesterol enzymatic kit, Boehringer Mannheim; Centrichem 600 autoanalyzer, Union Carbide.

The biochemical reactions of this test are:

Cholesterol esters $\xrightarrow{\text{cholesterol esterase}}$ cholesterol + fatty acids

Cholesterol + O_2 $\xrightarrow{\text{cholesterol oxidase}}$ Cholest-4-en-3-one+H_2O_2

H_2O_2 + phenol + 4-aminophenazone $\xrightarrow{\text{peroxidase}}$ Red dye + 2 H_2O_2

The hydrogen peroxide reacts in the presence of peroxidase with phenol and 4-aminophenazone forming a red dye. The intensity of the color formed is proportional to the cholesterol and is measured spectrophotometrically at 520 nm (Klose, Grief & Hagan, 1975; Trinder, 1969).

Reagents (Provided in the kit)

Buffer-chromogen: [illegible]M phosphate buffer, pH 7.2; 0.0075M, 4-aminophenazone; 2.0M, methanol; 0.8% w/v, hydroxypolyethoxydodecane; Solution 1: cholesterol esterase and peroxidase: 8.2 U/ml and 290 U/ml, respectively.

Solution 2: Cholesterol oxidase: 8.9 U/ml

Solution 3: 0.64M phenol in 1.7M ethanol. A stock working solution was prepared by mixing in sequence: 250 ml of

buffer-chromogen solution, with 4 ml of solution 1, with 4 ml of solution 2, and 5 ml of solution 3. This was then diluted upto 500 ml with distilled water.

For each series of measurements, a reagent blank with water instead of serum was carried out. The quantitation of cholesterol was determined from a standard curve made concurrently. For each measurement, 0.005 ml of sample or standard and 0.25 ml of stock working solution were pipetted into a cuvette, incubated for 10 minutes at 30°C and the absorbance read at 520nm. The difference in absorbance between that for the reagent blank and that for the sample or standard was used to calculate the quantity of cholesterol present.

Determination of Glucose in Serum

Materials

Glucose enzymatic colorimetric method kit, Boehringer Mannheim; Centrichem 600 autoanalyzer, Union Carbide.

The biochemical reactions of this test are:

$$\text{Glucose} + O_2 + H_2O \xrightarrow[\text{oxidase}]{\text{glucose}} \text{gluconate} + H_2O_2$$

$$2\ H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{peroxidase}}$$

$$\text{4-(p-benzoquinone-mono-imino) phenazone} + 4\ H_2O$$

The oxidation of glucose to gluconate with the formation of H_2O_2 is coupled to a reaction where the H_2O_2 reacts, in the presence of peroxidase, with 4-aminophenazone to give a red dye. The intensity of the color formed is proportional to the glucose and is measured spectrophotometrically at 510nm (Trinder, 1969).

Reagents (Provided in the kit)

Solution 1: phosphate buffer, 0.1M, pH 7.0; 4-aminophenazone, 0.00077M; glucose oxidase, 18 U/ml; peroxidase, 1.1 U/ml. Solution 2: phenol, 0.011M. A stock working solution was made by dissolving 100 ml solution 1 in 200 ml of distilled water and to that was added 30 ml of solution 2.

For each series of measurements, a reagent blank with water instead of serum was carried out. The quantitation of glucose was calculated from a standard curve made concurrently. For each measurement, 0.02 ml of sample or standard was mixed with 0.2 ml of stock working solution in a cuvette, incubated for 10 minutes at 37°C and the absorbance read at 520nm. The difference in absorbance between that for the reagent blank and that for sample or standard was used to determine the quantity of glucose present.

Determination of Insulin in Serum

Materials

Phadedas Insulin Test Kit, Pharmacia Diagnostics.

Method

Insulin was determined by a radioimmunoassay method (Thorell & Lanner, 1973).

Reagents (Provided in the kit)

Buffer: The buffer was prepared by dissolving the buffer substance in 200 ml redistilled water.

Sephadex-Anti-Insulin Complex: This was made by transferring quantitatively to a beaker containing a magnetic stirring rod the Sephadex-Anti-Insulin Complex using 100 ml of buffer solution in portions.

Insulin Standard: The standard was reconstituted in 4.0 ml of distilled water. After reconstitution, the solution contained 320 μU/ml.

Insulin ^{125}I: Ten (10) ml of buffer was used to reconstitute the lyophilized Insulin ^{125}I.

Working Decanting-Aid-Solution: This stock was prepared by diluting 5 ml of the Phadedas stock decanting-aid-solution to 175 ml with a .145M NaCl solution.

A series of plastic tubes were set up and labelled for duplicate assays of standards (5 μU/ml to 320 μU/ml) and of unknowns and two additional tubes for determination of total activity added. Into respective tubes was pipetted 0.1 ml of unknown or standard. To these tubes was added 0.1 ml of the Insulin ^{125}I solution. Into the two additional tubes was pipetted 0.1 ml of the Insulin ^{125}I solution. These tubes were capped immediately and used to determine total activity added. Care was taken to not dispense any of the Insulin ^{125}I onto the walls of these tubes. These were not incubated, centrifuged or washed.

The Sephadex-Anti-Insulin Complex suspension (1.0 ml) was pipetted into all tubes to which standards and unknowns had been added. The suspension was stirred continuously while it was dispensed into the tubes. All tubes containing standards and unknowns were incubated overnight at room temperature. During this incubation period, the tubes were mixed by vertical rotation to keep the particles in suspension. After the incubation was complete, the tubes of standards and unknowns were centrifuged at 2000 x g for two minutes in order to remove droplets from the stoppers. The stoppers were removed and 1.5 ml of the Working Decanting-Aid-Solution was added to all tubes except

those two tubes for total activity. The tubes were then centrifuged at 1500 x g for 2 minutes in swing-out bucket rotor in a Beckman TJ-5 centrifuge. The supernatant was then decanted off each tube ensuring that the last drop was removed. The radio-activity was determined in each tube for standards, unknowns, and total activity using a Picker Compac 120 gamma-counter. The background was determined by using an empty plastic centrifuge tube. Each tube was counted for one minute. Calculation of insulin concentration was done by the gamma-counter.

Protein Determination

1) Biuret Method. The method of Gornal, Bordawill & David (1949) was used.

Material

Bovine serum albumin, Sigma; Volumetric flask, Fisher Scientific.

Biuret Reagent

The biuret reagent consisted of 0.0548M sodium hydroxide, 0.0093M sodium potassium tartrate, 0.0083M potassium iodide, and 0.0035M copper sulphate. The final pH was about 12.

Stock Standard Solution of Bovine Serum Albumin

The stock standard solution of bovine serum albumin was made by dissolving 0.800 g of albumin in water and then making the volume up to 10 ml with water in a volumetric flask. Serial dolutions of 4.000 g/dl and 2.000 g/dl were prepared from the stock solution.

Method

Crude liver homogenates were first centrifuged at 3000 x g for 15 minutes at 4°C in a Beckman TJ-6 centrifuge. The resulting supernatant was retained and recentrifuged at 110,000 x g for 5 minutes at room temperature in a Beckman "Airfuge" ultracentrifuge. The resulting supernatant was used for protein determination.

Into separate 12 x 75 mm test tubes was pipetted 3.0 ml of biuret reagent. To the reagent was added 0.05 ml of liver sample or standard. A reagent blank of 3.0 ml of biuret reagent and 0.05 ml of H_2O was also prepared. Each tube was mixed and incubated for exactly 30 minutes at room temperature.

After 30 minutes, the resulting absorbances were read at 540nm using a Coleman Jr. II spectrophotometer. The instrument was first zeroed with the reagent blank at 540nm. A standard curve of absorbance at 540nm versus

concentration was plotted. The concentration of the unknowns was read from the standard curve.

2) Bradford's Method. The method of Bradford (1976) was used to quantitate microgram quantities of protein.

Material

Bovine serum albumin, Sigma; Volumetric flask, Coleman Jr. II spectrophotometer, Fisher Scientific.

Coomassie Blue Reagent

The Coomassie Blue reagent consisted of 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid.

Method

Test samples were diluted first to give an approximate concentration within the range of 0 to 50 mg/dl of protein. A stock standard solution of 50 mg/dl of protein was prepared using bovine serum albumin as the standard. Serial dilutions of 25, 12.5, 6.25 mg/dl were made from the stock standard solution.

