EFFECT OF MEAL-FEEDING ON THE RATES OF SYNTHESIS AND DEGRADATION OF RAT ADIPOSE TISSUE MALIC ENZYME

Dissertation for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
JAMES PAUL OLSON
1975

This is to certify that the

thesis entitled

EFFECT OF MEAL-FEEDING ON THE
RATES OF SYNTHESIS AND DEGRADATION
OF RAT ADIPOSE TISSUE MALIC ENZYME

presented by

James Paul Olson

has been accepted towards fulfillment
of the requirements for

Ph.D degree in Human Nutrition

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Date 10-9-75

O-7639

ABSTRACT

EFFECT OF MEAL-FEEDING ON THE RATES OF SYNTHESIS AND DEGRADATION OF RAT ADIPOSE TISSUE MALIC ENZYME

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Malic enzyme (L-Malate: NADP oxidoreductase (decarboxylating), EC 1.1.1. 40) was purified from rat liver. The enzyme was found to be homogeneous according to the following criteria of purity: purification to a constant specific activity, absence of other enzyme activities, electrophoresis on polyacrylamide gel, and immunodiffusion on agar.

Immunological analysis by double diffusion on agar and quantitative precipitin tests with anti-serum to rat liver malic enzyme indicate that the liver and epididymal fat pad malic enzymes are immunologically similar. The liver and adipose tissue malic enzymes of genetically lean and obese mice was shown to be immunologically similar to each other and to rat liver malic enzyme. Pig and chicken liver and adipose tissue malic enzyme showed a decreased cross-reactivity with anti-rat liver malic enzyme.

L-leucine-U-^{14}C was administered IP to label the epididymal fat pad malic enzyme of meal-fed (one 2 hour meal per day) and nibbling (pair-fed, control) rats. The half-lives, determined from the loss of radioactivity from malic enzyme, were 60- and 43 hours respectively in one experiment. The rate of synthesis of the enzyme appeared to be more important in controlling the tissue level of the enzyme than the rate of degradation. A second experiment indicated

half-lives of 198- and 75 hours for the adipose tissue malic enzymes of meal-fed and nibbling rats respectively. In this experiment, the rate constant of degradation of epididymal fat pad malic enzyme was found to be less in the meal-fed rat than in the nibbling rat.

Turnover analysis of protein fractions of the liver and adipose tissue of the meal-fed and nibbling rats was conducted. The half-lives of the protein fractions of liver and epididymal fat pad were the same in both meal-fed and nibbling rats. The values for the total protein fraction were found to be 96- and 113 hours in the first and second meal-feeding experiments, respectively. The corresponding half-life values of the soluble protein fraction of liver were 177- and 197 hours. The half-life values of both total and soluble protein fractions of the epididymal fat pad were 38 hours in the first experiment while that observed in the second experiment was 211 hours.

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By

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A DISSERTATION

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science and Human Nutrition

1975

To my wife, Patricia

ACKNOWLEDGEMENTS

The author wishes to thank Dr. G.A. Leveille, Chairman of the Department of Food Science and Human Nutrition, for placing the facilities of the department at his disposal.

Sincere thanks are extended to Dr. G.A. Leveille, Chairman and Professor, and Dr. D.R. Romsos, Associate Professor, for their guidance and advice during the course of this study and in preparation of this manuscript.

Acknowledgement and thanks are given to Ms. Kathy Muiruri for her excellent technical assistance which she readily extended to the author.

Special thanks are gratefully given to my wife, Pat, family, friends and colleagues for their unending support and encouragement throughout this project.

Acknowledgement is given to Mead-Johnson Company and the Michigan State University Research Station for providing financial support for this work.

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CoA	coenzyme A
EDTA	(ethylene dinitrilo) tetraacetic acid
EtOH	ethanol
K_d	rate constant of degradation
K_s	rate constant of synthesis
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced form
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
SDS	sodium dodecyl sulfate
-SH	sulfhydryl group
Tris	tris (hydroxymethyl) aminomethane
$T_{\frac{1}{2}}$	half-life

Introduction

Malic enzyme (L-Malate: NADP oxidoreductase (decarboxylating), EC 1.1.1.40) catalyzes the reaction: L-Malate + NADP $\overset{Mg^{++}}{\rightleftharpoons}$ pyruvate + CO_2 + NADPH. This has been postulated as being one of the two major sources of NADPH needed for lipogenesis (1). In a recent review, Romsos and Leveille have summarized evidence demonstrating that, under a variety of physiological and dietary states, malic enzyme activity usually parallels the rate of fatty acid synthesis (2). In chick and rat liver, changes in malic enzyme activity have been demonstrated, by immunological titration, to be due to changes in enzyme content rather than activation or inhibition of preformed enzyme (3-5). Differences in the rate of synthesis, rather than degradation, of malic enzyme have been implicated as being the primary cause of different malic enzyme contents in the livers of chicks and rats in various nutritional and physiological states (3,4,6).

The effect of periodicity of eating on the rates of synthesis and degradation of rat malic enzyme has never been studied. Adaptation to a single daily meal (meal-eating), as compared to ad libitum consumption (nibbling), caused a rise in the malic enzyme activity of rat epididymal fat pads (7). The rise in activity was positively correlated with, but occurred subsequent to, an increase in adipose tissue lipogenic capacity (7). In the meal-fed rat, the vast majority (95%) of the fatty acid synthesis occurred in the adipose tissue whereas the liver accounts for about one-third of fatty acid synthesis

in the nibbling rat (8). The increase in malic enzyme activity could be considered a reflection of a need for an increased rate of NADPH generation to sustain the elevated rate of lipogenesis in the meal-fed rat. The purpose of the present study has been to elucidate the underlying mechanism(s) controlling the level of malic enzyme in the epididymal fat pads of meal-fed and nibbling rats. Malic enzyme was purified from the livers of thyroxine-treated rats and specific antibodies to it were generated. The extent of cross-reaction between the anti-serum and rat epididymal fat pad malic enzyme was determined. Using immunological precipitation of labelled malic enzyme, the rates of synthesis and degradation of the enzyme in the epididymal fat pads of meal-fed and nibbling rats were estimated.

Review of the Literature

Malic enzyme (L-Malate: NADP oxidoreductase (decarboxylating), EC 1.1.1.40) was recognized independently by Moulder et al. (9), Lwoff et al. (10), Lwoff and Cailleau (11) and Ochoa et al. (12). The enzyme was initially identified and purified from avian liver by Ochoa et al. (12, 13). Since then, malic enzyme has been found in a wide variety of biological sources and has been isolated from unicellular (14), plant (15), avian liver (3, 13, 16), mammalian liver (4-6, 17, 18), mammalian heart (19), and mammalian brain (20) sources. Malic enzyme has also been the subject of numerous investigations into its intracellular distribution, isozymic forms, its role in metabolism and the effects of developmental, physiological and nutritional status on the tissue levels of the enzyme.

A. Intracellular Distribution of Isozymes of Malic Enzyme

The intracellular distribution of malic enzyme appears to be tissue and species specific. The enzyme is almost exclusively localized in the cytosol fraction of lipogenic tissues such as the liver of mouse, rat, guinea pig, rabbit, pig, sea gull and pigeon and in rat epididymal fat pad (21). Malic enzyme is also predominantly present in the cytosol fraction of the mouse and rat kidney and the bovine adrenal medulla (21). However, considerable activity has been detected in the mitochondrial fraction of hearts of mouse, rat, guinea pig, rabbit, pig, beef and sea gull; of the pig and beef adrenal cortex; of guinea pig, rabbit, pig and beef kidney; and of rat and beef brain (21, 22).

Isohashi et al. (5) demonstrated that the cytosol malic enzymes of various rat tissues were immunologically similar to rat liver malic enzyme. However, mitochondrial malic enzymes were immunologically distinct from that of rat liver cytoplasm. Mitochondrial and cytosol enzymes were also shown to be electrophoretically different for all species studied (19, 21, 23). Chromatographic behaviour on DEAE-cellulose and Sephadex G-200 was also different for the extra- and intra- mitochondrial malic enzymes of beef and rat heart (19, 24).

Differences have also been noted in the kinetic properties of the isozymes. Saito and Tomita (24) found that the apparent Km values for malate differed between the extra- and intra- mitochondrial enzymes of rat and beef heart. The Km values for the malic enzymes of beef heart cytosol fractions I and II and the mitochondrial fraction were 0.3, 0.8 and 1.1 mM, respectively. The corresponding values of cytosol- and mitochondrial malic enzymes of rat heart were 0.5 and 1.5 mM, respectively. Different Ki for dicoumarol inhibition of the rat heart isozymes were observed. Fifty per cent inhibition of the cytosol- and mitochondrial enzymes required 200 μM and 40 μM dicoumarol, respectively. Frenkel (25) found that the activity of the bovine heart mitochondrial malic enzyme did not follow Michaelis-Menton kinetics at low concentrations of malate but showed a distinct sigmoidicity at these concentrations. Succinate decreased the sigmoidicity but did not affect the maximum velocity of the reaction. The cytoplasmic isozyme of beef heart did not show any allosteric properties. The isozymes from bovine brain also demonstrated kinetic

differences (22). The mitochondrial malic enzyme was activated, at low malate concentrations, by either succinate, 2-mercaptosuccinate or L-aspartate. The cytosol enzyme was not affected by the dicarboxylic acids. Also, the concentrations of 5,5-dithiobis (2-nitrobenzoic acid) and Hg^{2+} required to inhibit the mitochondrial enzyme were much lower than those needed to inhibit the cytosol enzyme.

Saito and Tomita (26) demonstrated the presence of two immunologically distinct isozymes in rat liver cytosol. Immunodiffusion analysis showed the occurrence of a major and a minor precipitin band. The major band corresponded to the cytosol malic enzyme of rat adipose while the minor band corresponded to the cytosol enzyme of rat heart. The isozymes were designated as A (adipose tissue) and H (heart) types, respectively. The amount of A- type protein was apparently affected by hormonal and nutritional stimuli. The precipitin band of hepatic extracts of fasted rats was less sharp than those lines of hepatic extracts of rats fed glucose- or thyroid- containing diets; indicating lower malic enzyme concentration in livers of fasted rats. The precipitin band for the H-type protein was unaffected by the dietary and hormonal treatments. Three isozymes of the cytoplasmic chick liver malic enzyme have also been reported (3).

B. Metabolic Role of Malic Enzyme

In spite of numerous studies, the exact role of malic enzyme in metabolism has not been completely delineated. Depending on tissue and compartmental localization, several different roles have been suggested for malic enzyme.

Shortly after malic enzyme was identified in the liver cytosol fraction, investigators found that the enzyme, in the presence

of NADPH, could catalyze the reductive carboxylation of pyruvate to malate (27). This led to the assumption that malic enzyme might play a role in gluconeogenesis by converting pyruvate to malate. Malate could then be oxidized to oxaloacetate, the precursor of phosphoenol-pyruvate. However, the level of malic enzyme in rat liver under gluconeogenic conditions was insufficient to account for the rate of pyruvate conversion to glucose. Also, under conditions favoring gluconeogenesis, malic enzyme activity decreased (28).

After finding that the enzyme increased under conditions favoring lipogenesis (28), the above hypothesis was replaced with the suggestion that malic enzyme may play a role in lipogenesis by supplying NADPH for reductive synthesis. Flatt and Ball (29) demonstrated that, under conditions of enhanced lipogenesis, the pentose- pathway can provide only about 60% of the reducing equivalents required for fatty acid synthesis in rat adipose tissue. As an alternate source of reducing equivalents, the "transhydrogenation cycle" was postulated, in which hydrogen was transferred from NADH to NADPH. Young et al. (30) and Wise and Ball (31) suggested that citrate serves to transport acetyl CoA from the mitochondria to the cytoplasm where the ATP- dependent cleavage of citric acid by citrate lyase yields oxaloacetate and acetyl CoA. Acetyl CoA then is available in the cytoplasm for carboxylation to malonyl CoA, which is obligatory for fatty acid synthesis. The oxaloacetate can be converted to malate by malate dehydrogenase, oxidizing NADH. Malic enzyme catalyzes the conversion of malate to pyruvate and carbon dioxide with the release of NADPH. The cycle was completed by a suggestion from Ballard and Hanson (1) that adipose tissue pyruvate carboxylase catalyzes the

ATP- mediated carboxylation of pyruvate to oxaloacetate and ADP. By means of this cycle, NADPH can be generated from NADH with ATP supplying the necessary energy. Although the presence of cytosol pyruvate carboxylase in rat adipose tissue has been disputed (32), the pyruvate formed from malate could still enter the mitochondria to be metabolized.

Although the role of malic enzyme in lipogenesis has been generally accepted (2), recent studies (33, 34, 35) have led to the suggestion that liver malic enzyme may have alternate regulatory functions. Feeding a low-protein diet to rats induced liver malic enzyme but not lipogenesis or glucose 6-phosphate dehydrogenase activity. The rise in malic enzyme activity was positively correlated with glutathione reductase activity (35). The investigators suggested that malic enzyme may provide reducing equivalents to maintain glutathione in a reduced state. Reduced glutathione is needed to facillitate amino acid transport. Under conditions of restricted dietary protein intake, this could be important to the animal in view of the requirement for maximum nitrogen conservation.

The role of mitochondrial malic enzyme is obscure and, at present, largely unknown. Simpson and Estabrook (36) demonstrated the presence, after sonication, of a mitochondrial malic enzyme in bovine adrenal cortex. They found that the activity of the mitochondrial enzyme was sufficient to support, via malate reduction, 11 β-hydroxylation during steroid biosynthesis. The evidence supporting malic enzyme involvement in steroid biosynthesis is largely based on the stoichiometric appearance of pyruvate during 11 β-hydroxylation. However, nucleotide pyridine transhydrogenases (NADH$\rightarrow$ NADPH) may

also be supplying reducing equivalents for the 11 β-hydroxylation reaction (37). Pfeiffer and Tchen (38) reported that, with "intact" mitochondria of bovine adrenal cortex, low physiological concentrations of Ca^{2+} (1 mM) changed the mitochondrial ultrastructure, unveiled mitochondrial enzyme activity and stimulated extramitochondrial NADPH formation. The NADPH reduction was found to be dependent on malic enzyme, not transhydrogenase activity. The NADPH generated was suggested to provide part of that required for extramitochondrial steroid 21- and 17- hydroxylation reactions.

C. Developmental Patterns of Liver Malic Enzyme

The stage of an animal's development can be reflected by the level of malic enzyme in its liver (39, 40, 41). In the prenatal and unweaned rat, liver malic enzyme activity is low (39, 40). At twenty days of age, liver malic enzyme increases and rapidly attains adult levels (40). This increase coincides with the initiation of thyroid function (42) and with weaning, which shifts the animal from a high-fat milk diet to a high-carbohydrate diet. The rate of synthesis of rat liver malic enzyme increases 4-fold upon weaning (6). In the chick embryo, liver malic enzyme activity is absent (3) but rises to adult levels shortly after hatching (41). This increase coincides with a shift from a high-fat, yolk-lipid diet to a high carbohydrate diet upon hatching. The half-life of unfed, neonatal chick liver malic enzyme is long (350 hours) but decreases to 55 hours in the fed 8- and 11- day old chicks (3). In all these cases, hepatic malic enzyme activity parallels the lipogenic capacity of the animal's liver. Both the unweaned neonate and the unhatched embryo have very low hepatic lipogenic rates (3, 39, 40, 41). Upon weaning or hatching, hepatic lipogenic rates

are markedly increased. This serves as a means of storing excess ingested carbohydrate, which has now become the animal's main dietary source of energy.

D. Effect of Physiological State on Malic Enzyme

The absence of circulating insulin causes a decrease in adipose tissue malic enzyme levels, as observed in alloxan-diabetic rats and pigs (31, 43). The rate of synthesis of liver malic enzyme decreases in alloxan-diabetic rats (6). The lipogenic action of insulin is absent in diabetes (44), and, as a consequence, malic enzyme activity of adipose tissue decreases (31).