To each tube was added 0.100 ml of standards and test samples. To each tube was added 2.5 ml of the Coomassie

Blue reagent. A reagent blank was prepared by substituting 0.100 ml of distilled water for the test volume and adding 2.5 ml of the Coomassie Blue reagent. All tubes were mixed by gentle inversion and allowed to stand at room temperature for at least 2 minutes but not longer than 1 hour. The spectrophotometer was zeroed with the reagent blank at 595nm. The test samples and standard were read at the same wavelength. A standard curve of absorbance at 595nm versus protein concentration in mg/dl was plotted. The concentration of protein in the test sample was read from the curve and multiplied by the dilution factor to determine the actual protein concentration.

Statistical Analyses

Data for Zucker fat rats and lean rats were analyzed by Student's t test. Means are followed by their standard errors (SEM). Correlation coefficient and slopes were calculated by linear regression analysis using a Sharp pocket computer programmed for linear regression analysis.

Simple effects analysis (Howell, 1982) was used to determine if significant differences exist between ob/ob and lean mice at all ages from 23 days and older for liver and adipose tissue PChE activity.

One-way analysis of variance was used to determine if statistical differences exist between the groups fed different diets. Then, to test for which groups were different from each other, Scheffe's multiple comparison procedure (Neter & Wasserman, 1974) was used.

Student's t test was also used to analyze the results for the effect of a specific inhibitor of PChE on epinephrine-stimulated lipolysis and the effect of a specific inhibitor of PChE on the lipolytic activity of purified lipase *in vitro*. Means are followed by their standard deviations.

RESULTS

Serum and Liver PChE Activity, and other Biochemical Characteristics of Zucker Fat Rats

The results in Table 2 show that the mean ± SEM of PChE activity in the serum and liver, respectively, are 4020 ± 290 micromoles/min/L and 11.10 ± 1.01 micromoles/min/g wet weight in the fa/fa rats; 2320 ± 120 and 7.9 ± 0.75 in the lean rats. Serum triglycerides and insulin as well as body weight and food intake are significantly higher in the fat rats when compared to lean controls. In contrast, serum glucose, serum cholesterol, and adipose tissue PChE activity are not significantly different between the two groups.

The Mutual Relationship Between Various Physiological and Biochemical Characteristics of Zucker Fat Rats

Linear regression analysis was done on the data from Table 2 to determine the mutual relationship between any two of the variables. The results for the correlation coefficients are presented in Table 3. The data indicated a good positive correlation between serum PChE activity and liver PChE activity (r=0.55), serum triglycerides (r=0.53),

body weight ($r = 0.60$) and food intake ($r = 0.60$); however a weak correlation was found with serum insulin ($r = 0.47$), glucose ($r = -0.31$) and cholesterol ($r = 0.06$). Food intake has good positive correlations with serum triglycerides ($r = 0.70$) and body weight ($r = 0.70$).

Table 2: A comparison of various analyses between the Zucker fat (fa/fa) rats and lean (Fa/Fa) rats.

TYPE OF ANALYSIS	FAT RATS (13)[x]	LEAN RATS (14)[x]
Serum PChE*	4020.0 ± 290	2320.0 ± 120
Liver PChE+	11.1 ± 1.01	7.90 ± 0.75
Adipose PChE+	2.08 ± 0.29	2.26 ± 0.22
Serum Triglycerides (mg/dl)	534.0 ± 104.0	96.1 ± 15.4
Serum Cholesterol (mg/dl)	90.4 ± 11.5	68.7 ± 7.6
Serum Glucose (mg/dl)	278.0 ± 35.2	305.0 ± 20.6
Serum Insulin (μU/ml)	57.1 ± 7.8	11.3 ± 0.6
Body Weight (g)	530.0 ± 120.5	321.0 ± 22.8
Food intake (g/day)	29.9 ± 7.4	19.9 ± 1.0

[x]Number of animals in each group

*Micromoles of thiocholine formed/min/L serum; +micromoles of thiocholine formed/min/g wet weight. All the values are significantly higher ($P < 0.01$) in the fat rats than the leans except for adipose tissue PChE, cholesterol and glucose.

Table 3: Correlation co-efficient of serum PChE activity and food intake versus any one of the following variables.

VARIABLE	SERUM PChE	FOOD INTAKE
1. Liver PChE	0.55	-
2. Serum Triglycerides	0.53	0.70
3. Serum Insulin	0.47	0.18
4. Serum Cholesterol	0.23	0.16
5. Serum Glucose	-0.31	-0.06
6. Body Weight	0.60	0.70
7. Food Intake	0.60	-

Age Related Changes in Liver PChE Activity in ob/ob Mice: Relationship to Body Weight and Food Intake

The relationship between liver PChE activity and age in ob/ob mice and lean littermates is shown in Figure 1. Simple effects analysis for liver PChE activity showed that significant differences ($P < 0.05$) exist between ob/ob mice and lean littermates at all ages from 23 days and older. The change in activity between 23 and 38 days was greatest in ob/ob mice. In lean mice, liver PChE activity began to increase at 27 days. Figure 1 also shows that body weight was significantly higher in the ob/ob mice beginning at 23 days when compared to lean littermates. Food intake was also significantly greater in the ob/ob mice at 27 days of age.

Age Related Changes in Adipose Tissue PChE Activity in ob/ob Mice

The relationship between adipose tissue PChE activity and age in ob/ob mice and their lean littermates is shown in Figure 2. Adipose tissue PChE activity was shown, by simple effects analysis, to be significantly lower ($P < 0.05$) in ob/ob mice at all ages from 23 days and older when compared to lean mice of the same age. Both ob/ob and lean mice showed a similar pattern in adipose tissue PChE activity

in relation to age. In both groups, adipose tissue PChE activity decreased between 23 and 38 days of age. The decrease was greater between 23 and 27 days of age for the ob/ob mice, and between 27 and 38 days of age only a small decrease was observed. In contrast, adipose tissue PChE activity of the lean mice showed a sharp drop between 23 and 27 days of age, but between 27 and 38 days of age a lower decrease occurred.

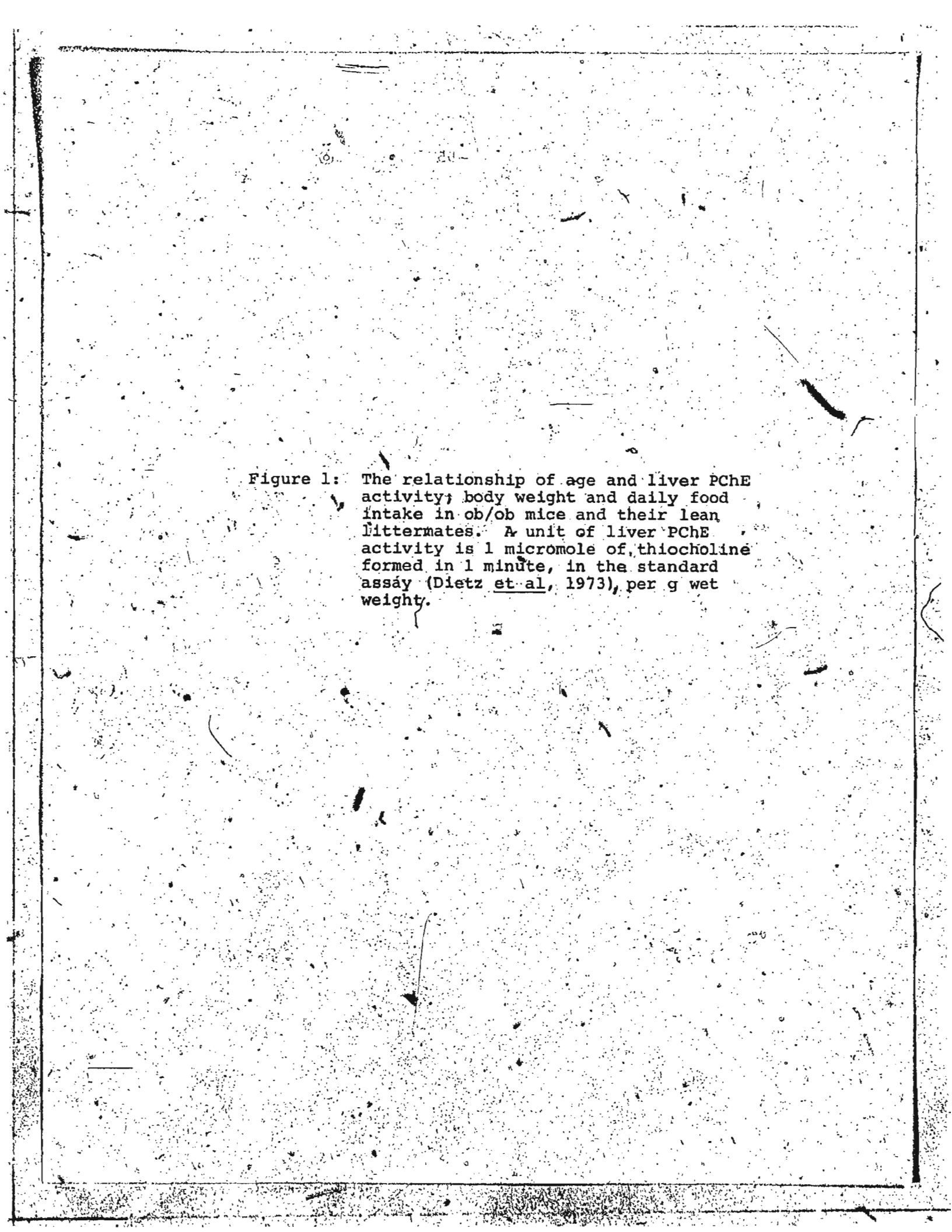
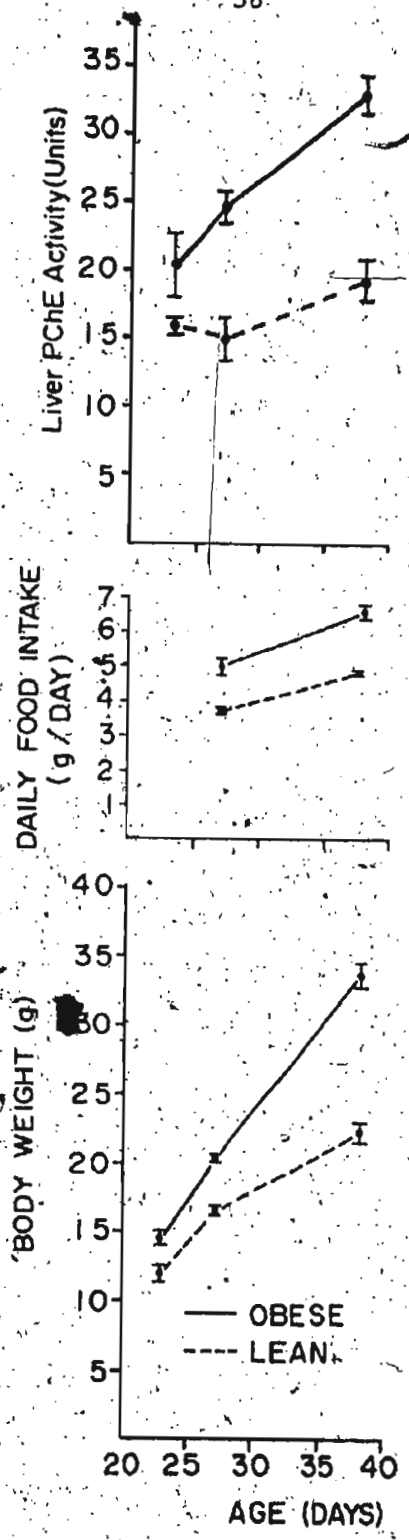


Figure 1: The relationship of age and liver PChE activity, body weight and daily food intake in ob/ob mice and their lean littermates. A unit of liver PChE activity is 1 micromole of thiocholine formed in 1 minute, in the standard assay (Dietz et al, 1973), per g wet weight.


Liver PChE Activity(Units)
35
30
25
20
15
10
5
DAILY FOOD INTAKE (g/DAY)
7
6
5
4
3
2
1
BODY WEIGHT (g)
40
35
30
25
20
15
10
5
OBESE
LEAN
20 25 30 35 40
AGE (DAYS)

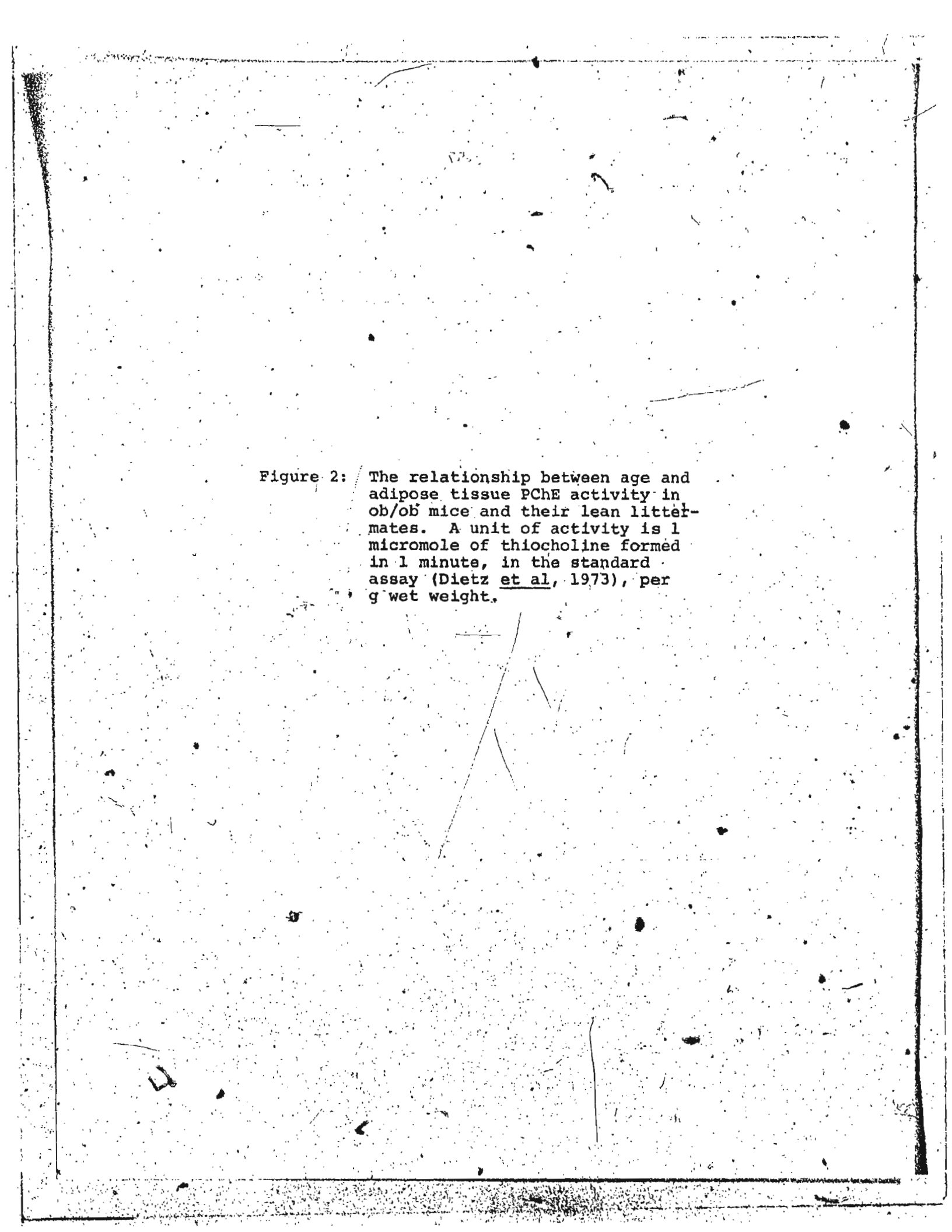
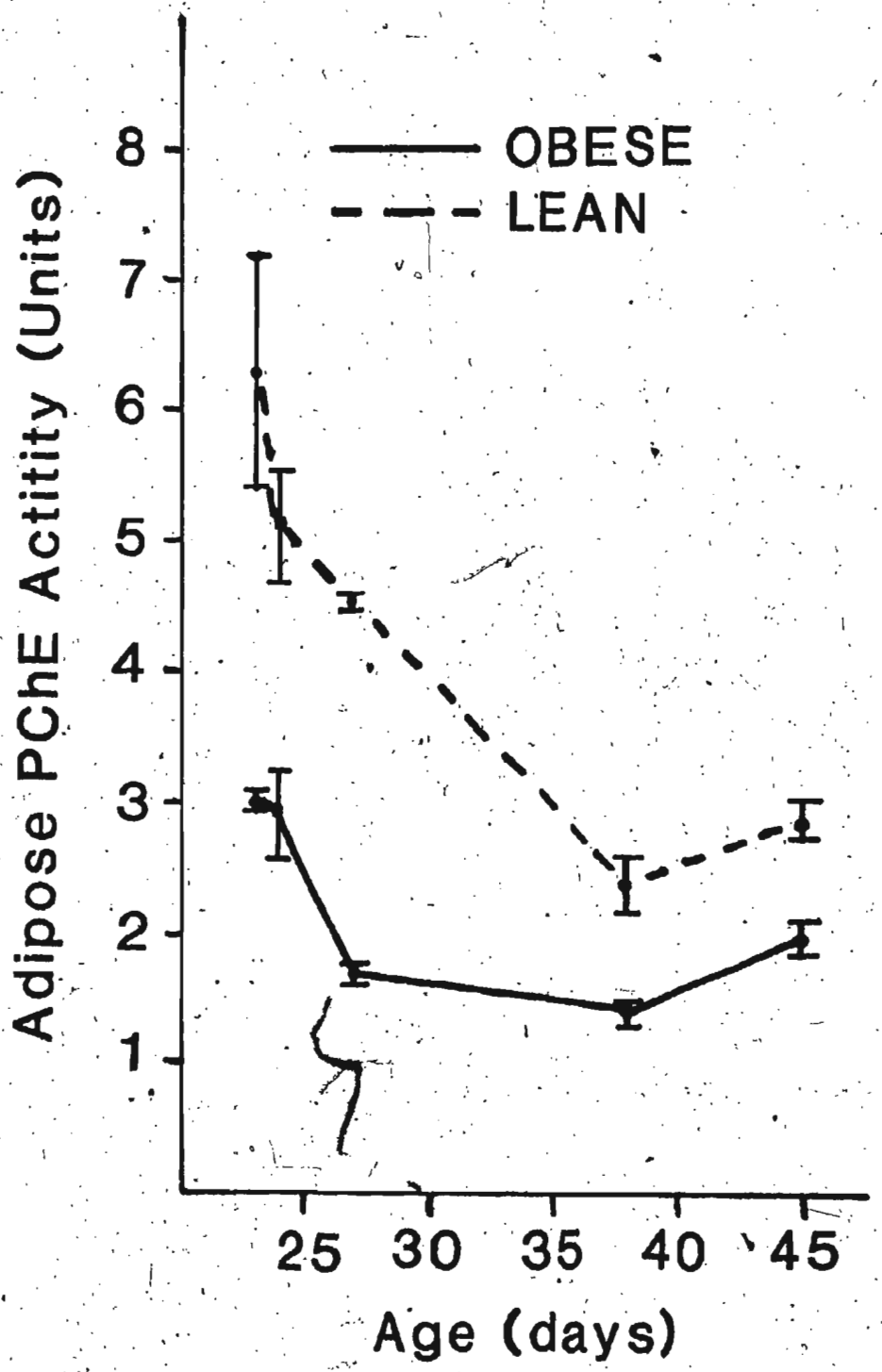