Fasting and then refeeding a high-carbohydrate diet induces an increase in malic enzyme activity to levels greater than those observed in ad libitum fed animals (45, 46). When compared to lean animals, this seems to be impaired in obese animals. Liver and adipose tissue malic enzyme of obese mice and adipose tissue malic enzyme of obese pigs decrease upon fasting but the increase upon re-feeding is slower than in lean animals (47, 48). It also appears that lipogenesis does not decrease as much during fasting of obese mice as during fasting of lean animals (49).

E. Effect of Diet on Malic Enzyme Activity

Dietary manipulation has been shown to alter the level of malic enzyme in the liver and adipose tissue of several species (2). Depending on the dietary regime imposed on the animal, fluctuations in malic enzyme activity usually correspond to changes in lipogenic activity of the tissue and species studied. There is, however, an exception to this generality that will be discussed later.

Fasting has been shown in numerous investigations (3, 4, 6,

18, 26, 50-55) to decrease both tissue malic enzyme activities and lipogenesis in rats, mice, pigs and chicks. In the rat, fasting decreases liver and adipose tissue malic enzyme by 30 and 50% respectively of that of high-carbohydrate diet fed controls (6,50). Lipogenesis in these tissues is decreased by 99 and 50% (7). The rate of synthesis of malic enzyme is decreased in the liver of fasted rats (4, 6). Although a 24-hour fast markedly decreased lipogenesis in mouse liver and adipose tissue, malic enzyme activity of these tissues was unaffected (51). Baker and Huebotter (52) and Jansen et al. (53) also reported decreased lipogenesis in hepatic and adipose tissues of fasting mice. In the pig, fasting reduced adipose tissue malic enzyme activity and lipogenesis by 60 and 99% respectively (54). Goodridge showed that a 24-hour fast decreased lipogenesis by 94% while a 72-hour fast decreased malic enzyme activity by 70% in the livers of 20 day old chicks (55). Silpananta and Goodridge (3) reported that the half-life of liver malic enzyme was 28 hours for fasted chicks and 55 hours for fed chicks.

Feeding a high-fat diet to rats, pigs and chicks decreases tissue lipogenesis and malic enzyme activity. Increasing dietary fat content from 10 to 30% causes a 80 and 90% decrease in rat liver and adipose tissue malic enzyme activity respectively (7,56). Lipogenesis decreased by 80 and 67% respectively. Pig adipose tissue malic enzyme activity and lipogenesis are decreased by 40 and 67%, respectively, by increasing dietary lipid content from 2.4 to 13.4% (57). High fat ingestion (20% dietary lipid) in the chick reduced liver malic enzyme activity to 14% of the level observed in chicks fed 2% dietary fat (58). Lipogenesis was virtually completely inhibited by a 20% fat diet.

In the rat, feeding a high-protein diet decreases malic enzyme

40% in the adipose tissue (7, 58, 59) while lipogenesis was decreased by 60%. Increasing the dietary protein from 19 to 34% decreased pig adipose tissue malic enzyme activity and fatty acid synthesis by 50 and 25%, respectively (57).

Malic enzyme is induced under dietary conditions that increase lipogenic rates. In the rat, fasting and refeeding a high carbohydrate diet causes liver malic enzyme to rise to levels greater than those in livers of ad libitum-fed rats (18, 45, 46). Repeated fasting- refeeding gives an even higher malic enzyme activity (60). Fat-feeding during refeeding abolishes the observed malic enzyme increase (7). Hepatic lipogenesis is also increased by fasting and refeeding (7) as are malic enzyme activity and lipogenesis in rat adipose tissue (61). Synthesis of rat liver malic enzyme was increased 9-fold in fasted-refed animals (51,53). Fatty acid synthesis in rat epididymal fat pad is not decreased by fasting but markedly increased upon feeding. Malic enzyme activity was raised in the liver and epididymal fat pads of intermittently-fed mice (51). Gorging also induces liver and adipose tissue malic enzyme activity (52). Chick liver malic enzyme activity and lipogenesis are enhanced by fasting and refeeding (55, 62). Fasted and refed pigs showed a 50 and 10% increase in adipose tissue malic enzyme activity and lipogenesis, respectively, over those of ad libitum-fed animals (54).

High-carbohydrate, low-fat or fat-free diets increase malic enzyme activity in liver and adipose tissue of rats and mice and in chick liver (3,4,63,64). However, Derr and Zeive (65) have shown that a large dose of carbohydrate gives the same proportionate increase in liver malic enzyme of both fed and fasted rats. They claim that the

observed rise, suggested to be an adaptation to the high-carbohydrate diet by other investigators, is simply a short term response to an overload of carbohydrate being presented to the liver at one time.

The feeding of thyroid hormones induces rat liver malic enzyme to levels several fold greater than those of untreated animals (6, 66). In separate investigations, Murphy and Walker (6) and Li et al. (18) reported a two-to four fold increase in the rate of synthesis of liver malic enzyme of thyroxine-fed rats. Li et al. (18) found no difference in the rate of synthesis of liver proteins. Although liver malic enzyme is readily induced by thyroid hormone feeding, the adipose tissue enzyme is increased only by long-term feeding of the hormone (66). Flatt (17) found that hyperthyroidism in the rat increased the turnover of the bulk of soluble adipose tissue proteins while the rate of turnover of malic enzyme was unchanged. Young (66) suggested that increased activities of lipogenic enzymes, due to thyroid hormone feeding, reflected enhanced lipogenesis of rat liver and epididymal fat pads.

Hepatic malic enzyme activity was enhanced when fasted rats were fed a protein-free diet (67). Frenkel et al. (33) and Stark and Frenkel (34) found that liver malic enzyme activity increased when rats were switched from a diet containing 18% lactoalbumin to one containing 0.5% lactoalbumin. However, the activities of liver glucose 6-phosphate dehydrogenase and NADP- isocitrate dehydrogenase did not rise in rats fed the low-protein diet (33, 34, 67). Stark et al. (35) also found that feeding a low-protein diet to rats did not increase the rate of hepatic lipogenesis even though malic enzyme activity was markedly increased. They suggested a possible alternate metabolic role in

glutathione reduction, as described above, for rat liver malic enzyme. In contrast, Leveille and Yeh (62) found that both malic enzyme and lipogenesis were enhanced in the livers of chicks adapted to intermittent protein-free diets.

F. Effect of Periodicity of Eating on Malic Enzyme

As well as being affected by dietary composition, malic enzyme activity and lipogenesis respond to patterns of food ingestion. Adaptation to meal-feeding (ingestion of a single 2 hour meal per day) causes marked physiological and biochemical alterations in the liver and adipose tissues of the rat. The metabolic effects of meal-feeding, as compared to nibbling (ad libitum ingestion), have been extensively studied and reviewed by Leveille in 1970 (7).

The majority of metabolic and biological changes observed in the meal-fed rat represent adaptations to the ingestion of its entire daily intake in a single meal. The digestive tract increases in size and hence increases its physical capacity and absorptive area (7). This indicates a development, in the meal-fed rat, of the capacity to ingest larger quantities of food, to absorb nutrients more rapidly and hence to present greater amounts of glucose and other nutrients to the tissues for disposal than the nibbling rats. Romsos and Leveille (68) recently reported that hexokinase activity in the intestinal mucosa from meal-fed rats was significantly higher 13 hours after the meal than that observed in nibblers. Intestinal pyruvate kinase activity was similar in the two groups. They suggest the intestine mucosal hexokinase increase is a response to meal ingestion and that the increase is gradual. Adaptations enabling tissues to accomodate the influx of glucose include a more rapid accumulation of glycogen in tissues of the meal-fed rat than in those

of nibbling rats (7), a greater capacity to release insulin (7), and a better glucose tolerance than in nibbling rats (69). Wiley and Leveille (70) found a greater insulin sensitivity in the meal-fed rat and suggested that insulin plays an important role in the improved glucose tolerance of the meal-fed rat. The meal-fed rat must store energy during the meal period to be used during the ensuing period of fast (71). Seventy per cent of the ingested energy is stored as glycogen and lipid; the majority being lipid. The stored glycogen appears to be rapidly utilized when the animal reaches the post-absorptive state, and, about fourteen hours after the start of the meal, plasma free fatty acids subsequently rise to a fasting level and an RQ indicative of lipid oxidation is observed (72). The increase in adipose tissue glycogen synthetase activity is greater, upon feeding, in fasted meal-fed than in fasted nibbling rats(73). The increase is suggested to be due to activation of an inactive form of glycogen synthetase. The meal-fed rat also exhibits a greater apparent efficiency in utilizing ingested energy (74). Following an initial adjustment period rats fed a single, daily two-hour meal ingested only about seventy-five per cent of the amount of food consumed by ad libitum-fed control animals but gained weight at the same rate. In a long-term experiment, Leveille (75) showed a greater longevity in meal-fed rats than in nibbling rats. He attributed the increase in life span to the body weight, and presumably lower body fat, of the meal-fed rat.

In conjunction with the increased glucose load presented to the tissues of the meal-fed rat, a major metabolic alteration observed is an increased rate of lipogenesis in both liver and adipocytes. Tepperman and Tepperman (76) have shown that meal-feeding increases

the hepatic capacity for fatty acid biosynthesis. Leveille (77) has demonstrated that the lipogenic capacity of adipose tissue from meal-fed rats far exceeds that of liver. In conjunction with this, in vivo experiments have shown that adipose tissue of the meal-fed rat is responsible for over 95% of the fatty acid formed subsequent to meal ingestion (8). Recently this increased lipogenic capacity of adipose tissue of meal-fed rats over that of nibbling rats has been shown to be due to a true metabolic adaptation rather than a change in the adipocyte cell size or number (51, 78).

The increased rate of lipogenesis in liver and adipocytes of meal-fed rats, as compared to nibbling rats, is also well correlated with an enhancement of the activities of enzymes directly or indirectly involved in fatty acid synthesis and carbohydrate metabolism (79). However, the increase in lipogenesis occurs prior to the increase in activity of malic enzyme and the pentose pathway dehydrogenases of the adipocytes (7). Of the enzymes studied, the number with enhanced activities was much greater in the adipose tissue than in the liver (65, 79, 80). A comparison of the activities of adipocyte enzymes of meal-fed and nibbling rats (7) showed a significant increase in the activity of many adipocyte enzymes in meal-fed animals. The adipocyte enzymes which exhibited a 100% or greater increase in activity were: hexokinase, citrate cleavage enzyme, acetyl CoA carboxylase, fatty acid synthetase, glucose 6-phosphate dehydrogenase, pyruvate carboxylase, and malic enzyme. These enzymes could be considered as being rate-limiting in the adipocyte of nibbling rats. A substantial increase in the activity of several rate-limiting enzymes of a metabolic pathway would reflect an increase in the overall activity of the pathway and,

consequently, would allow an increased amount of substrate to flow through the pathway.

Of the adipocyte enzymes exhibiting enhanced activities in meal-fed rats, malic enzyme demonstrated a five-fold increase in activity (63). The enzyme's participation in the postulated "transhydrogenation cycle" to generate the additional NADPH required under conditions of heightened lipogenic rates has been discussed above. It is conceivable that, under the increased lipogenic rates in the adipocyte of meal-fed rats, an increase in the transhydrogenation cycle would be induced in order to generate the necessary additional reducing equivalents required to sustain the elevated rates of fatty acid synthesis. Although citrate cleavage enzyme is not part of the transhydrogenation cycle, its heightened activity could serve to initiate and partially sustain the cycle. The enhanced activity of malic enzyme correlates well with the increased activity of two enzymes intimately involved in fatty acid synthesis, namely acetyl CoA carboxylase and fatty acid synthetase, in the adipocytes of meal-fed rats.

G. Methods of Analysis of Protein Synthesis and Degradation Rates

Although malic enzyme is not in the direct pathway of lipogenesis, its enhanced activity in the adipose tissue of meal-fed rats reflects increased rates of fatty acid synthesis. The activity of an enzyme can be increased by elevating the total amount of enzyme present in the cell. It is probable that there has been an increase in the amount of malic enzyme in the adipose tissue of the meal-fed rat.

Schimke and Doyle (81) have recently reviewed the synthesis and degradation of proteins and have suggested that the level of a

protein in a cell is controlled by the combined rates of synthesis and degradation of that protein. An increase in the cellular levels of an enzyme can be due to alterations in the rate of its de novo synthesis, a decreased rate of degradation of the enzyme or both. These changes, as brought about by the effect of some modifier, would result in the establishment of new equilibrium at a different level of enzyme protein concentration in the tissue.

The rates of synthesis and degradation of a given cellular protein are affected by many mechanisms (81), a few of which will be mentioned here. Synthesis of a protein is affected by such factors as: (1) the rate of entry of amino acids into the amino acid pool from which protein synthesis occurs; (2) the rate of m RNA synthesis; (3) the rate of charging of t RNA with amino acids; (4) the levels of functional ribosomes and (5) the availability of energy for protein synthesis. Degradation may be affected by one or more of several possible degradation mechanisms such as: (1) conformational changes in the protein which make it susceptible to protease breakdown; (2) organelle degradation in which the entire organelle, such as lysosomes, degrades at once and (3) alterations in the activity of the degrading system.

There are several methods which can be used to determine changes in protein levels in tissues but the most common technique used is measurement of the rate of tracer isotopes (labelled amino acids in this case) incorporation into or release from cellular proteins and specific proteins of a given tissue. Schimke et al. (82, 83) have worked out suitable procedures to study the turnover of arginase and tryptophan pyrrolase in rat liver. These methods entail generation of

a specific antibody against a purified enzyme or protein from a tissue. This antibody is used to specifically precipitate the enzyme or protein from homogenates of this tissue taken at various time intervals after injecting animals with radioactively labelled amino acids. By this means the level of the label in the precipitate would correlate with the amount of specific protein precipitated and, hence, the amount of specific protein in the cell.

The most common method used involves single isotope administration (pulse method). The method involves a single dose of an isotopically labelled protein precursor. This will constitute a pulse which, theoretically, should instantaneously enter the protein precursor pool of the tissue in question, be rapidly incorporated into the tissue proteins, and, upon release, should not be returned to the precursor pool.

Label in tissue protein will increase with time to a maximum (indicating incorporation). Subsequently, the amount of label in tissue proteins will decline as the labelled proteins are gradually degraded and replaced with proteins synthesized from unlabelled amino acids. The most important assumption involved in this technique is that there should be no reutilization of isotope (84). The decay of label from protein is not only a function of the rate at which a labelled protein is degraded to its constituent amino acids, but also a function of the rate at which such isotopes reenter the amino acid pool from which new protein is synthesized (81). Schimke and Doyle (81) stated that reutilization results in estimated rates of degradation that are slower than the actual rates, the degree of error being a function of the degree to which the isotope is reutilized.

Another assumption involved in the single isotope administration technique is that the radioactivity isolated in the protein sample is representative of all of the specific protein present in the sample (84). The protein samples isolated must be shown to be pure. The presence of a minor contaminant can decrease the accuracy of the estimation of the degradation rate of a given protein.

The rates of synthesis and degradation of a given enzyme can be estimated from a plot of the counts per minute of the immunoprecipitate per milligram of protein (total tissue protein or a specific protein) against the time period following injection of labelled amino acid. As reported in the literature (81), all proteins thus far examined are synthesized according to zero order kinetics and degraded according to first order kinetics. These relationships may be expressed as: $(dE/dt)_s = k_s$ and $(dE/dt)_d = -k_dE$ where $(dE/dt)_s$ and $(dE/dt)_d$ are, respectively, the rates of synthesis and degradation and E is the enzyme concentration. In the steady state the net rate of synthesis is zero and the relationship can be expressed as $k_s - k_d E=0$. The first order rate constant for degradation, k_d, may be calculated from the slope of the decay curve of the plots obtained. The equation is slope = $2.303/k_d$. Knowing k_d, k_s may be obtained by measuring the concentration of the enzyme in the tissue, to give E, which can be obtained from measurements of the enzyme activities in the tissue homogenates. The rate constant for synthesis, k_s, will provide an estimate of the amount of enzyme protein synthesized per gram of tissue protein per hour. The rate constant for degradation, k_d, can be used in the half-life formula ($T_{\frac{1}{2}} = 0.693/k$) to estimate the half-life of loss

of label from the enzyme.