Figure 2: The relationship between age and adipose tissue PChE activity in ob/ob mice and their lean littermates. A unit of activity is 1 micromole of thiocholine formed in 1 minute, in the standard assay (Dietz et al, 1973), per g wet weight.


OBESE
LEAN
Adipose PChE Actitity (Units)
8
7
6
5
4
3
2
1
25
30
35
40
45
Age (days)

Electron Microscopy Studies of Liver PChE in the ob/ob Mouse

Electron microscopy studies (Figure 3) of liver preparations revealed an intense staining for PChE located in the rough endoplasmic reticulum of the ob/ob mice which was not intense in the liver of the lean. Iso-OMPA completely inhibited the staining for cholinesterase, indicating that the staining is entirely due to PChE.

Liver PChE Activity under Different Dietary Conditions

Table 4 gives the mean and SEM for liver PChE, final body weight, daily food intake, and daily energy intake under various dietary conditions. First, one-way analysis of variance was used to test the null hypothesis that there exist no differences among the groups fed various diets for the variables liver PChE activity, daily food intake, daily energy intake, and final body weight. The F-values of the analysis of variance lead to the conclusion that not all groups are identical for the variables. Since testing all possible pairs of means in the usual way affects the probability of rejecting a null hypothesis, Scheffe's multiple comparison procedure was used. By this procedure, it was possible to determine which groups are different

from each other. When this multiple comparison procedure was applied, an experiment-wise error rate, α, was used to indicate the overall significance. The choice of α is determined in part by the number of comparisons and is larger if the number of possible comparisons is larger.

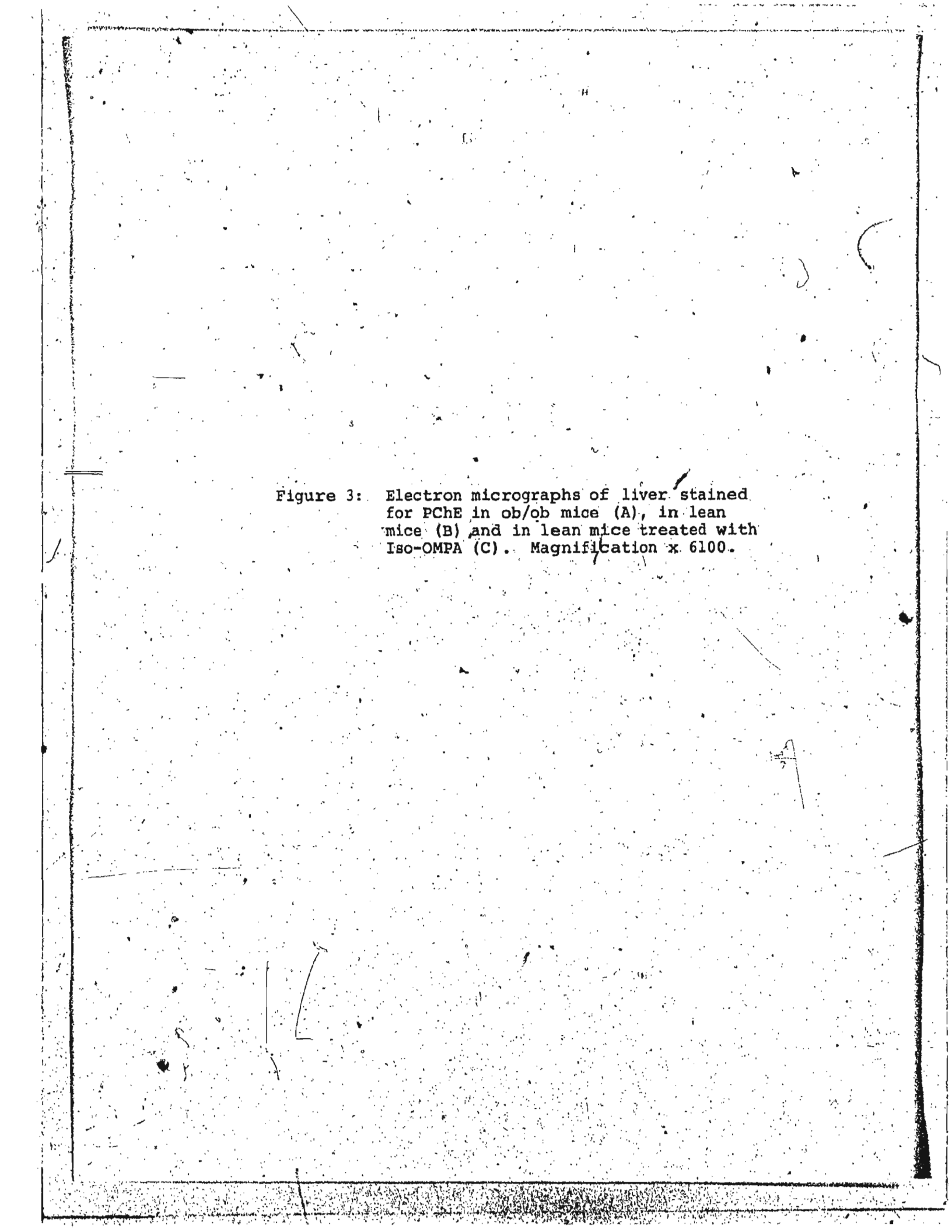
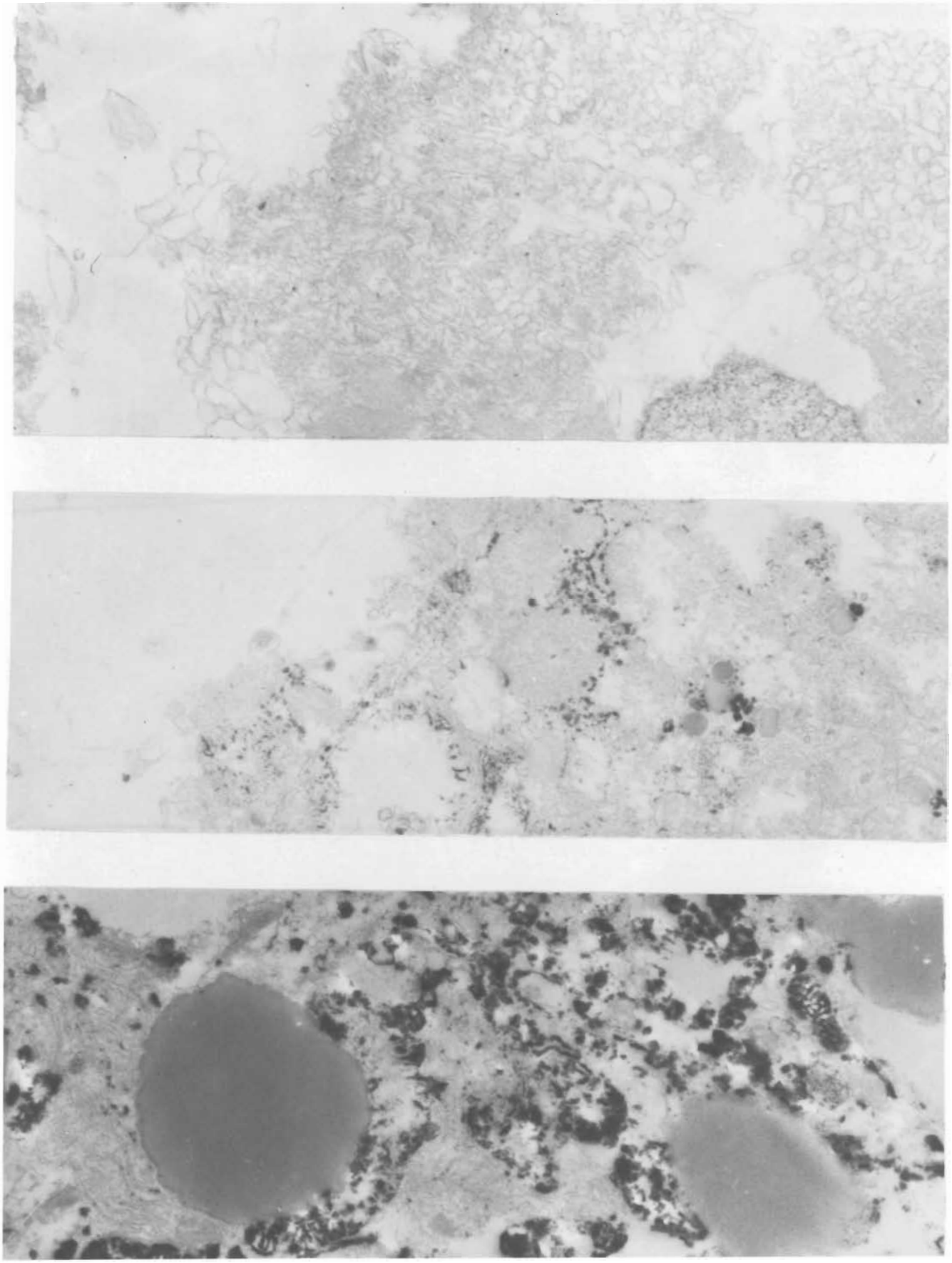