Continuous administration of isotope can also be used to estimate rates of protein degradation (81). This technique involves the feeding of a diet containing labelled amino acids of constant specific activity. The rate constant of degradation can be determined from the time course of approach of isotope labelling to that of the specific activity of the administered isotope. The major assumption of this technique is that the isotope should saturate instantaneously the free amino acid pool from which the protein(s) is synthesized (84). A problem with this assumption may be the existance, in a given tissue, of more than one protein precursor pool which are not in equilibrium. This method allows the estimation of per cent protein replaced with labelled protein in any sample. Schimke (82) used this technique in determining the rate of degradation of rat liver arginase.

Analysis of the time course of change of enzyme activity can indicate the relative contribution that changing rates of synthesis and degradation make in altering enzyme levels (81). The assumptions involved in this technique are that the rate constants for synthesis and degradation change immediately with application and removal of the stimulus, and that the rate constants immediately revert back to their former values upon removal of the stimulus. It is also assumed that a change in enzyme activity constitutes a change in enzyme content. This is questioned in a recent paper, in which Hizi and Yagil (85) found no differences in the rates of synthesis or degradation of mouse liver glucose 6-phosphate dehydrogenase of mice on fat-free or high-fat diets. They suggest that, since the immune serum precipitated equivalent amounts of protein in both tissues,

glucose 6- phosphate dehydrogenase existed in active and inactive forms in mouse liver. The inactive form would be activated upon some, yet unfound, physiological stimulus. Haining (86) stated that, in many studies following decreases of liver enzyme activity after removal of a stimulus, the level of enzyme present is due to simultaneous degradation and synthesis of the protein. Hence, first-order reaction kinetics, as those used in isotope decay studies, are not valid for this type of study. He outlined formulae which took into account both synthesis and degradation and showed that, in the case of tryptophan oxygenase, the "true" rate constant of degradation was much greater than the "apparent" rate constant of degradation of the enzyme.

The rate of synthesis of a specific protein can be estimated by determining the specific activity of the isotope in both the free amino acid pool and the protein over a given period of time (81). Single dose and continuous administration of isotope techniques have been used. Murphy and Walker (6), using a single isotope dose, estimated the rate of synthesis of rat liver malic enzyme in rats at various stages of development and nutritional status. They suggested that alterations in the rate of synthesis, rather than rate of degradation, of malic enzyme has more effect on changing the level of the enzyme during thyroxine treatment or weaning.

The effect of changing rates of synthesis and degradation in altering the level of malic enzyme has been studied in rat liver (4,6, 18), rat epididymal fat pad (17) and in chick liver (3). Gibson et al. (4) showed that a change in the rate of synthesis was the major determinant of net malic enzyme variation in livers of fasted re-fed rats. Starvation caused liver malic enzyme to decrease to low levels and the

rate of synthesis became zero. Refeeding a fasted animal caused a 5- to 6- fold increase in both the enzyme level and its rate constant of synthesis over the normal, fed state. The degradative rate constant of the enzyme was similar in rats that were fed a normal diet, starved or refed a high-carbohydrate diet after starvation. The half-life of liver malic enzyme decreased from 51 hours in the rats fed a normal diet to 41 hours in starved rats and 30 hours in refed rats. The dietary treatments had a similar effect on the rate constants of synthesis and degradation and the half-life of rat liver citrate lyase and fatty acid synthetase.

Murphy and Walker (6) studied the rate of synthesis of rat liver malic enzyme as affected by stage of development, fasting, alloxan diabetes and thyroxine. They did not study the rate of degradation of rat liver malic enzyme as the low level of the enzyme in rat liver and its slow turnover rate made adequate isotope incorporation and degradation analysis difficult. They suggest that due to the low incorporation of isotope into the liver malic enzyme of adult rats, changes in enzyme activity arise mainly from alteration in the rate of enzyme synthesis. L-(4,5-^{3}H) leucine incorporation into rat liver malic enzyme was measured thirty minutes after intraperitoneal administration of the amino acid. In all cases, differences in enzyme levels were paralleled by changes in the rate of synthesis of the enzyme. Thyroxine- feeding caused a 4- to 5- fold increase in liver malic enzyme activity and in the relative rate of synthesis in young adult rats while alloxan-diabetes and fasting caused a significant decrease in enzyme activity and in its synthetic rate. Unweaned rats had comparatively low liver malic enzyme levels and rates of synthesis

whereas weaning caused an eight-fold increase in activity and a six-fold increase in the apparent rate of synthesis of the enzyme.

Li et al. (18) also studied the rate of synthesis of rat liver malic enzyme. They reported that the increase in enzyme activity following fasting and refeeding a high-carbohydrate diet and thyroxine administration was due in part to an increase in the rate of malic enzyme synthesis. The rate of synthesis of liver malic enzyme was 8- to 9- fold greater in refed rats than that of chow-fed rats.

Flatt (17) reported that the malic enzyme of rat epididymal fat pads had a half-life of 59 hours when L-alanine-U-^{14}C was used and 89 hours when L-leucine-U-^{14}C was employed. In this experiment, the fat pads had been exteriorized, exposed to label for 30 minutes and returned to the peritoneal cavity without damage. One fat pad was removed 2 days later and the other 6 days later and the level of label in malic enzyme measured. Intraperitoneal administration of isotope allowed circulation throughout the carcass and abolished the above difference in turnover rates observed with alanine or leucine. Thyroid treatment was found to increase the turnover of the bulk of the soluble adipose tissue proteins while the turnover of malic enzyme was unchanged.

Silpananta and Goodridge (3) have studied the rates of synthesis and degradation of chick liver malic enzyme. The rate of synthesis of chick liver malic enzyme increased with age; a 50-fold increase occurring between 1- and 11- days of age. There was no difference between the rate of synthesis and the half-life of the enzyme (55 hours) in 8- and 11- day old chicks. However, the rate of synthesis and half-life (28 hours) decreased upon fasting 10-day-old

chicks for 48 hours. The half-life of unfed neonatal chicks was very long (350 hours). This indicates a very low degradation rate for the enzyme, but may just be an energy conserving mechanism of the unfed neonate. Silpananta and Goodridge suggested that the rate of degradation was less important than the rate of synthesis in controlling the concentration of malic enzyme. Activation of inactive forms of malic enzyme was not observed to account for the observed changes in total enzyme activity.

Materials and Methods

A. Materials

Liver and epididymal adipose tissue were obtained from male Sprague-Dawley rats for use in the malic enzyme purification and turnover experiments. Hepatic and adipose tissue samples for immunological species specificity studies were obtained from mice (Ob/+, male and female), rabbits (New Zealand, male), chickens (White Leghorn, female), pig (Yorkshire, female), cow (Hereford, female), and lamb (Merino, male) and hepatic tissue from dog (mongrel, male). Male New Zealand rabbits were used for the development of antibodies to rat liver malic enzyme. Normal rabbit serum was obtained by the exsanguination of adult New Zealand rabbits (male and female).

L-arginine-guanidino-^{14}C (50 m Ci/m mole) and L-leucine-U-^{14}C (312 m Ci/m mole) were obtained from Schwartz-Mann Ltd. (New York City). L-lysine-U-^{14}C (306 m Ci/m mole) and Omnifluor were obtained from New England Nuclear Corp. (Boston, Mass.). D-glucose-U-^{14}C (240 m Ci/m mole), sodium bicarbonate-^{14}C (0.1 m Ci/m mole) and PCS (Phase Combining Solvent) were obtained from Amersham-Searle (Arlington Heights, Ill.). Casein and rat salt mixture were obtained from United States Biochemicals (Cleveland, Ohio). Solka-flok was obtained from Brown Co.(Berlin, New Hamp.) while cerelose was obtained from CPC International (Englewood Cliffs, N.J.). All other chemicals were of reagent grade quality.

In all experiments, the rats were fed the same basic diet. This diet consisted of 20% casein, 66.1% carbohydrate (cerelose,

glucose monohydrate), 5.0% fat (corn oil), 0.3% DL-methionine, 4.0% rat mineral mix (87), 0.4% rat vitamin mix, 0.2% choline chloride, 4.0% fiber (Solka-flok). The only variation in the diet was the addition of 1.0% thyroid powder to induce malic enzyme for the purposes of purification. Diet composition and vitamin mix are tabulated in Appendix I.

B. General Methods

Absorbance measurements were made with either a Gilford 240 spectrophotometer or a Gilford 300 automatic recording spectrophotometer.

Protein concentrations were measured by the method of Lowry et al. (88) using bovine serum albumin as a reference protein. A modified method (Appendix II) was used to measure the protein concentration of NaOH-solubilized samples. For pure enzyme preparations, the absorbance at 280 nm was also used to measure protein concentration.

Since Tris (tris (hydroxymethyl) aminomethane) has a high temperature coefficient for pH, the temperature at which the pH was measured is given in parentheses in those cases where temperature is not obvious.

Carbon-14 measurement was carried out in a refrigerated Packard Tri-Carb (Packard Instrument Co., Downer's Grove, Ill.) liquid scintillation counter. ^{14}C- labelled samples were suspended in 10 ml of PCS cocktail or, in the case of the fatty acid synthesis samples, in 10 ml of toluene scintillation-cocktail. Quenching was estimated by the channels ratio method. Per cent efficiency of counting was calculated from a standard curve obtained from quenched

standards. All values are expressed as disintegrations per minute.

One way analysis of variance was carried out for the enzymatic activities and lipogenic capacities of the liver and epididymal fat pads of the meal-fed and nibbling rats. Differences were considered to be significant if they attained a probability of $P \leq 0.05$.

Enzyme Assays

Unless otherwise stated, all assays were conducted at 25°C, and the rate of change of NADP concentration was measured at 340 nm. A unit of enzyme activity is defined as 1 n mole of NADP reduced per minute under the assay conditions used. The results are expressed as units per milligram protein.

For the routine assay of malic enzyme (EC 1.1.1.40) activity, a modification of the procedure of Ochoa et al. (89) was used. The rate of formation of pyruvic acid and carbon dioxide was measured spectrophotometrically by following the reduction of NADP in the presence of excess L-malate and $MnCl_2$. The reaction mixture (3.0 ml) contained 60 μ moles triethanolamine (pH 7.4 at 25°C), 3.6 μ moles $MnCl_2 \cdot 6\ H_2O$, 0.21 μ moles NADP, 2.5 to 10 units of enzyme, 0.45 μ moles L-malate (pH 7.4). The reaction was initiated with the addition of L-malate.

The double-substrate procedure of Glock and McLean (90), as modified by Ball and Jungas (91), was used to assay glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phospho-gluconate dehydrogenase (EC 1.1.1.44). Acetyl CoA carboxylase (EC 6.4.1.2) activity was measured by the $NaH^{14}CO_3$ fixation procedure, as described by Gregolin et al. (92). Fatty acid synthetase was assayed by the

procedure of Gibson and Hubbard (93).

Fatty Acid Synthesis

Lipogenic capacity was determined for adipose tissue by incubating a weighed piece of tissue (approximately 100 mg) in Ca^{2+}-free Krebs-Ringer bicarbonate buffer containing 25 m Moles glucose, 0.20 μ Ci glucose-U-^{14}C and 0.1 unit of porcine insulin per milliliter. For hepatic tissue the Krebs-Ringer bicarbonate buffer contained 100 mM glucose, 0.30 μ Ci glucose-U-^{14}C and 0.1 unit of porcine insulin per milliliter. The procedures for the tissue incubation, isolation of fatty acids and assay of radioactivity has been previously described by Leveille (94) and are detailed in Appendix III.

Zone Electrophoresis

Discontinious polyacrylamide gel electrophoresis was performed at 5°C on 4.5 or 5.0% acrylamide disc gels in a Buchler gel electrophoresis chamber at 5 mA per tube according to the methods of Ornstein (95) and Davis (96). The conditions and buffer systems used are described in the text. The gels were stained for protein with Coomasie Blue. Destaining was carried out overnight in circulating 7% acetic acid- 5% methanol at 50°C. The malic enzyme stain used was that described by Henderson (23).

Sodium dodecyl sulfate gel electrophoresis was carried out on pure malic enzyme dissociated with 1% sodium dodecyl sulfate- 1% β-mercaptoethanol for 30 minutes at 37°C. Sodium dodecyl sulfate-free gels (5.0 or 6.0% acrylamide) were pre-run against sodium-dodecyl sulfate containing buffer for 1.0 hour prior to addition of the dissociated protein sample. The gels were stained for protein with Coomassie Blue and destained overnight at 50°C with circulating 7%

acetic acid-5% methanol.

Immunological Analysis of Malic Enzyme with Specific Anti-Serum

Anti-serum to rat liver malic enzyme was prepared in rabbits by subcutaneous injection of the rat liver enzyme emulsified in Freund's complete adjuvant. The injection schedule involved three weekly injections (1 to 2 mg protein per injection) of recrystallized enzyme followed by a "booster" injection (1 to 2 mg protein) three months after the first injection. Immune serum (anti-rat liver malic enzyme) was obtained from clotted blood drawn weekly (starting the fifth week after the first injection) and was stored at -5°C until used.

Immunological analysis of malic enzyme with specific anti-serum was carried out using a modified two-dimensional gel diffusion (Ouchterlony) method as described by Campbell et al. (97). The agar plate contained 0.8% agar (w/v)-0.01% trypan blue (w/v) (pH 7.5 at 20°C). The reaction was carried out for 4 to 6 days at room temperature.

Quantitative precipitin analysis was used to measure the extent of cross reaction of the anti-rat liver malic enzyme with the pure enzyme and liver and adipose tissue extracts of rats (in different physiological states), mice, rabbit, chicken, pig, dog (liver only), lamb and cow. Serial dilutions (ranging from 1:1 to 1:32 dilutions in 0.5 ml volumes) of the anti-serum in 50 mM Tris, 5 mM EDTA, 100 mM KCl, 2 mM -SH (pH 7.4 at 5°C) were allowed to react with a sample aliquot containing 250 units of malic enzyme activity. A control reaction, consisting of 250 units of malic enzyme activity in 0.5 ml of the above buffer, was also made for each enzyme or tissue preparation. The samples were allowed to react for 30 minutes

at 30°C and then overnight at 2°C. The samples were then centrifuged at 50,000 x g for 30 minutes at 0 to 5°C and the supernatants were assayed for malic enzyme activity. The results are expressed as per cent loss of enzyme activity (100%- % control activity) versus per cent dilution of the anti-serum. Measurement of the amount of protein precipitated by the enzyme-anti-serum reaction was accomplished by a modification of the Lowry protein method and is described in Appendix II.

C. Purification of Rat Liver Malic Enzyme

The procedure for purification of rat liver malic enzyme is a communication from Dr. J.P. Flatt and is as follows.

General Comments on Isolation of Liver Malic Enzyme

All steps were carried out at 0 to 5°C unless otherwise indicated.

Step I. Induction of Liver Malic Enzyme

Rat liver malic enzyme was induced in 15 male Sprague-Dawley rats (200 to 225 gm) by a double fasting-high carbohydrate refeeding regime. In each cycle, the animals were fasted for 48 hours followed by feeding a 60% carbohydrate, 20% protein diet containing 1% thyroid powder for three days.

Step II. Extraction

The animals were sacrificed by decapitation and the livers were quickly removed, chilled, pooled and weighed. The livers were homogenized for 2 minutes in a Waring blender in 3 volumes (w/v) of 250 mM sucrose, 50 mM Tris, 20 mM Mg acetate, 0.5 mM EDTA (pH 7.4 at 5°C) at 5°C. The homogenate was centrifuged at 50,000 x g for 30 minutes at 0 to 5°C. The supernatant was recentrifuged at 100,000 x g for 1 hour at 0 to 5°C.

Step III. Heat Treatment

Magnesium acetate (1 M) was added to the 100,000 x g supernatant to a concentration of 0.1 M. Dithiothreitol was added to a concentration of 0.1 mM. The pH was adjusted, with stirring, to pH 7.35 to 7.40 with the addition of 1 M Tris (pH 10 at 5°C). The extract was then quickly heated to and kept at 51°C in a water bath (75-80°C) for 5 minutes. After cooling on ice, the heat treated extract was centrifuged at 50,000 x g for 20 minutes at 0 to 5°C. The supernatant was kept at 2°C overnight.

Step IV. Alcohol Fractionation

The supernatant was cooled, with stirring, to 0°C by refrigerated ethylene glycol circulating through a jacketed, foil-covered beaker. Cold 95% ethanol (-70 C) was added to a concentration of 20% (v/v) over a period of 1 hour. During this time the temperature of the sample was lowered to -10°C. After the alcohol addition, the sample was centrifuged at 25,000 x g for 30 minutes at -10°C. The supernatant was then brought to 45% ethanol concentration (v/v), by the addition of cold ethanol, over a period of 1 hour and the temperature lowered to -20°C. After stirring slowly for 30 minutes at -20°C, the sample was centrifuged at 25,000 x g for 30 minutes at -15°C and the supernatant discarded. The precipitated protein was suspended in a small amount (4 ml/200 ml centrifuged sample) of ice cold water and an identical volume of 50 mM Tris, 5 mM EDTA (pH 7.4 at 5°C) was added. The suspension was transferred to a centrifuge tube with three rinses, in small volumes, of 25 mM Tris, 25 mM EDTA (pH 7.4 at 5°C) and centrifuged at 50,000 x g for 30 minutes at 0 to 5°C.

Step V. Crystallization

The slurry supernatant (16 mg/ml) was dialyzed for 48 hours against 1 liter of 0.1 M Mg acetate, 0.05 M Tris, 1 mM EDTA, 1 mM dithiothreitol, 8% ethanol (v/v) (pH 7.4 at 5°C). Dialysis was continued for a further 48 hours using the above buffer without ethanol. The dialysate was centrifuged at 20,000 x g for 20 minutes at 0 to 5°C. The pellet was suspended in 50 mM Tris, 0.5 mM EDTA (pH 7.4 at 5°C) and recentrifuged. The resulting pellet was solubilized in 0.2 M Tris, 0.8 M NaCl, 0.5 mM EDTA (pH 7.4 at 5°C) and immediately diluted with an equal volume of cold water. The solubilized enzyme was centrifuged at 50,000 x g for 20 minutes at 0 to 5°C and the pellet discarded.

The second crystallization of the enzyme (4 mg/ml) was carried out in the same manner except that only 4% ethanol (v/v) was added during the initial dialysis.

A third crystallization of the enzyme, for the purposes of storage, was carried out. The enzyme was dialyzed for 24 hours in the absence of ethanol; the buffer changed and dialysis was continued for 24 hours. The dialysate was centrifuged at 50,000 x g for 20 minutes at 0 to 5°C and the pellet suspended in the dialyzing buffer. The enzyme was stored at 0°C with minimal loss of enzyme activity over a period of 6 months.

D. Label Incorporation into Rat Tissue Proteins and Malic Enzyme

The following description details the procedures used in determining the effects of meal-feeding, as compared to nibbling, on liver and epididymal fat pad protein and malic enzyme turnover. Included are several experiments, listed in the order of occurrence,

that were performed to determine and solve the problems that were encountered during the turnover experiments.

Dietary Regime

Male Sprague-Dawley rats (200-225 gm) were adapted to the previously described diet until the average weight was 250 gm. The animals were then paired on the basis of body weight. On the day of initiation of meal-feeding, all food cups were removed from the cages at 10:00 A.M.. Assuming that the rats designated as meal-eaters had eaten nothing between 8:00 and 10:00 A.M., the corresponding paired nibblers were given nothing to eat. On the next day, and on all subsequent days during the experimental period, the meal-eaters were given a food cup (of known weight) at 8:00 A.M. and allowed to eat until 10:00 A.M.. At this time, the food cups were removed, weighed and recorded. The difference in weight of each food cup was recorded and this amount was presented to the corresponding paired nibbling rat. Each nibbling rat was housed in a cage modified to hold a mechanical feeder that distributed the food evenly over a period of twenty-four hours. This ensured that the nibbling rat was consuming the food uniformily throughout the day and was not experiencing periods of peak consumption.

Protein Precipitation with Trichloroacetic Acid (TCA)

This procedure is used throughout all experiments and is, hence, described separately. In every experiment, 5.0 ml of 30% trichloroacetic acid was added to an 1.0 ml aliquot of the liver and epididymal fat pad homogenates and extracts. These were then set aside in a refrigerator until the end of the experiment. The samples were centrifuged at 50,000 x g for 20 minutes at 0 to 5^{o}C. The

supernatants were decanted and saved. The precipitate was suspended in 5.0 ml of TCA and heated at 80°C for 1.5 hours in a water bath. This step removed nucleic acids, which are soluble in hot TCA. After centrifugation at 50,000 x g for 10 minutes at 0 to 5°C, the pellet was successively washed with 5.0 ml of acetone: ethanol (1:1) and 5.0 ml of petroleum ether. These steps removed the lipid present in the pellet. The resulting pellet was solubilized in 2 ml of 2 N NaOH with heat at 70°C until all the protein was solubilized. Aliquots (0.05 and 0.10 ml) were diluted with 0.9 ml water, and suspended in 10 ml of PCS. Label incorporation into the tissue protein fraction was measured in the liquid scintillation counter. The solubilized protein from the homogenates represented total cellular protein while that from extracts represented soluble cellular protein. Results for label incorporation into protein were expressed as dpm per milligram protein. In all experiments, except the last experiment, the protein content of the TCA-precipitated protein was measured by the method of Lowry et al. (88). Unfortunately, the protein yield, according to the method results, was not complete; yielding from 40 to 70% of the amount of protein present in an unprecipitated sample. This could be due to a myriad of reasons, some of which could be loss of amino acids and peptides that would be measured by the method of Lowry, solubilization of hydrophobic proteins by TCA, TCA interference with the Lowry protein method, or incomplete solubilization of the pellet with NaOH. A comparison of degradation data, based on Lowry protein measurements of tissue extracts or TCA precipitates, showed little or no difference in the rates of degradation of proteins. In the last experiment results are expressed as dpm per milligram of tissue protein.

The supernatants from the initial TCA precipitation were used to measure the amount of label present in the TCA-non precipitable fraction. TCA was removed from the supernatants with three 5.0 ml washes of petroleum ether. Aliquots (0.3 ml) of the supernatants were suspended in 10 ml of PCS and label measured in the liquid scintillation counter. Results are expressed as dpm per milliliter of homogenate.

Malic Enzyme Precipitation with Specific Anti-serum

Incorporation of L-Arginine-guanidino-^{14}C into Malic Enzyme

A preliminary turnover experiment was carried out using L-arginine-guanidino-^{14}C as the labelled amino acid. Label, 35 μ Ci per animal, was administered intraperitoneally at 1200 hours, to 12 pairs of pair fed meal-eaters and nibblers that had adjusted to the dietary regime for 4 weeks. The animals were sacrificed, two pairs at a time, at 1,6,12,24,48 and 72 hours after injection. For each animal several samples, each containing known amounts (250 units) of malic enzyme from the epididymal fat pad or heat-treated liver extract, were mixed with 0.5 ml specific anti-serum; incubated for 30 minutes at 30°C and then at 2°C overnight. Non-specific precipitation of label was measured by carrying through, for each sample, a blank consisting of 250 units of malic enzyme with 0.5 ml serum which contained no malic enzyme antibodies. One sample of each of the above reactions was set aside for determination of precipitated protein. The enzyme-antibody complex was filtered, under vaccuum, through Millipore filters. After washing with 20 ml of cold saline, the filter was placed in PCS and label in malic enzyme was measured in the liquid scintillation counter. Correction, by subtraction, was made for non-specific

precipitation of label. Results were expressed as dpm per milligram of protein precipitated by the antibody. Precipitated protein was measured by the modified Lowry protein method described in Appendix II. TCA-precipitation of tissue protein was carried out as described. A comparison of the effects of meal-eating and nibbling on the rate of fatty acid synthesis and several enzyme activities was carried out immediately prior to and following the degradation experiment. This was done to correlate the effects of meal-feeding and nibbling on malic enzyme activity with other physiological effects.

Effect of Intravenous and Intraperitoneal Routes of Administration

A comparison of the effects of injecting L-arginine-guanidino-^{14}C by IV or IP routes on label incorporation into tissue protein and malic enzyme was conducted. Two rats (400 and 420 gm) fasted 72 hours and refed a high carbohydrate diet containing 1% thyroid powder for 48 hours, were injected, either IV or IP with 25 μ Ci and sacrificed 24 hours after injection. TCA-precipitation of protein was carried out as described. Malic enzyme was precipitated with anti-serum, as before, but the enzyme-antibody complex was collected by centrifugation at 50,000 x g at 0 to 5°C, washed 3 times with 0.5 ml of cold saline, solubilized with 0.5 ml of 60% (v/v) formic acid, suspended in 10 ml PCS and counted. Modified Lowry protein analysis (Appendix II) was carried out on the protein precipitates solubilized with 0.5 ml of 1 N NaOH. Results were expressed as dpm per unit malic enzyme activity.

Effect of Length of Malic Enzyme Anti-serum Reaction

Quantitative precipitin tests were conducted to compare the

effect of the length of time the malic enzyme and antibody were allowed to react on the completeness of precipitation of malic enzyme. Two rats, fasted 48 hours and refed a high carbohydrate diet containing 1% thyroid powder for 48 hours, were sacrificed, and heat-treated extracts from the pooled livers and extracts from the pooled epididymal fat pads were prepared. Malic enzyme activity of the liver extracts was determined and aliquots containing 250 units of malic enzyme were mixed with 6 sets of serial dilutions of the anti-serum. All sets were allowed to incubate for 30 minutes at 30°C. Three sets were incubated at 2°C overnight prior to centrifugation while the other 3 were centrifuged immediately. The supernatants of all samples were decanted and assayed and the pellets were solubilized for protein determination.

Effect of Initial Blank Reaction on Non-Specific Precipitation of Label

A third experiment was performed to determine whether the use of an initial blank serum reaction, prior to the malic enzyme-anti-serum reaction, would reduce the level of non-specific label precipitation during the reaction. Two rats (229 and 243 gm), adapted to meal-feeding for 4 weeks, were injected IP, at 1200 hours, with 35 μ Ci of L-arginine-guanidino-^{14}C (in saline) and sacrificed 24 hours later. TCA precipitation of protein was carried out as described earlier. Immunological precipitation of malic enzyme and label measurement was performed basically as described in the immediately preceeding experiment but with several modifications. Malic enzyme activity in the tissue preparations was measured, and the volume of extract corresponding to 1.25 times that needed to yield 250 units of malic enzyme activity

was measured out and mixed with 0.5 ml of blank serum. Several such mixtures were allowed to incubate for 30 minutes at 30°C, then centrifuged at 50,000 x g for 30 minutes at 0 to 5°C, and a volume of supernatant equivalent to 250 units of malic enzyme activity (accounting for prior dilution with serum) from each sample was mixed with 0.5 ml of anti-serum or more blank serum. This mixture was incubated for 30 minutes at 30°C and centrifuged at 50,000 x g for 30 minutes at 0 to 5°C. The supernatants were decanted and assayed for malic enzyme activity to assure that the anti-serum had removed all the enzyme present or that none had precipitated or been denatured during the blank reactions. The pellets were solubilized for counting or Lowry protein measurements, as described above. The procedures of using an initial blank reaction and 30 minute incubations were used in all subsequent experiments.

Effect of Multiple Injection of Label

In an attempt to increase the amount of radioactivity incorporated into liver malic enzyme, which, in all previous experiments, had been just above that of the non-specific precipitate, IP injection of multiple doses of labelled amino acid was attempted. Three small doses (12 μ Ci per dose) of L-arginine-guanidino-^{14}C over a period of 3 hours, were injected into 2 rats (296 and 375 gm) that had been meal-fed for 4 weeks. Upon sacrificing, 24 hours after the last injection, extracts were made from the pooled livers and pooled epididymal fat pads. Malic enzyme was precipitated and processed by the techniques described in the immediately preceding experiment. A comparison was made of the effect of washing the enzyme-antibody pellet with saline or with 1 M NaCl. TCA precipitation of protein was carried out as described.

A similar experiment was also carried out using multiple injections (8 μ Ci per dose) of L-lysine-U-^{14}C (in saline). Two rats (252 and 264 gm), fed a high carbohydrate diet containing 4% thyroid powder for 48 hours, were sacrificed 24 hours after the last injection. Malic enzyme was precipitated and processed by the previously described techniques. TCA precipitation of protein was carried out as described.

Effect of Specific Amino Acid Administered

The incorporation of L-arginine-guanidino-^{14}C or L-lysine-U-^{14}C into tissue proteins and malic enzyme was also studied. Two pairs of paired meal-eaters and nibblers that had been on the dietary regimen for 7 weeks (pair A, 403 gm (meal-eater) and 420 gm (nibbler)); (pair B, 433 gm (meal-eater) and 408 gm (nibbler)) were injected IP with 25 μ Ci per animal of L-arginine-guanidino-^{14}C (in saline) (pair A) or L-lysine-U-^{14}C (in saline) (pair B). The animals were sacrificed 24 hours after injection. Malic enzyme in the tissue extracts was precipitated and processed by the previously described techniques. TCA-precipitation of protein was carried out as described.

L-leucine-U-^{14}C (in saline) incorporation in tissue proteins and malic enzyme was studied. Two pairs of paired meal-eaters and nibblers that had been on the dietary regimen for 9 weeks (pair A, 465 gm (meal-eater) and 439 gm (nibbler)); (pair B, 454 gm (meal-eater) and 439 gm (nibbler)) were each injected IP with 75 μ Ci per kg body weight of L-leucine-U-^{14}C. The animals were killed 24 hours after injection. Malic enzyme in the tissue extracts was precipitated and processed by the previously described techniques. TCA-precipitation of protein was carried out as described.

Incorporation of L-Leucine-U-^{14}C into Malic Enzyme

An experiment was conducted, using L-leucine-U-^{14}C as the labelled amino acid, to determine and compare the rates of degradation of the liver and epididymal fat pad proteins and of malic enzyme from meal-fed and nibbling rats. Label (in saline) was administered IP, at 1200 hours, with 100 μ Ci per kg body weight to 2 separate groups, consisting of 8 pairs of pair-fed meal-eaters and nibblers each, that had adjusted to the dietary regimen for 10 weeks. The animals were sacrificed, 2 pairs at a time, at 1,24,48 and 72 hours after injection. For each pair of animals, several samples, each containing 1.25 times the amount of malic enzyme from the pooled heat-treated liver extracts to yield 500 or 1000 units of enzyme activity or from pooled epididymal fat pads extracts to yield 250 or 500 units of enzyme activity, were mixed with 0.5 ml (with 500 units of malic enzyme) or 1.0 ml (with 1000 units of malic enzyme) of blank serum, incubated for 30 minutes at 30°C and centrifuged at 50,000 x g for 30 minutes at 0 to 5°C. A volume of supernatant equivalent to the desired amount of malic enzyme activity (accounting for prior serum dilution) from each sample was mixed with 0.5 or 1.0 ml of anti-serum or blank serum. This mixture was incubated for 30 minutes at 0 to 5°C. The supernatants were decanted and assayed for malic enzyme activity to ensure that the anti-serum had removed all of the enzyme present or that none had precipitated or been denatured during the blank reactions. For each tissue extract, the enzyme activity in the blank reaction supernatant was used for calculating the data on the basis of label incorporation per unit of enzyme activity. The malic enzyme-antibody pellets were solubilized for counting or for Lowry protein measurements, as described above. TCA precipitation of protein was carried out as described.