Figure 3: Electron micrographs of liver stained for PChE in ob/ob mice (A), in lean mice (B) and in lean mice treated with Iso-OMPA (C). Magnification x 6100.



(C)

(B)

(A)

Table 4: Pseudocholinesterase (PChE) activity in the liver, final body weight, daily food intake and daily energy intake under various dietary conditions in albino mice. Mice were fed chow, high protein, high carbohydrate or high fat diets for 5 days. Mice were fed the restricted diet for 11 days. Detailed description of the diet is found on pages 23-25.

Group Number	N	Diet	PChE activity micromoles/min/g wet weight	Final body weight,g	Food intake g/day	Energy Intake KJ/day
1	8	Chow ad lib.	9.26 ± 0.9	33.0 ± 0.4	5.3 ± 0.5	95.5 ± 9.22
2	6	Restricted chow	6.15 ± 0.4	27.1 ± 0.3	2	36.0
3	5	High protein ad lib.	15.06 ± 1.2	34.3 ± 1.0	5.02 ± 0.3	89.8 ± 4.61
4	3	High carbohydrate ad lib.	9.87 ± 1.0	36.6 ± 1.2	6.10 ± 0.2	109.0 ± 3.35
5	5	High fat ad lib.	6.21 ± 0.3	33.6 ± 0.8	3.56 ± 0.2	94.4 ± 4.19

Results are mean ± standard error of mean. N is the number of animals.

Since the F-test led to the rejection of the null hypothesis that the diets are identical at the 0.05 level of significance, I chose an error rate of $\alpha = 0.20$, 0.10, and 0.05, respectively. The results of Scheffe's multiple comparison procedure are given in Table 5.

Relationship between Liver PChE Activity and Casein Content in the Diet

Figure 4 shows that liver PChE activity increased with each increase in the casein content of the diet. A strong positive correlation exists between liver PChE activity and the casein content of the diet. A linear regression model was fitted to the data and revealed that liver PChE activity (e) was related to casein content of the diet (p) by the equation $e = 0.25p + 11.76$ ($r=0.73$).

Liver Protein Levels under Different Dietary Conditions

The differences in liver PChE activity, measured as micromoles per minute per g wet weight of tissue, found between high protein, high carbohydrate, and high fat diets do not reflect different levels of liver protein. The extracts, made at a standard concentration of 1:4 (w/v)

of tissue to homogenizing medium for measuring PChE activity, did not contain significantly different concentrations of protein (high protein diet, 3.04 ± 0.17; high carbohydrate diet, 2.56 ± 0.24; high fat diet, 2.6 ± 0.24).

Table 5: Scheffe's multiple test between the diets for liver PChE activity, final body weight, daily food intake and daily energy intake.

	Restricted	Chow ad lib.	High Protein	High Carbohydrate	High Fat
Restricted	-				
Chow ad lib.	$1^0,2^0,3^b,4^a$	-			
High protein	$1^0,2^0,3^a,4^a$	$1^x,2^x,3^a,4^x$	-		
High carbohydrate	$1^0,2^0,3^b,4^a$	$1^x,2^b,3^x,4^c$	$1^x,2^b,3^a,4^x$	-	
High fat	$1^0,2^0,3^x,4^a$	$1^a,2^x,3^b,4^x$	$1^b,2^x,3^a,4^x$	$1^a,2^b,3^b,4^c$	-

The notations 1,2,3 and 4 are to denote the variables daily food intake, daily energy intake, liver PChE activity and final body weight, respectively.

Superscript a indicates the pairs are significant at $\alpha = 0.05$.

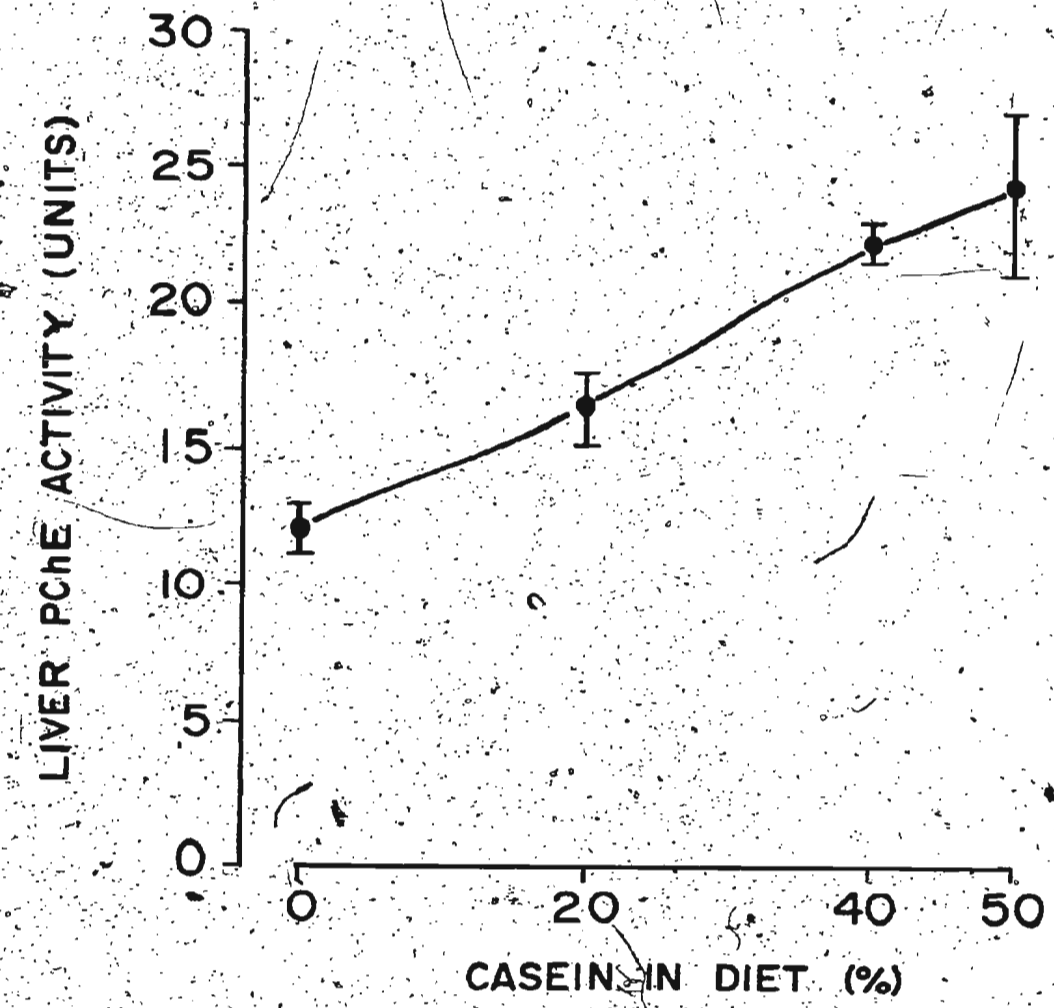
Superscript b indicates the pairs are significant at $\alpha = 0.20$.