A comparison of the effects of meal-feeding and nibbling on the rate of fatty acid synthesis and several enzymes was conducted following the degradation experiment. This was done to correlate the effects of meal-feeding and nibbling on malic enzyme activity with other physiological effects.

A replication of the above experiment, using L-leucine-U-^{14}C as the labelled amino acid, was conducted to determine and compare the rates of degradation of the liver and epididymal fat pad proteins and epididymal fat pad malic enzyme from meal-fed and nibbling rats. Label (in saline) was administered IP, at 100 μ Ci per kg body weight, to 24 pairs of pair-fed meal-eaters and nibblers each which had adjusted to the dietary regimen for 4 weeks. Six pairs of animals were sacrificed at 1,24,48 and 72 hours after injection. The label incorporation into epididymal fat pad malic enzyme was measured using the same procedure as previously described. The malic enzyme-antibody pellets were solubilized for counting or for Lowry protein measurements, as described above. Results were expressed as dpm per unit of malic enzyme activity. TCA precipitation of protein was carried out as described, but results were expressed on the basis of the protein content of untreated homogenates and extracts. Lowry protein analysis (Appendix II) was carried out on 1.0 ml aliquots of NaOH- treated tissue homogenates and extracts.

Calculation of Isotope Incorporation Data

The incorporation data from the last two meal-feeding experiments was used to calculate the linear regression and slopes of the loss of label from tissue proteins and malic enzyme with time. Values for protein and malic enzyme half-lives ($T\frac{1}{2}$) and rate constants of degradation and synthesis were calculated from the above data using the formulae outlined by Schimke in his review article (81).

Results

A. Purification of Rat Liver Malic Enzyme

A summary of a typical purification procedure of rat liver malic enzyme is given in Table 1. The overall purification was 139-fold and eighty-two per cent of the original enzyme activity was recovered. Decreased enzyme activity in Fractions III and IV may have been due to incomplete removal of ethanol from the sample.

Homogeneity of the purified malic enzyme was demonstrated by several means: (a) purification to constant specific activity, (b) absence of other enzymatic activities, (c) immunological analysis, and (d) polyacrylamide gel electrophoresis.

The enzyme retained constant specific activity following crystallization and recrystallization (Table 1). The final specific activity of 12490 units/mg protein was observed in two separate purification experiments. Crystallization of malic enzyme was always easily and repeatedly achieved by dialysis.

Often contaminants in an enzyme preparation are other enzymes which are, metabolically, closely related. Twice crystallized malic enzyme was assayed for 4 enzymes involved in lipogenesis. Percentage contamination is expressed as the ratio of activity of the contaminating enzyme to that of malic enzyme, using the assays described in Materials and Methods. The percentage contamination was less than 0.001% for glucose 6 -phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, acetyl CoA carboxylase, and fatty acid synthetase. Enzyme specificity for NADP, as a coenzyme, was shown by the fact that NAD could not be

Table 1

Purification of Rat Liver Malic Enzyme

Fraction	Total Enzyme Activity	Yield	Specific Activity	Purification
	units	%	units/mg protein	fold
I. Extract	1009	100	90	1.0
II. Heat Treatment	994	98.5	190	2.11
III. 22-45% ETOH Fraction	723	71.7	750	8.33
IV. 1st Crystallization	638	63.2	11140	123.8
V. 2nd Crystallization	826	81.9	12490	138.8

used, in place of NADP, in the decarboxylation of malate.

Anti-serum to rat liver malic enzyme from rabbits was used in a modified Ouchterlony double diffusion method to determine the purity of the liver enzyme preparation. Only a single precipitin band appeared with the 1:5 diluted crystalline enzyme (41500 units/ml) and this line fused with the single precipitin lines that developed with rat liver extract (670 units/ml) (Figure 1). The presence of a single precipitin band with extract suggests that the antibody was specific for malic enzyme. Rat epididymal fat pad extract (160 units/ml) was also included; but no precipitin band was observed. This may be due to low enzyme concentration in the extract.

By means of an indirect immunological titration of anti-rat liver malic enzyme with pure rat liver malic enzyme and extract preparations of rat liver and epididymal fat pad, it was possible to determine quantitatively the extent of cross-reaction. A constant amount of enzyme or extract preparation (250 units/reaction) was added to doubly diluted amounts of anti-rat liver malic enzyme. After incubation and centrifugation, the supernatants were assayed for residual antigen by measuring the malate decarboxylation activity in the supernatant. The results, shown in figure 2, demonstrate that anti-liver malic enzyme completely inhibited malic enzyme activity in the purified enzyme preparation as well as in rat liver and epididymal fat pad extracts.

Polyacrylamide gel electrophoresis of the pure enzyme at pH 8.4, resulted in a single band with no detectable contamination. Histochemical stain for the enzyme in the gel also yielded only one band (Figure 3). Purified malic enzyme that had been dissociated

Figure 1

Double Diffusion Analysis of Purified Rat Liver Malic Enzyme, Rat Liver and Epididymal Fat Pad Homogenates

Double diffusion analysis of purified rat liver malic enzyme (M), rat liver homogenate (L) and epididymal fat pad homogenate (F). The center well contained anti-rat liver malic enzyme. Conditions are described in Materials and Methods.

Figure 1

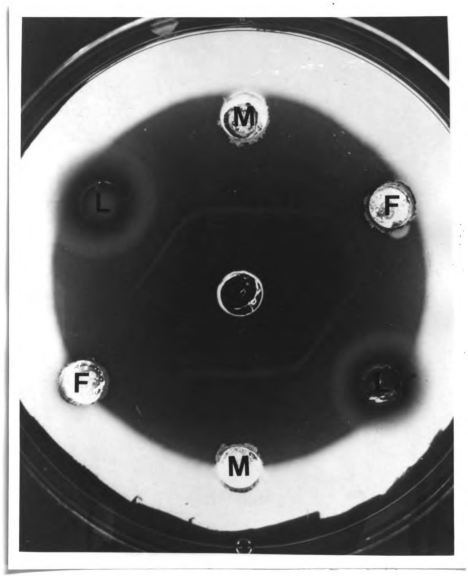


Figure 2

Precipitation of Purified Rat Liver Malic Enzyme and the Malic Enzyme from Extracts of Rat Liver and Epididymal Fat Pad with Anti-Serum to Rat Liver Malic Enzyme

Precipitation of purified rat liver malic enzyme (●) and the malic enzyme of extracts of rat liver (o) and epididymal fat pad (Δ) with anti-serum to rat liver malic enzyme. Six doubling dilutions of anti-serum were prepared in 50 mM Tris, 5 mM EDTA, 100 M KCl, 2 mM β-mercaptoethanol (pH 7.4 at 5°C). To each 0.5 ml of diluted antibody, 250 units of malic enzyme activity of purified enzyme or from tissue extract was added. After incubation at 30°C for 30 minutes and at 2°C for 16 hours, the precipitates were removed by centrifugation at 50,000 x g for 30 minutes and malic enzyme was assayed in each supernatant.

Figure 2

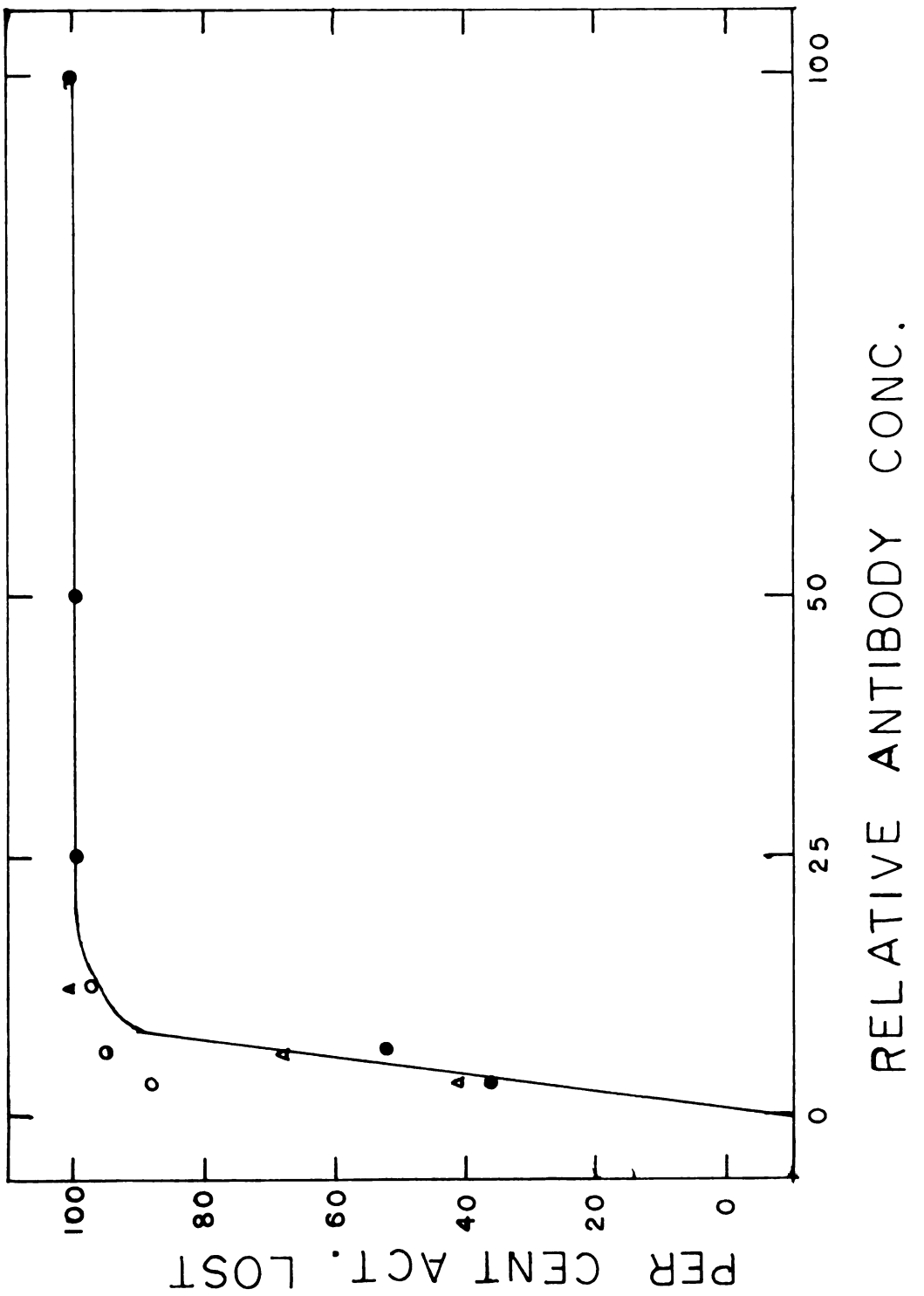
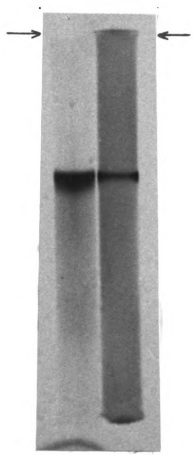

PER CENT ACT. LOST
100
80
60
40
20
0
0
25
50
100
RELATIVE ANTIBODY CONC.

Figure 3

Polyacrylamide Gel Electrophoresis of Malic Enzyme

Purified malic enzyme (33 μg/gel) was applied to gels, containing 4.5% acrylamide, prepared with Tris-SO_4 (1.5 M), pH 8.4 at 25°C, for the running gel and Tris-SO_4 (0.30 M), pH 8.4 at 25°C, for the stacking gel. Electrophoresis was performed, using Tris-borate (0.065 M); pH 8.4 at 25°C as the tank buffer, at 25°C for approximately 1.5 nours at a current of 4 m a per gel. After electrophoresis, gels were stained for protein with 0.25% Coomassie blue. Malic enzyme activity was demonstrated by the method of Henderson (23).

Figure 3



by incubation with 1% sodium dodecyl sulfate, 1% β-mercaptoethanol for 30 minutes at 37°C also showed only one band with no detectable contamination (Figure 4).

B. Immunological Analysis of Rat Liver Malic Enzyme

Immunological analysis of liver and adipose tissue malic enzymes of rats and several other species were performed using anti-rat liver malic enzyme. The analyses were carried out by means of double diffusion on agar plates and quantitative precipitin tests.

A comparison of quantitative precipitin tests for malic enzyme of liver and epididymal fat pad extracts of meal-fed and nibbling rats (250 units/reaction) shows no differences in the extent of cross-reaction (Figure 5). Both double diffusion and precipitin analyses suggest that physiological status has no effect on the antigenicity of rat malic enzyme.

Anti-serum to rat liver malic enzyme was used in immuno-chemical analyses of the liver and adipose tissue malic enzymes of several species. In the double diffusion in agar of anti-rat liver malic enzyme against purified malic enzyme (0.5 mg/ml) and the malic enzymes of the livers of obese (Ob/Ob) (2390 units/ml) and lean (Ob/+) or (+/+) mice (760 units/ml) and the epididymal fat pads of obese (80 units/ml) and lean mice (230 units/ml), there was good cross-reaction between the anti-serum and the purified liver malic enzyme and the liver malic enzymes of obese and lean (Ob/+) mice (Figure 6). The continuity between the precipitin lines of rat liver malic enzyme and the mouse liver malic enzyme suggests a fairly high degree of similarity between the two enzymes. It also suggests that the different specific activities of the obese and lean (Ob/+) mouse

Figure 4

Polyacrylamide Gel Electrophoresis of Malic Enzyme in the Presence of Sodium Dodecyl Sulfate (SDS)

SDS- dissociated malic enzyme (20 μg/gel) was applied to gels, containing either 5 or 6% acrylamide, prepared with Tris-SO_4 (1.5 M), pH 8.4 at 25°C. The gels were pre-run for 1 hour using Tris-glycine (0.065 M)- 1% SDS- 1% β-mercaptoethanol (pH 8.4 at 25°C) as a tank buffer. Electrophoresis was performed at 25°C for approximately 1 hour at a current of 5 m a per gel. After electrophoresis, the gels were stained for protein using 0.25% Coomassie blue.

Figure 4

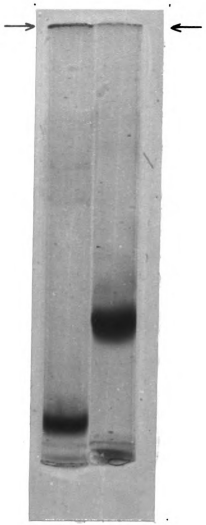


Figure 5

Precipitation of the Malic Enzyme from Extracts of Liver and Epididymal Fat Pad from Meal-fed and Nibbling Rats with Anti-Serum to Rat Liver Malic Enzyme

Precipitation of the malic enzyme of extracts of meal-fed rat liver (o) and epididymal fat pad (●) and of nibbling rat liver (Δ) and epididymal fat pad (▲) with anti-serum to rat liver malic enzyme. Six doubling dilutions of anti-serum were prepared in 50 mM Tris, 5 mM EDTA, 100 mM KCl, 2 mM β-mercaptoethanol (pH 7.4 at 5°C). To each 0.5 ml of diluted antibody, 250 units of malic enzyme activity from tissue extract was added. After incubation at 30°C for 30 minutes and 2°C for 16 hours, the precipitates were removed by centrifugation at 50,000 x g for 30 minutes and malic enzyme was assayed in each supernatant.