Superscript c indicates the pairs are significant at $\alpha = 0.10$.

Superscript 0 indicates that for the variables daily food intake and energy intake, there is a significant difference between the restricted group and the other groups because the variance is zero for the restricted group.

Superscript x indicates there is no significant difference.

Figure 4: Effect of different levels of protein on the activity of liver pseudocholinesterase (PChE) in mice. A unit of enzyme activity is 1 micromole of thiocholine formed in 1 minute, in the standard assay (Dietz et al, 1973), per g wet weight of tissue. The results are the mean ± standard error of the mean. A minimum of six animals were used in each group. Each diet was fed for 5 days.


LIVER PChE ACTIVITY (UNITS)
30
25
20
15
10
5
0
0
20
40
50
CASEIN IN DIET (%)

Iso-OMPA Inhibition of Epinephrine-stimulated Lipolysis in Vitro

The results in Table 6 show the mean ± the standard deviation of the release of glycerol from isolated rat adipocytes under the various incubation conditions: The release of glycerol in the presence of 5×10^{-5}M epinephrine (1843 ± 1040 nmole/1 hr./ml packed cell volume) was significantly higher than in the basal condition (649 ± 294 nmole/1 hr./ml packed cell volume). When epinephrine was incubated with propranolol (10^{-5}M), a significant inhibition of the release of glycerol was observed. Similarly, when epinephrine was incubated with Iso-OMPA (10^{-5}M), a significant inhibition of the release of glycerol was also seen.

Table 6: Inhibition of epinephrine-stimulated lipolysis by Iso-OMPA in isolated rat adipocytes.

Incubation Condition	Release of Glycerol nmole/1 hr./ml packed cell volume
Basal	694 ± 294
Epinephrine, (5×10^{-5}M)	1843 ± 1040*
Epinephrine + Iso-OMPA (10^{-5}M)	408 ± 316
Epinephrine + Propranolol, (10^{-5}M)	377 ± 56*

Results are the mean ± the standard deviation of 4 observations. Statistical significance of difference between basal and experimental (Student's t test, based on difference between means) is indicated by *($P < 0.05$).

PChE Activity in Isolated Rat Adipocytes under Various Incubation Conditions

Adipocyte PChE activity under basal and in the presence of 5×10^{-5}M epinephrine were not significantly different (Table 7). However, when the cells were incubated in the presence of 10^{-5}M propranolol, a significant inhibition of PChE activity occurred as compared to the basal condition. PChE activity was, as expected, significantly inhibited when 10^{-5}M Iso-OMPA was present in the incubation mixture.

Table 7: PChE activity in isolated rat adipocytes under various incubation conditions.

Inculation Condition	PChE Activity nmole/hr./mg protein
Basal	311.8 ± 78.0
Epinephrine, (5×10^{-5}M)	296.8 ± 36.7
Epinephrine + Iso-OMPA, (10^{-5}M)	3.0 ± 4.2*
Epinephrine + Propranolol, (10^{-5}M)	38.1 ± 50.9*

Results are the mean ± the standard deviation of 4 observations. Statistical significance of difference between basal and experimental (Student's t test, based on difference between means) is indicated by *($P < 0.05$).

Purified Horse Serum PChE lacks Lipolytic Activity

The results in Table 8 show the lipolytic activity for the purified lipase preparation and for the purified horse serum PChE. The lipolytic activity of the purified lipase was 483 ± 109 μmole/min/L. In contrast, no lipolytic activity was observed for the purified horse serum PChE.

Table 8: The lipolytic activity of purified horse serum PChE *in vitro* as compared to that for purified lipase.

Sample	Lipolytic activity micromoles/min/L
Purified Lipase	483 ± 109
Purified horse serum PChE	0

Results are the mean ± the standard deviation of 4 observations.

The Lipolytic Activity of Purified Lipase is not affected by a Specific Inhibitor of PChE, Iso-OMPA

The effect of Iso-OMPA on the lipolytic activity of purified lipase, *in vitro*, is given in Table 9. No significant inhibition of lipolytic activity was observed when the purified lipase was incubated in the presence of the specific inhibitor of PChE, Iso-OMPA.

Table 9: Effect of Iso-OMPA on the lipolytic activity of purified lipase *in vitro*.

Sample	Lipolytic activity micromoles/min/L
Purified lipase only	483 ± 109
Purified lipase + Iso-OMPA, (10^{-5}M)	442 ± 70

Results are the mean ± the standard deviation of 4 observations. There is no significant difference between the two samples by Student's t test.

DISCUSSION

PChE in the sera is increased in over 70% of hyperlipoproteinemic and obese patients. In genetically obese (ob/ob and db/db) mice, increased serum and liver PChE activity is a consistent observation. Decreased adipose tissue PChE activity is also an established finding in these animal models. However, on a per cell basis, the activity would be higher. Moreover, mice given gold thioglucose to induce obesity showed the same kind of alterations in PChE activity as is observed in ob/ob and db/db mice. Therefore, abnormal levels of PChE are not restricted to genetic models of obesity and diabetes.

These obese animals could serve as a model to study PChE in relation to its involvement in lipid metabolism since they have well defined alterations in lipid synthesis and catabolism. These abnormalities include 1) increased lipogenesis, and 2) decreased oxidation of fatty acids. Moreover, genetically obese rodents are frequently used as models of obesity and human diabetes mellitus (Bray & York, 1979). In mice with genetic obesity and hyperglycemia (ob/ob mice), high blood glucose levels, high plasma insulin levels, and marked obesity are present at least during one period of the developing syndrome. The fa/fa rat becomes obese but does not develop hyperglycemia. This strain is of interest for diabetes research because of its marked peripheral insulin resistance (Herberg & Coleman, 1977). In this study, I used two widely accepted experimental

models of obesity and diabetes to study the interrelationships between obesity and diabetes, and PChE. The first was the ob/ob mouse and the second was the genetically obese Zucker fat (fa/fa) rat.

The altered levels of PChE activity in serum, liver and adipose tissue of obese individuals have been concluded to be due to high calorie intake. These conclusions were made on the basis of results in hyperphagic obese mice and lean mice fed a high calorie diet. In both studies, PChE activity was altered in response to overeating. One aspect that is fundamental to the discussion of obesity is energy balance. It is clear that obesity, the excessive accumulation of fat (Rabinowitz, 1970), results from either the ingestion or more food energy than the body needs or utilizes, or the expenditure of less energy than normal (Bray, 1979). Overeating is observed in both obese animals and man during some stage of developing obesity (Herberg & Coleman, 1977; Bray, 1980). Therefore, obese individuals ingest greater amount of macronutrients as well as calories when compared to non-obese individuals during some stage of the development of the obesity.

PChE Alterations in Zucker Fat Rats

Similar to the obese patients (Chu et al, 1978;

Cuciuanu et al, 1968) and mice (Kutty, Huang & Kean, 1981), this study showed that serum PChE activity is significantly higher in fa/fa rats when compared to the lean controls (Table 2). I have thus shown that PChE activity is altered in another experimental model of obesity and have further illustrated that altered PChE activity is a consistent observation in obesity. Hyperphagia is a common characteristic in both obese mice and fa/fa rats (Bray & York, 1979). Obese patients are also known to overeat (Bray, 1980). Previous studies by Kutty, Huang & Kean (1981) and Kutty, Kean, Jain & Huang (1983) indicated that a high calorie diet is responsible for the increase in serum and liver PChE activity in obese mice since these mice overeat all macronutrients including protein.