Figure 5

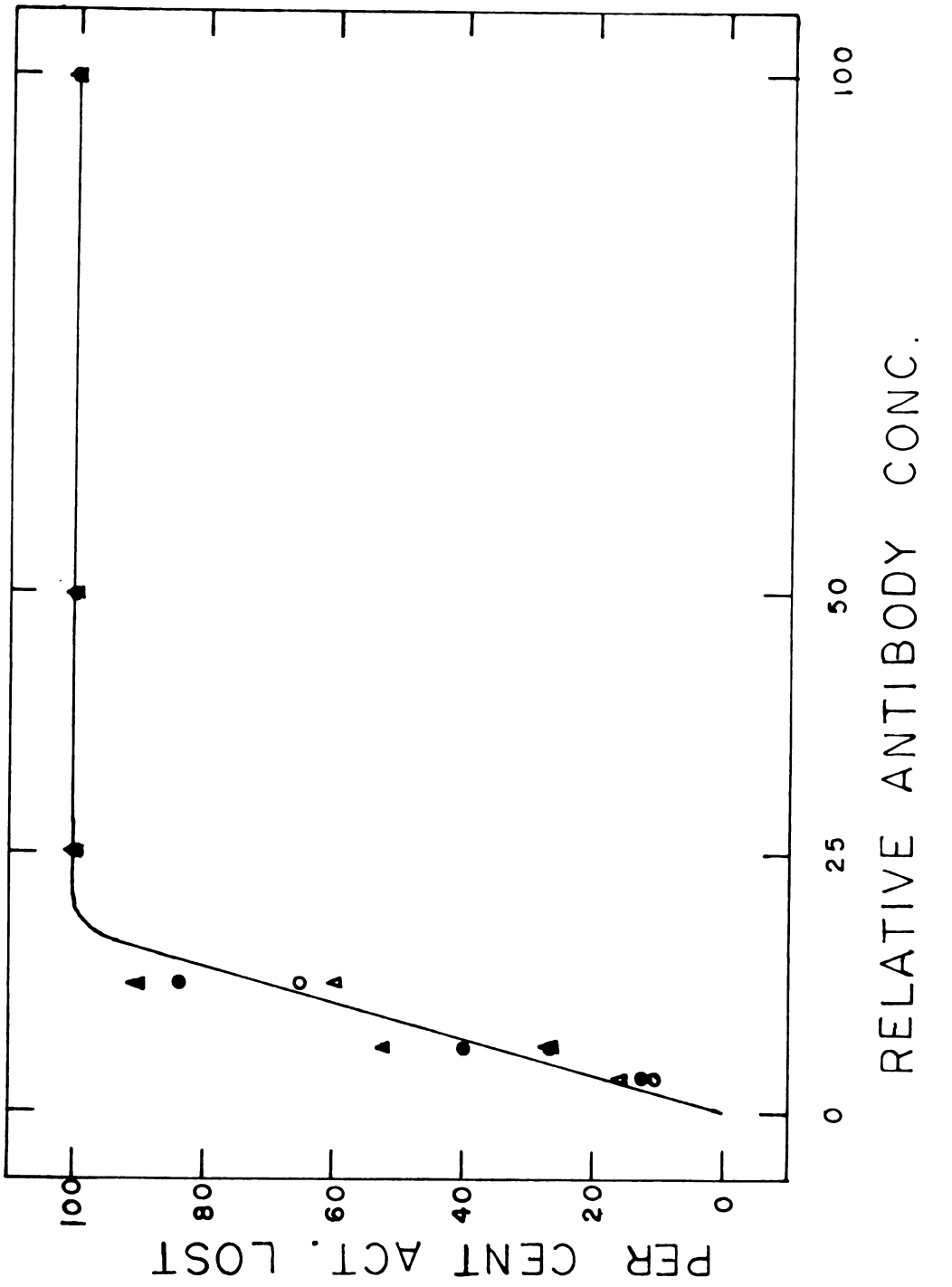
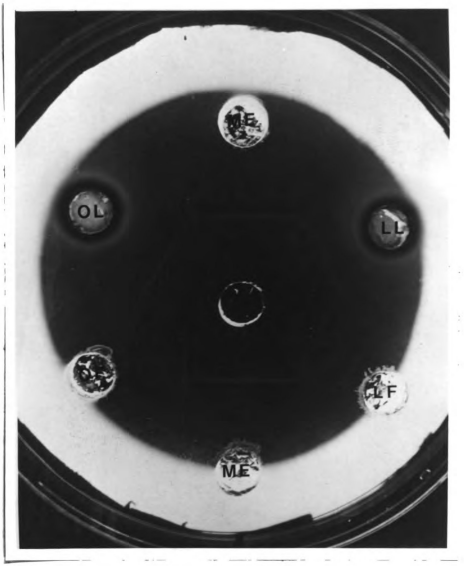

PER CENT ACT. LOST
0
20
40
60
80
100
0
25
50
100
RELATIVE ANTIBODY CONC.

Figure 6

Double Diffusion Analysis of Purified Rat Liver Malic Enzyme and the Malic Enzyme of Liver and Epididymal Fat Pad from Obese and Lean Mice

Double diffusion analysis of purified rat liver malic enzyme (ME) and the malic enzyme from liver (OL) and epididymal fat pad (OF) of obese mice and liver (LL) and epididymal fat pad (LF) of lean mice. The center well contained anti-rat liver malic enzyme. Conditions are described in Materials and Methods.

Figure 6



liver malic enzymes may be due to tissue concentration differences rather than molecular differences. The lack of cross-reaction between the anti-serum and the mouse epididymal fat pad malic enzyme may be due to an insufficient enzyme concentration in the tissue extracts to yield a precipitin band.

In the quantitative precipitation of the anti-serum to rat liver malic enzyme with rat liver and epididymal fat pad extracts and liver and epididymal fat pad extracts from obese (Ob/Ob) and lean (Ob/+) mice (125 or 250 units/reaction), little or no variation was found in the extent of cross-reaction between anti-rat liver malic enzyme and tissue extracts of the two species (Figure 7). Double diffusion analysis of rat and mouse liver malic enzyme and precipitin analysis of the malic enzyme present in the liver and epididymal fat pad of rats and mice are antigenically similar.

Double diffusion on agar analysis failed to demonstrate any cross-reaction between anti-serum to rat liver malic enzyme and extracts of dog liver (30 units/ml), chicken liver (24700 units/ml), chicken adipose tissue (20 units/ml), rabbit liver (50 units/ml), rabbit adipose tissue (10 units/ml), pig liver (80 units/ml), pig adipose tissue (280 units/ml), cow liver (10 units/ml), cow adipose tissue (20 units/ml), lamb liver (10 units/ml) and lamb adipose tissue (10 units/ml). This could be due to the low malic enzyme concentration in the tissue extracts. The lack of interaction between the anti-serum and the chicken liver malic enzyme could be due to low antibody specificity for that enzyme.

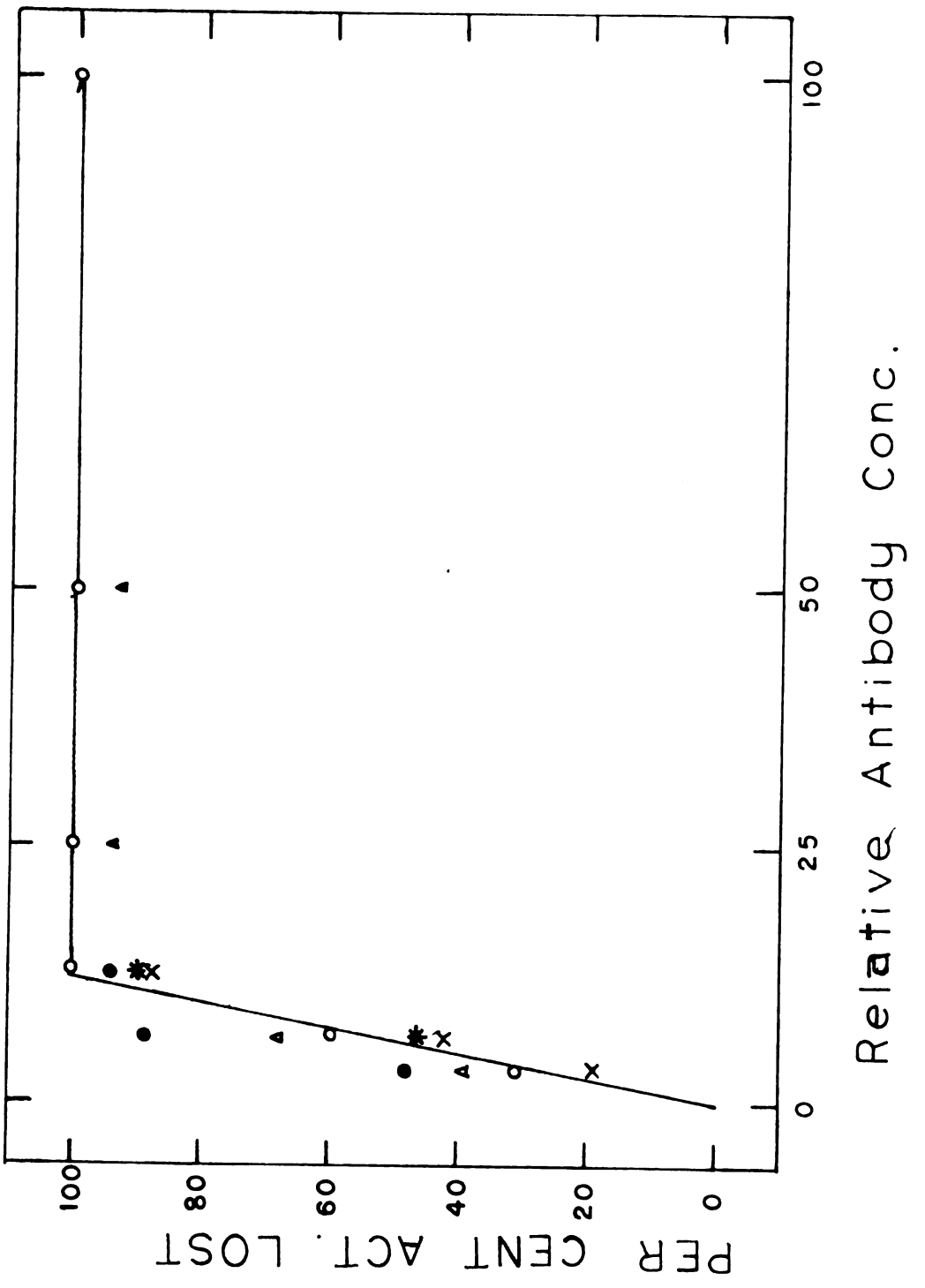
However, the quantitative precipitin test of anti-rat liver malic enzyme with the above extracts (125 or 250 units/reaction)

Figure 7

Precipitation of the Malic Enzyme from Extracts of Liver and Epididymal Fat Pad from Rats, Obese Mice and Lean Mice with Anti-Serum to Rat Liver Malic Enzyme

Precipitation of the malic enzyme from extracts of rat liver (*) and epididymal fat pad (X), liver (o) and epididymal fat pad (●) of obese mice and liver (Δ) and epididymal fat pad (▲) of lean mice with anti-serum to rat liver malic enzyme. Six doubling dilutions of anti-serum were prepared in 50 mM Tris, 5 mM EDTA, 100 mM KCl, 2 mM β-mercaptoethanol (pH 7.4 at 5°C). To each 0.5 ml of diluted antibody, 250 units of malic enzyme activity from tissue extract was added. After incubation at 30°C for 30 minutes and 2°C for 16 hours, the precipitates were removed by centrifugation at 50,000 x g for 30 minutes and malic enzyme was assayed in each supernatant.

Figure 7


PER CENT ACT. LOST
100
80
60
40
20
0
0
25
50
100
Relative Antibody Conc.

showed a variable degree of cross-reactivity between anti-serum and the malic enzymes of the species studied. The extent of cross-reaction was about the same for dog liver and pig liver and adipose tissue malic enzyme but much lower for chicken liver and adipose tissue malic enzyme (Figure 8). The extent of cross-reactivity for the liver and adipose tissue malic enzyme of rabbit, cow and lamb was very low, virtually non-existant. The low enzyme content of the tissue extracts of the last three species and the instability of the enzymes during incubation (as estimated with the blank reaction) made estimation of cross-reactivity with anti-rat liver malic enzyme difficult.

C. Label Incorporation into Rat Tissue Proteins and Malic Enzyme

Meal-Feeding Experiment I:

Incorporation of L-Arginine-guanidino-^{14}C into Malic Enzyme and Proteins of Meal-Fed and Nibbling Rats

While subjected to the dietary regimen for four weeks, the pair-fed meal-eaters and nibblers gained weight at the same rate (Figure 9). Daily food consumption of the meal-eaters (Figure 10) was low initially but rose steadily and stabilized by the end of the second week.

The specific activities of malic enzyme (Table 2), and glucose-6-phosphate dehydrogenase and the lipogenic capacity of the epididymal fat pads of the meal-fed rats were significantly greater ($P \leq 0.05$) than those of the nibbling rats (Table 3). There was no difference between the hepatic enzyme activities and lipogenic capacities and the adipose acetyl CoA carboxylase activities of the meal-fed and nibbling rats.

Incorporation of L-arginine-guanidino-^{14}C into tissue proteins

Figure 8

Precipitation of Rat Liver Malic Enzyme and the Malic Enzyme from Liver and Adipose Tissue Extracts of Several Species with Anti-Serum to Rat Liver Malic Enzyme

Precipitation of the malic enzyme of rat liver (o) and epididymal fat pad (●), dog liver (x), pig liver (*), pig adipose tissue (▼), chicken liver (Δ) and chicken adipose tissue (▲) extracts with anti-serum to rat liver malic enzyme. Six doubling dilutions of anti-serum were prepared in 50 mM Tris, 5 mM EDTA, 100 mM KCl, 2mM β-mercaptoethanol (pH 7.4 at 5°C). To each 0.5 ml of diluted antibody, 250 units of malic enzyme activity from tissue extract was added. After incubation at 30°C for 30 minutes and 2°C for 16 hours, the precipitates were removed by centrifugation at 50,000 x g for 30 minutes and malic enzyme was assayed in each supernatant.

Figure 8

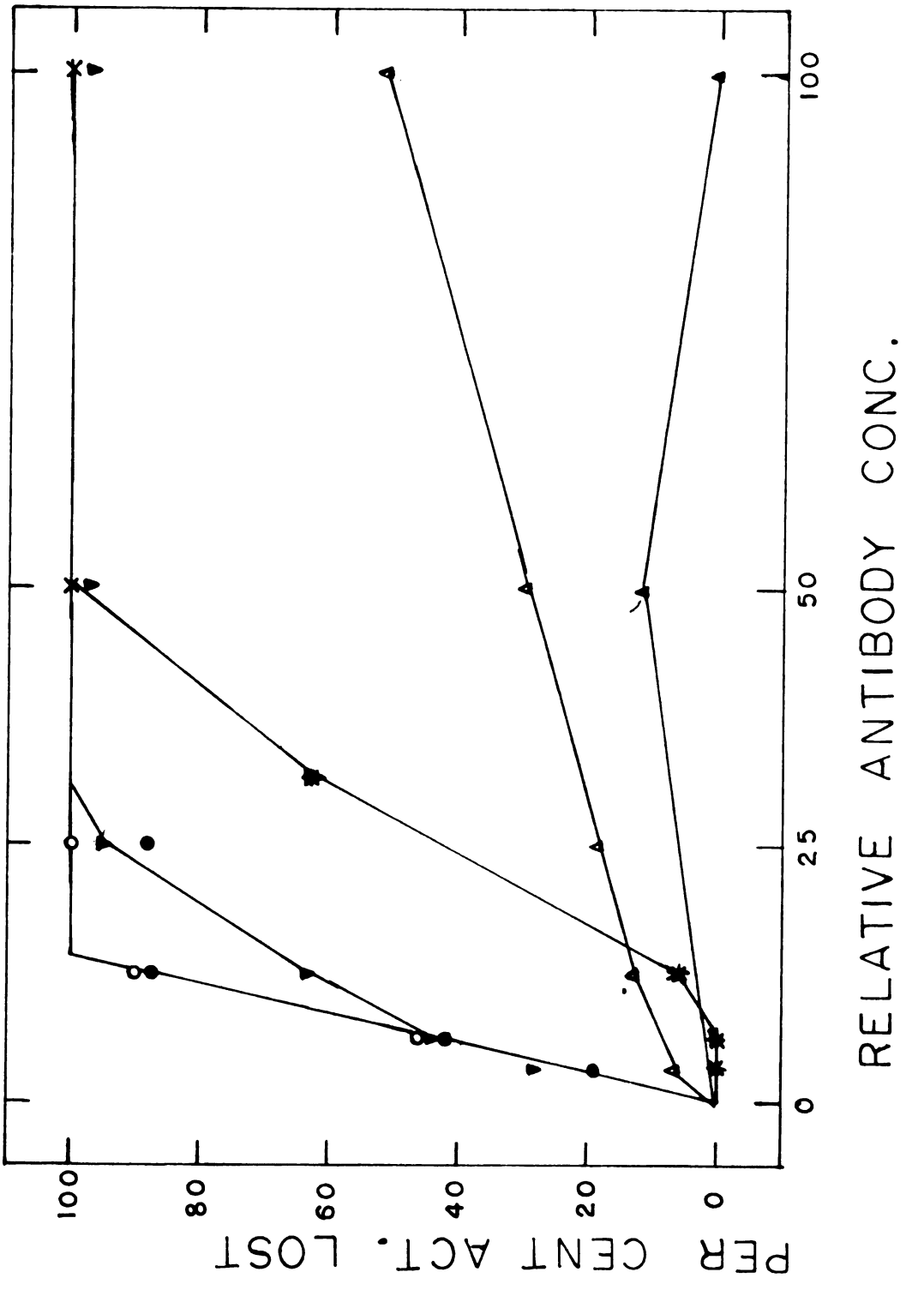

PER CENT ACT. LOST
0
20
40
60
80
100
0
25
50
100
RELATIVE ANTIBODY CONC.