A relationship between PChE and nutrition has also been suggested earlier. For example, Gerebtzoff (1959) demonstrated that in mice, which had been starved for 24 hours, the liver PChE activity was increased after refeeding. The increase in activity peaked at 4 hours after refeeding and then the activity decreased. Waterlow (1950) observed a marked increase in serum PChE activity after malnourished children had been treated with a high milk diet to improve their nutrition. A good positive correlation between serum PChE and food intake in these fa/fa rats adds further proof

that there is a relationship between the enzyme and nutrition (Table 3). Serum PChE has been concluded to have its origin in the liver (Terzani, Natalizi & Macinucci, 1968; Sawyer & Everett, 1947) and this study also substantiates that conclusion.

Serum PChE has been reported to be increased in patients with hypertriglyceridemia (Cuciuanu et al, 1968; Jain et al, 1983). In this latter study, Jain et al, (1983) examined results from 304 hyperlipoproteinemic patients. They found that about 40% had type IV hyperlipoproteinemia and increased serum PChE activity. Similar results were observed in patients with type IIB hyperlipoproteinemia. Both type IV and IIb hyperlipoproteinemias are characterized by having increased serum very low-density lipoproteins (VLDL). Zucker fa/fa rats are not only a good model to study obesity but also hypertriglyceridemia. The increased triglycerides in Zucker fa/fa rats is apparently due to and manifested by enhanced production of VLDL by the liver (Schonfeld & Pfleger, 1971; Wang, Fukuda & Ontko, 1984). One of the major reasons for the overproduction of VLDL in the liver is due to excessive caloric intake including protein calories. PChE is also produced in excess under similar circumstances. However, a direct metabolic role for PChE in VLDL metabolism cannot be suggested at the present

time. On the other hand, there is evidence to show that PChE can stabilize low density lipoproteins (LDL) which are mainly derived from VLDL (Behr, Batsch, Forte & Bensadoun, 1981). Further studies are needed to establish a direct role for PChE in lipogenesis, VLDL synthesis and LDL turnover.

Serum PChE has a good positive correlation with food intake, body weight and serum triglycerides. Similarly, food intake has a good positive correlation with triglycerides and body weight (Table 3). For the reasons mentioned above, it appears that in fa/fa rats, hypercaloric nutrition is the common cause for increased PChE activity and also for hypertriglyceridemia. However, the exact relationship between these variables in relation to PChE at the fundamental biochemical level remains to be resolved.

In contrast to that observed in ob/ob mice, adipose tissue PChE activity was not reduced in fa/fa rats (Table 2). In both these animals, PChE activity was expressed in terms of wet weight. The mean cell size of epididymal adipocytes of Zucker fa/fa rats was found to be 3-4 times larger than in leans (Cleary, Brasel & Greenwood, 1979). The mean cell size of epididymal adipocytes in ob/ob mice have been reported (Johnson & Hirsch, 1972) to be approximately 6-8 times larger than in the lean mice. Therefore,

one would suggest that the absolute enzyme activity per cell should be higher in more enlarged adipocytes. This is because there are fewer number of adipocytes for the same mass of adipose tissue in the obese animals when compared to the lean animal.

Liver and Adipose Tissue PChE Activity in Young ob/ob Mice: Relationship to Characteristics of Postweanling ob/ob Mice

It is known that the hyperphagic phase associated with the development of obesity in such animal models as the ob/ob mouse is evident almost immediately after weaning, (Bray & York, 1979). Lin, Romsos and Leveille (1977) reported that the intake of a stock diet was depressed in ob/ob mice until day 35 after which hyperphagia was observed. Before weaning, milk intake is normal (Lin et al, 1977). Kutty, Kean, Jain & Huang (1983) have previously proposed that in obese mice, the increase in plasma PChE activity occurs in conjunction with hyperphagia. In this study, I found that liver PChE was significantly higher in ob/ob mice as early as 23 days when compared with lean controls. In contrast, adipose tissue PChE activity is significantly less in ob/ob mice and this is also evident at 23 days of age (Figure 1). However, adipose tissue PChE activity would

be higher if the results were expressed as per cell. Moreover, adipocytes are 6-8 times larger in obese mice whereas the decrease in PChE only 2 times lower in the obese mouse.

Overt obesity does not occur until about 25 to 28 days of age or even later in ob/ob mice (Bray & York, 1979) and hyperphagia is not likely to be present at 23 days of age (Lin, et al, 1977). Insulin first rises around 3-4 weeks (Dubuc, 1976) but insulin resistance is not evident until the sixth week of age in muscle of ob/ob mice (Grundleger, Godbole & Thenen, 1980). Moderate hyperglycemia is observed in the weaned ob/ob mouse although there is an initial hypoglycemia at days 14-17 in the face of rising insulin (Dubuc, 1977).

In this study, liver PChE activity was also found to show a parallel increase with body weight and food intake especially in the ob/ob mice (Figure 1). This increase at 23 days can be seen at an earlier age period than was observed before in plasma which started at 35 days of age (Kutty, Kean, Jain & Huang, 1983). The results for the fa/fa rats also provide evidence that the increase in serum and liver PChE activity is a function of hypercaloric nutrition. The fa/fa rat cannot be visually detected before 4 weeks of age which is similar to that for the ob/ob mouse

(Lin et al, 1977; Bray & York, 1979). Like the ob/ob mouse, the fa/fa rat is not hyperphagic before weaning (Stern & Johnson, 1977). Furthermore, the obesity of ob/ob mice is not dependent on hyperphagia as increased body fat is detectable by 17-21 days although the animals are not visually detectable as "obese" or hyperphagic (Dubuc, 1976; Bray & York, 1979). Chlouverakis (1970) also showed that ob/ob mice can become obese even in the absence of excessive food intake. Apart from the dietary influences, PChE may have a direct effect on lipogenesis as has been suggested previously by Ballantyne & Bunch (1967). They found that PChE is associated with tissues which are known to have a large turnover of lipids. Increased lipogenesis is present in preweanling ob/ob mice and this is before any visable signs of obesity such as increased body weight (Thurlby & Trayhurn, 1980). This also might explain the increase in PChE in the liver of the ob/ob mouse before hyperphagia is evident. The results here show that liver and adipose tissue PChE are also altered before overt obesity is evident in the ob/ob mouse.

Electron Microscopic Examination of Liver PChE in the ob/ob Mouse

The electron microscopic studies of liver (Figure 3) from the ob/ob mouse provided information on the subcellular site of the enzyme and also the apparent underlying cause for the elevation of liver PChE activity.

The results of the electron microscopic studies demonstrated that in the ob/ob mouse, the intense PChE activity is associated with the rough endoplasmic reticulum. Furthermore, the enzyme activity was much greater in the liver of the ob/ob mouse in comparison with that in the lean mouse. An increased rate of synthesis was therefore indicated in the obese animal.

Dietary Induction and Repression of PChE

As noted above, the observations of Gerebtzoff (1959) and Waterlow (1950) have suggested a relationship between PChE and nutrition. This is also substantiated by results in the rat. Harrison & Brown (1951) showed that liver PChE activity decreased in starved rats. Henderson et al (1971) also showed that both plasma and liver PChE activity decreased

within 1 day of starvation in young male rats.

Increased serum and liver PChE activity in obese mice and Zucker fa/fa rats is apparently due to hypercaloric nutrition. It was thus important to determine if a specific type of diet such as protein, carbohydrate, or fat alone, or in a combination may be the cause for inducing elevation of PChE.

Diet causes either induction or repression, depending on the type of diet fed. Changes in the proportion of protein, carbohydrate and fat in the food taken by animals can have marked effects on given liver enzyme levels (Olson, 1975).

In general, diets high in protein or carbohydrate increase liver enzymes associated with the degradation of the macronutrient and/or enzymes for conversion of the macronutrient to a storage fuel in the body (Aebi & Berger, 1980). This is chiefly fat (Krebs, 1972). Diets high in fat decrease liver enzymes associated with synthesis of fat (Romsos & Leveille, 1974).

In this study, a number of different diets were used, and when the number of possible comparisons is larger, the error rate will be greater. Thus, Scheffe's multiple comparison procedure was used to determine where significant differences existed for the variables between the pairs of

different groups used in this study. First, I have shown that when mice are fed a regular mouse chow, restricted to about 50% of ad libitum comsumption, there is a significant reduction in liver PChE activity (Table 5). Further studies indicate that a maximum induction of the enzyme in the liver occurs with high levels of protein intake. On the other hand, with the high carbohydrate diet, in spite of the higher energy intake, the induction was significantly lower than with the high protein regimen (Table 5) and almost equaled results for animals fed a regular mouse chow (Table 4). The regular mouse chow and the high carbohydrate diet were not significantly different in protein concentration. It therefore appears that the effects of the liver PChE activity are due to the protein content. Leto et al (1976) showed that liver PChE activity in rats, that had been fed a 26% casein diet, was significantly higher than in rats fed a 4% casein diet. When protein was absent from the diet of rats, a significant decrease in the liver enzyme was observed (Barrows & Roeder, 1961). Similarly, when patients with kwashiorkor were given milk protein supplements, serum PChE increased two-fold, on the average, as compared to pretreatment levels (Burch, et al, 1957).

In contrast to the induction effects of the high protein diet, the high fat diet appeared to cause a repression of liver PChE (Table 4). Alternatively, the low protein content of the high fat diet may be the cause of the low PChE activity. Moreover, other essential nutrients in the diet are also decreased and this may have had an effect. In this study, I showed that liver PChE activity rises in parallel with the increase in casein from 0% to 50% casein (Figure 4). Sucrose may not produce either repression or induction of PChE because levels were the same in the livers of animals fed chow and high carbohydrate ad libitum (Table 4). It is clear that the changes do not appear to be due to calorie, that is energy, intake per se. This is demonstrated by the fact that no significant differences exist between the diets except for the results in the high carbohydrate-fed mice.

Physiological Role of PChE

To date, there is no clearly established physiological role for PChE. My present observations are consistent with the suggestion that this enzyme may be involved in lipid metabolism (Clitherow et al, 1963). These authors suggested that enhanced PChE induction would take place during accel-

erated fatty acid metabolism to remove intermediate choline esters that would be formed (Clitherow et al, 1963). High protein diets are known to cause increased lipogenesis (Aebi & Berger, 1980). Obese animals have increased lipogenesis (Bray & York, 1979). Higher than normal levels of dietary protein may also increase the tendency to obesity. Donald, Pitts & Pohl (1981) demonstrated this when they showed that adult rats fed high levels of protein in their diet had twice the absolute amount of fat as those fed a low protein diet. The diets were isocaloric and the food intake between the two groups was not different (Donald et al, 1981). Moreover, obese mice consume more protein than lean mice (Chee, Romsos, Bergen & Leveille, 1981) and regulation of protein intake was shown to be affected by the nonprotein macronutrient source (Chee, Romsos & Bergen, 1981). Protein intake was lower if fat was the source and higher when it was carbohydrate. However, it is not clear at what molecular level the exact induction processes take place or what specific intermediates of protein metabolism cause the induction of PChE. It is also interesting to note that high fat diets repress lipogenesis (Romsos & Leveille, 1974). In addition, Cawthorne & Cornish (1979) showed that both lean and obese mice are affected in the same way by a high fat diet. The rate of lipogenesis was

decreased in the liver in both the lean and the ob/ob mice. Moreover, in patients with hypertriglyceridemia of endogenous origin, as found in types IIb and IV hyperlipoproteinemias, serum PChE activity was found to be increased (Jain, et al, 1983).

PChE and Epinephrine-Stimulated Lipolysis

There are three components to the activation of lipolysis in adipocytes by adrenergic agonists such as epinephrine (Faih & Garcia-Scisz, 1983). First, epinephrine binds to the beta-adrenoreceptor at the cell membrane to activate adenylate cyclase for the production of cyclic adenosine monophosphate(cAMP). The cAMP binds to the regulatory subunit of a protein kinase. This protein kinase phosphorylates triacylglycerol lipase. Phosphorylation activates tryacylglycerol lipase and this results in an increase in lipolysis.

An association between PChE and lipolysis has been made. The results of this study will be discussed in relation to two of the key components involved in activation of lipolysis in adipose tissue by adrenergic agonists: the beta-adrenoreceptor and the triacylglycerol lipase.

The results showed that the purified PChE exhibited

no lipase activity towards a substrate readily hydrolyzed by the purified lipase (Table 8). PChE is known to hydrolyze tributyrin (Mendel & Rudney, 1943) but as shown in this study, PChE has no activity for the triolelin used in this study. Dixon (1948) did show that diisopropyl fluorophosphonate (DFP) does inhibit lipases. DFP is an inhibitor of PChE (Silver, 1974). Thus, the inhibition of lipolysis by such organophosphorus, PChE inhibitors, such as DFP, would appear to act by inhibitng lipases. However, the concentration of DFP required to inhibit lipase is 1,000 times greater than that to obtain differential inhibition of PChE (Silver, 1974). Furthermore, DFP inhibits other enzymes such as trypsin and chymotrypsin (Dixon & Webb, 1964). These results would suggest a nonspecific affect of DFP on lipases. The results in this study also showed that iso-OMPA, a selective inhibitor of PChE, has no effect on the lipolytic activity of the purified lipase (Table 9). This would reaffirm that specific inhibitors of PChE, at concentrations selective for inhibition of PChE, have no effect on the lipolytic activity of lipases.

How then does selective inhibition of PChE relate to inhibition lipolysis as suggested earlier by Szendzikowki et al (1961/62) and Colville et al (1964)? A relationship between the adipocyte beta-adrenoreceptor and PChE can be

inferred by the inhibitory effect of propranolol on PChE and lipolysis. Propranolol is widely known as a beta-adrenoreceptor blocking agent which inhibits the lipolytic action of catecholinamines such as epinephrine (Gilman, Goodman & Gilman, 1980). Marmo (1971) showed that propranolol inhibits serum PChE activity in rats. Whittaker, Britten & Wicks (1981) demonstrated that propranolol could be used as a differential inhibitor of plasma PChE in man. In this study, I have shown that propranolol inhibited PChE activity, like Iso-OMPA, in rat adipocytes as well as blocking epinephrine-stimulated lipolysis (Table 7). In addition, the specific inhibitor of PChE, Iso-OMPA, significantly inhibited epinephrine-stimulated lipolysis like the beta-adrenoreceptor blocking agent propranolol (Table 6). Propranolol is not a quaternary ammonium compound like substrates for PChE, and it does not structurally resemble Iso-OMPA (Gilman et al, 1980; Silver, 1974). Propranolol may then act at another site such as an allosteric site. The presence of an allosteric site on PChE has been suggested for PChE from rabbit liver (Rush, Main, Kilpatrick & Faulkner, 1981). By whatever mechanism, propranolol inhibits PChE, its effects suggest that there is a complementarity of the structure of PChE and of the beta-adrenoreceptor. However, the exact relationship of PChE and

epinephrine-stimulated lipolysis at the molecular level has to be resolved. For example, does specific inhibition of PChE in adipose tissue have any effect on the production of cAMP?

Conclusions

In conclusion, I have demonstrated an increase in serum PChE activity in Zucker fa/fa rats similar to that observed in ob/ob mice and obese humans. This reaffirms that PChE is altered in obesity. Liver PChE activity is also increased and this is similar to that observed in obese mice. However, adipose tissue PChE activity is not decreased in the Zucker fa/fa rats and this can be explained by the relative differences in fat cell size in these two different animal models of obesity and diabetes. The increase in serum enzyme activity in these fa/fa rats appears to be related to hypercaloric nutrition. Thus, I propose that serum PChE might serve as a potential marker to assess the nutritional state of an individual. More specifically, evidence has been put forth here that liver PChE activity is a function of the level of protein in the diet. Liver PChE activity is elevated as early as 23 days of age in the ob/ob mouse whereas adipose tissue PChE activity is decreased.

Electron microscopic studies established that liver PChE is associated with the rough endoplasmic reticulum of obese mice and that increased synthesis of the enzyme is the apparent cause of the increased activity of liver PChE in obese mice. Further confirmation of increased synthesis as the cause of enhanced enzyme activity in obesity or due to increased protein intake requires studies using immuno-precipitation techniques which were not possible at the time of this study. Specific inhibition of PChE causes a decrease in epinephrine-stimulated lipolysis like that observed by beta-adrenoreceptor for blocking agents as propranolol. However, this effect is apparently not due to inhibition of lipase. The inhibiting effects of the beta-adrenoreceptor blocking agent, propranolol, on PChE activity and epinephrine-stimulated lipolysis in isolated rat adipocytes suggest that there are complementary structural features between PChE and the beta-adrenoreceptor of rat adipocytes.

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