Figure 9

Body Weight of Meal-eating and Nibbling Rats

Body weight of meal-eating (o) and nibbling (x) rats. Each point represents the mean for 18 meal-fed or nibbling rats.

Figure 9

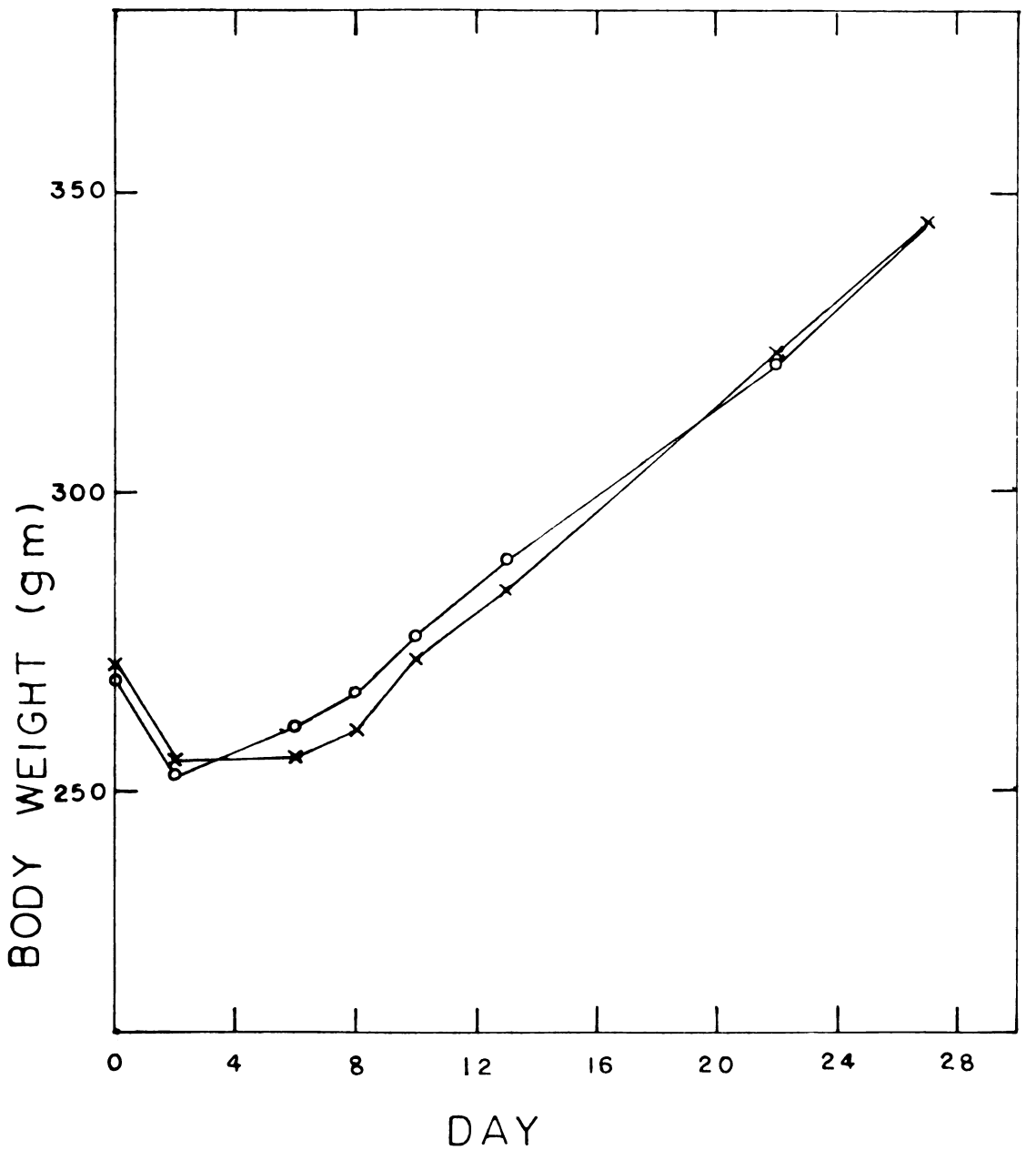


Figure 10

Food Consumption of Meal-eating Rats

Food consumption of meal-eating rats. Each point represents the mean for 18 meal-fed rats.

Figure 10

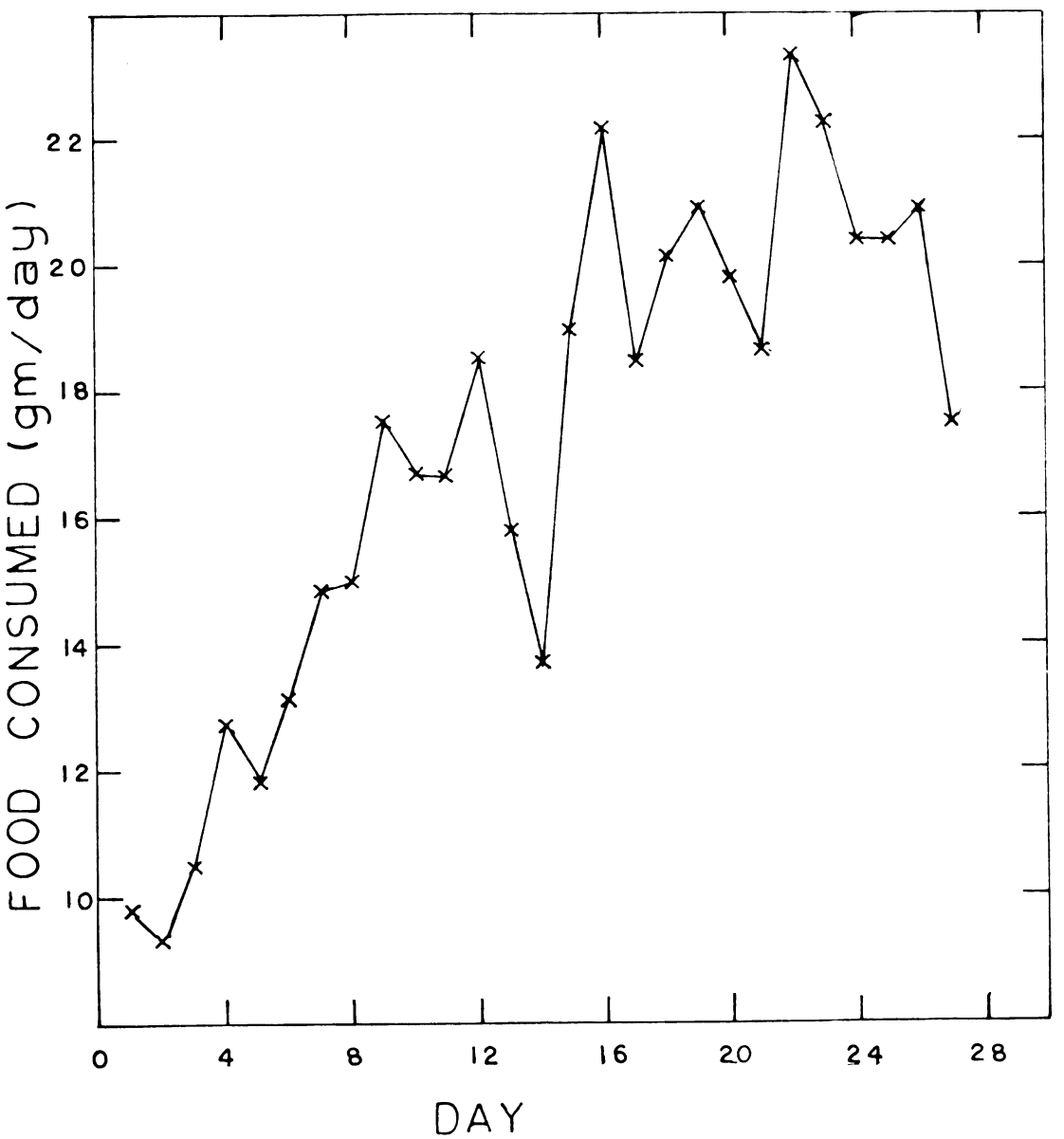


Table 2

Hepatic and Epididymal Adipose Tissue Malic Enzyme Activities of Meal-Fed and Nibbling Rats.[1,2]

Treatment	Liver	Epididymal Fat Pad
Meal-Eaters	16 ± 4	244 ± 60*
Nibblers	15 ± 5	160 ± 74*

1. Enzyme activities are expressed as nanomoles of substrate utilized per minute per milligram of soluble protein at 25°C.

2. Mean ± SD for 12 rats.

* Asterisk indicates that the difference between meal-fed and nibbling values is significant ($P \leq 0.05$).

Table 3

Hepatic and Epididymal Adipose Tissue Enzyme Activities and Lipogenic Capacities of Meal-Fed and Nibbling Rats

		Liver		Epididymal Fat Pad	
		Meal-Eaters	Nibblers	Meal-Eaters	Nibblers
Malic Enzyme[1,3]	A	12.3 ± 4	12.0 ± 3	206 ± 41	143 ± 34
	B	6.8 ± 1	8.6 ± 4	123 ± 3	88 ± 38
G-6-P DH	A	36.2 ± 14	21.6 ± 7	151 ± 31*	93 ± 16*
	B	29.6 ± 1	36.6 ± 19	155 ± 12*	59 ± 50*
Acetyl CoA Carboxylase[1,3]	A	23 ± 7	21 ± 0.4	28 ± 9	25 ± 4
	B	16 ± 5	20 ± 0.8	44 ± 0.4	25 ± 8
Fatty Acid Synthesis[2,3]	A	678 ± 165	503 ± 153	19098 ± 2423*	9652 ± 738*
	B	938 ± 149	3016 ± 742	20521 ± 3769*	13996 ± 8273*

1. Enzyme activities are expressed as nanomoles of substrate utilized per minute per milligram of soluble protein at 25°C.

2. Fatty acid synthesis is expressed as nanomoles of D-glucose-U-^{14}C converted to fatty acids per g wet tissue per 2 hours.

3. Mean ± SD for 3 rats.

* Asterisk indicates that the difference between meal-fed and nibbling values is significant ($P \leq 0.05$).

was usually maximal within one hour following IP injection and followed an exponential decay curve over the subsequent 72 hours. The half-lives ($T\frac{1}{2}$) of the liver and epididymal fat pad total and soluble protein were determined from a plot of dpm/mg of protein (ordinate) against hours following injection (abscissa) (Figures 11 and 12). A comparison of the $T\frac{1}{2}$'s for protein of meal-fed and nibbling rats is shown on Table 4. These values can only be considered as estimates because of the poor replication and animal variability. Hence, rate constants for synthesis and degradation were not calculated. Label present in the protein precursor pool was maximal within one hour following IP injection and rapidly decreased to minimal levels within 24 hours after injection.

L-arginine-guanidino-^{14}C incorporation into liver and epididymal fat pad malic enzyme appeared maximal within one hour following IP injection. However, plotting a curve of the loss of label from malic enzyme with time proved impossible due to a high degree of variability in the dpm/mg protein precipitated after correction for non-specific precipitation of label. A plot of dpm/mg protein precipitated (ordinate) against hours following injection (abscissa) shows the poor correlation to an exponential curve obtained for adipose tissue (Figure 13). Estimated half-life of the malic enzyme from meal-fed rat epididymal fat pad was 32 hours. The estimated half-life of the malic enzyme of nibbling rat epididymal fat pad was 30 hours. The poor results are due to poor reproducibility (due to insufficient replication), animal variability, low label incorporation into the enzyme and very high counts present in the non-specific precipitation of label. In the liver, malic enzyme represents a very small proportion of the total

Figure 11

Turnover of Total and Soluble Protein Fractions of Livers from Meal-fed and Nibbling Rats

Turnover of total (x) and soluble (o) protein fractions from livers of meal-fed (A) and nibbling (B) rats. The rats were injected with L-arginine-guanidino-^{14}C and killed after the specified intervals.

Figure 11

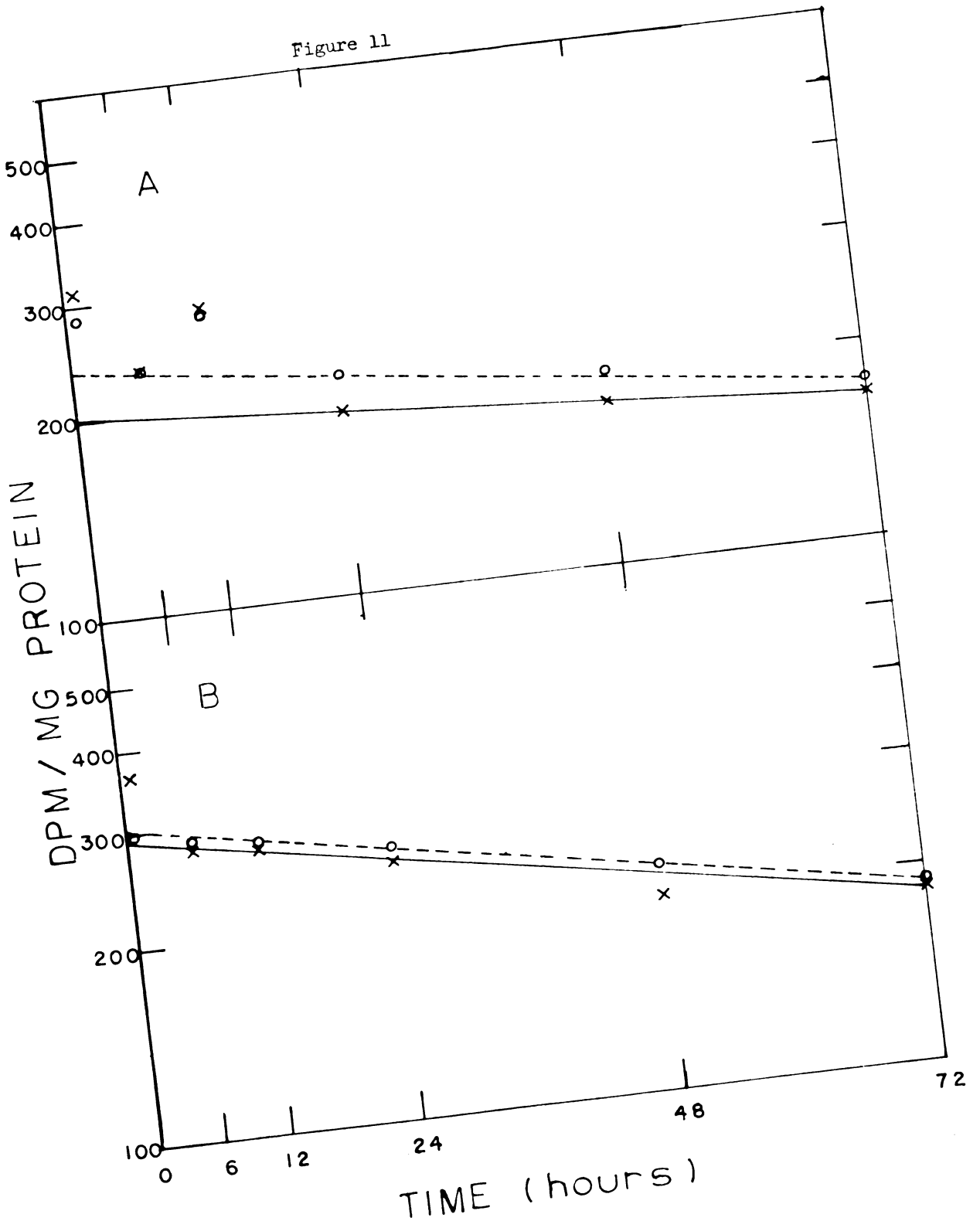


Figure 12

Turnover of Total and Soluble Protein Fractions of Epididymal Fat Pads from Meal-fed and Nibbling Rats

Turnover of total (x) and soluble (o) protein fractions of epididymal fat pads from meal-fed (A) and nibbling (B) rats. The rats were injected with L-arginine-guanidino-^{14}C and killed after the specified intervals.

Figure 12

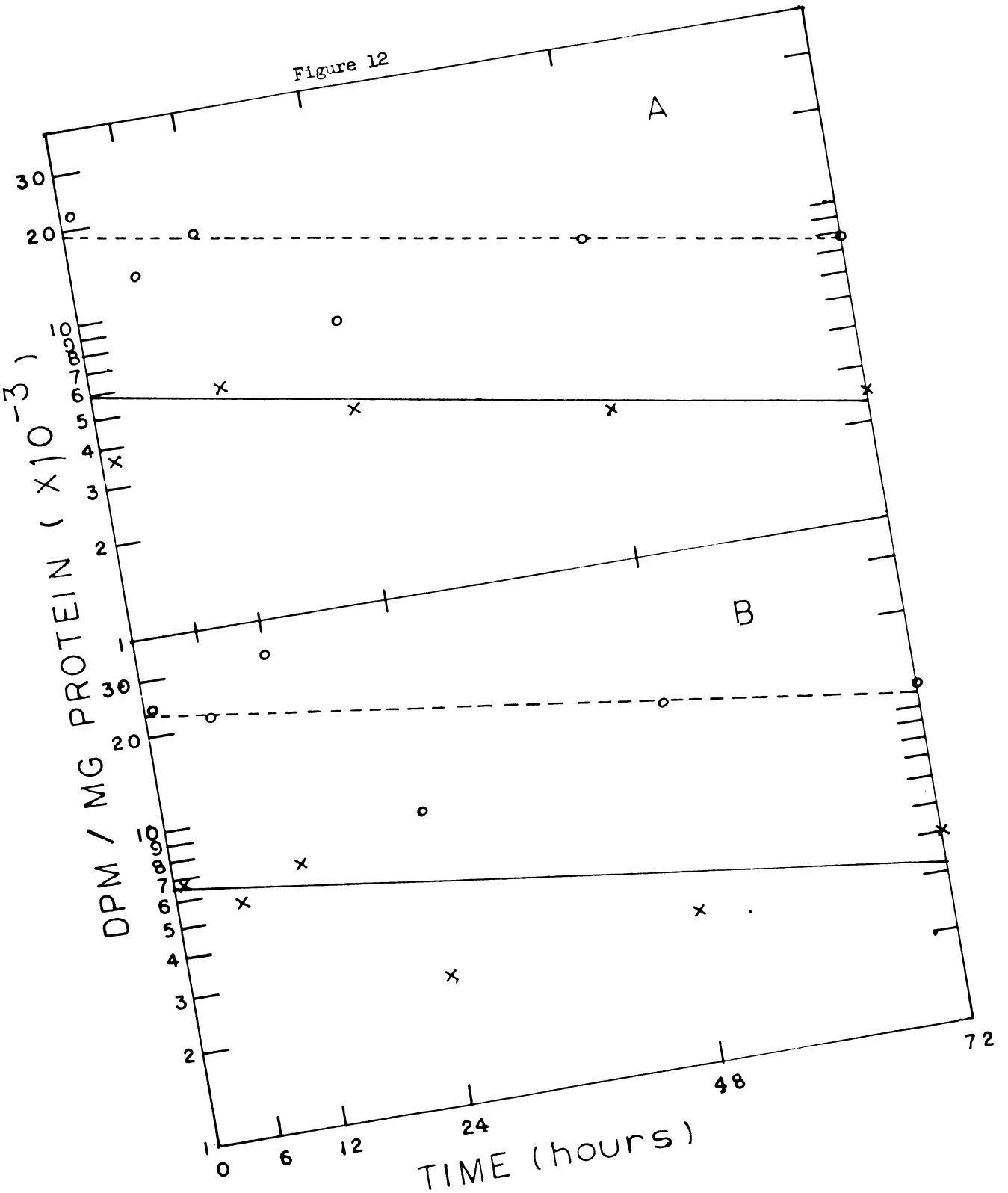

A
B
30
20
10
9
8
7
6
5
4
3
2
1
DPM / MG PROTEIN (X10 -3)
0
6
12
24
48
72
TIME (hours)

Table 4

$T\frac{1}{2}$ of Total and Soluble Protein Fractions of the Livers and Epididymal Fat Pads of Meal-Fed and Nibbling Rats.

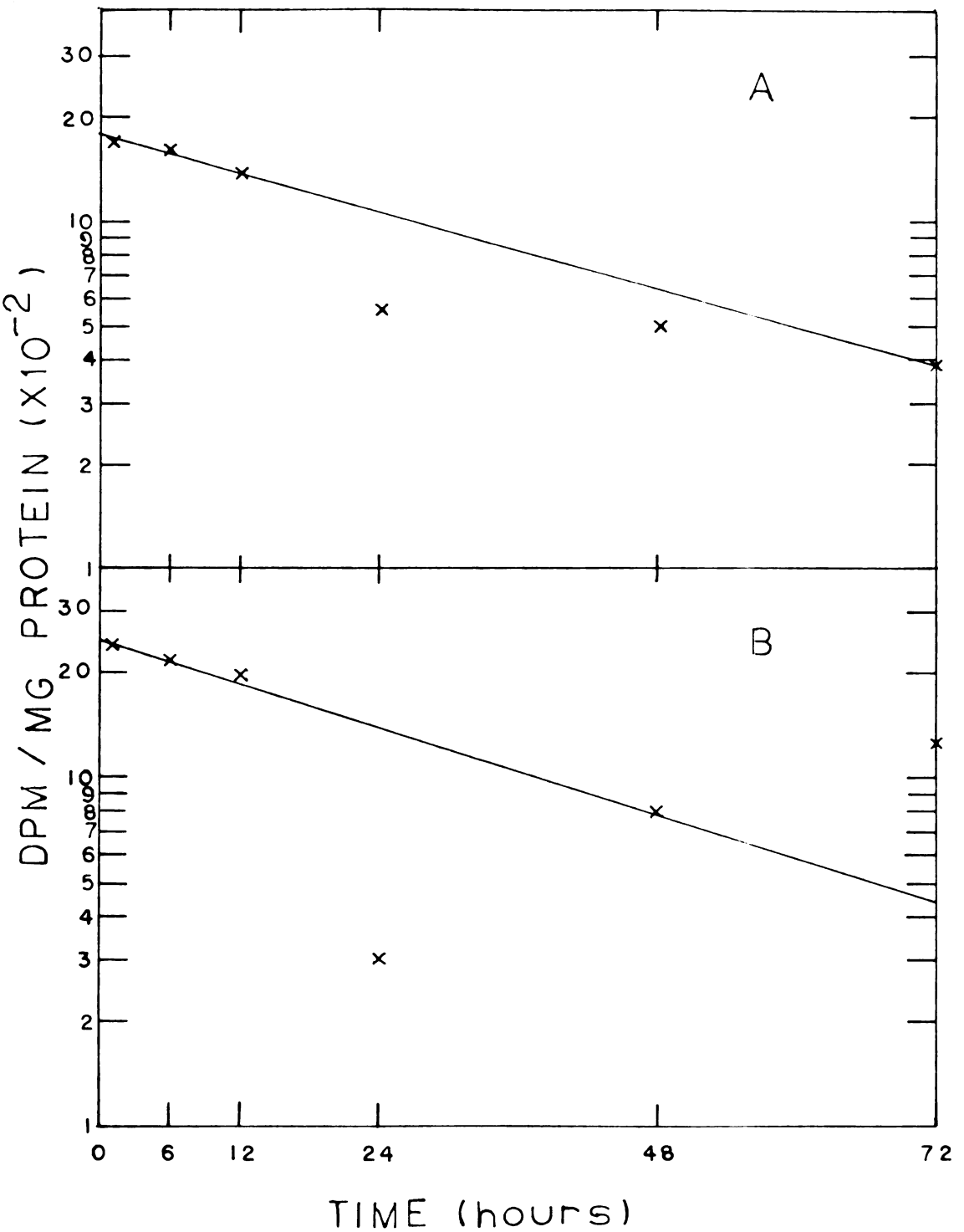
	$T\frac{1}{2}$ (hours)	
	Liver	Epididymal Fat Pads
A. Meal-Eaters		
i) Total Protein	231	55
ii) Soluble Protein (TCA)	131	45
iii) Soluble Protein (Tissue)	116	55
B. Nibblers		
i) Total Protein	107	74
ii) Soluble Protein (TCA)	104	62
iii) Soluble Protein (Tissue)	126	70

Figure 13

Turnover of Malic Enzyme Epididymal Fat Pads from Meal-fed and Nibbling Rats

Turnover of malic enzyme of epididymal fat pads from meal-fed (A) and nibbling (B) rats. The rats were injected with L-arginine-guanidino-^{14}C and killed after the specified intervals.

Figure 13


A
B
DPM / MG PROTEIN (X10^{-2})
30
20
10
9
8
7
6
5
4
3
2
1
0
6
12
24
48
72
TIME (hours)

protein fraction and label incorporation into this enzyme may be very low in proportion to total incorporation. Hence, accurate measurement of label incorporation into the enzyme may not be possible.

L-Arginine-guanidino-^{14}C: IV vs. IP Injection and Effect of Dose

A comparison of the effects of IV and IP administration of 25 μ Ci/animal of L-arginine-guanidino-^{14}C was made (Table 5). Little difference was observed in the level of label incorporation into liver total and soluble proteins twenty-four hours after injection. Label in the protein precursor pool (TCA-soluble fraction) was higher following IP injection than after IV injection. The incorporation of label into epididymal fat pad total and soluble proteins was higher following IP injection. However, label in the protein precursor pool was similar for IV and IP injection. The administration of a greater amount of L-arginine-guanidino-^{14}C (35 μ Ci/animal) increased label incorporation into the total and soluble protein fractions and the protein precursor pool of both liver and epididymal fat pads.

A 2-fold greater incorporation of label into liver malic enzyme was obtained with IV injection than with IP injection (Table 6). However, IP injection gave a 10-fold greater incorporation into epididymal fat pad malic enzyme than IV injection. In all cases, a very large proportion of the counts were present as non-specifically precipitable label and the differences in the effects of IV and IP injections were rendered meaningless. The higher dose (35 μ Ci/animal) actually yielded a lower gross and net label incorporation into liver and epididymal fat pad malic enzyme.

The net incorporation into liver malic enzyme was very small

Table 5

Incorporation of L-Arginine-guanidino-^{14}C into Tissue Protein and Protein Precursor Pools: Effect of Route of Administration and Dose

	Total Proteins (dpm/mg protein)	Soluble Proteins (dpm/mg protein)	Amino Acids (dpm/ml homogenate)
Liver			
i) IV (25 μ Ci/animal)	110 ± 4	86 ± 3	686 ± 32
ii) IP (25 μ Ci/animal)	100 ± 11	90 ± 0.3	1095 ± 29
iii) IP (35 μ Ci/animal)	369 ± 6	336 ± 2	1529 ± 22
Epididymal Fat Pad			
i) IV (25 μ Ci/animal)	330 ± 9	411 ± 30	152 ± 8
ii) IP (25 μ Ci/animal)	1587 ± 32	1627 ± 446	199 ± 10
iii) IP (35 μ Ci/animal)	3682 ± 73	3666 ± 96	191 ± 7

Table 6

Incorporation of L-Arginine-guanidino-^{14}C in Malic Enzyme: Effect of Route of Administration, of Dose and of Using Untreated[1] or $(NH_4)_2SO_4$ Precipitated Treated Serums[2]

	ME-Ab Rx (dpm/unit enzyme)	Blank Rx (dpm/unit enzyme)	Δ (dpm/unit enzyme)
Liver			
a) IV Untreated Serum (25 μ Ci/animal)	682	338	344
b) IP Untreated Serum (25 μ Ci/animal)	814	685	129
c) IP Untreated Serum* (35 μ Ci/animal)	99 ± 2	89 ± 0	10
d) IP Treated Serum* (35 μ Ci/animal)	70 ± 1	65 ± 1	6
Epididymal Fat Pad			
a) IV Untreated Serum (25 μ Ci/animal)	255	217	38
b) IP Untreated Serum (25 μ Ci/animal)	3864	3465	399
C) IP Treated Serum* (35 μ Ci/animal)	318 ± 6	139 ± 13	179

1. The term "untreated serum" indicates serum obtained from coagulated rabbit blood.

2. The term "treated serum" indicates the protein fraction of rabbit serum that was precipitated by adding solid $(NH_4)_2SO_4$ to 50% saturation at 0°C, collected by centrifugation and dialyzed against saline.

* Asterisk indicates use of an initial blank reaction.

as the non-specific precipitation was still proportionately large. The use of $(NH_4)_2SO_4$- treated serum was inconsequential in reducing non-specific precipitation. The non-specific label precipitation was much smaller for the epididymal fat pad extract-anti-serum reaction. As a consequence, labelled malic enzyme represents a larger proportion of the total precipitated label than in previous studies. This is partly due to the fact that a large part of the non-specifically precipitated label was removed using an initial blank reaction prior to malic enzyme reaction with anti-serum. Also the length of the malic enzyme-anti-serum reaction was restricted to 30 minutes; thereby preventing non-specific label precipitation due to protein denaturation that may be occurring during the overnight incubation. The feasibility of using a 30 minute reaction is shown in Figure 14 which compares the extent of cross-reaction of rat liver extract (1940 units/ml) reacted with several serial dilutions of anti-rat liver malic enzyme for 30 minutes or overnight. Precipitation of enzyme was similar for both systems. Both the above modifications were incorporated in all subsequent immunological studies.

L-Arginine-guanidino-^{14}C and L-Lysine-U-^{14}C:
Administration of Multiple Doses

L-arginine-guanidino-^{14}C or L-lysine-U-^{14}C was administered in 3 equal doses injected IP at hourly intervals. This was an attempt to increase the specific activity of label incorporated into tissue proteins and malic enzyme. Label incorporation into liver proteins was more than 10-fold greater for L-lysine-U-^{14}C (25 μ Ci/animal) than for L-arginine-guanidino-^{14}C (35 μ Ci/animal) yet was similar for the epididymal fat pad proteins (Table 7). This could be a reflection of

Figure 14

Effect of Time on Precipitation of Malic Enzyme from Rat Liver and Epididymal Fat Pad Extracts with Anti-Serum to Rat Liver Malic Enzyme

Reaction times: rat liver, 30 minutes (o) and overnight (●) and rat epididymal fat pad, overnight (x). Six doubling dilutions of anti-serum were prepared in 50 mM Tris, 5 mM EDTA, 100 mM KCl, 2 mM β-mercaptoethanol (pH 7.4 at 5°C). To each 0.5 ml of diluted antibody, 250 units of malic enzyme activity from tissue extract was added. The overnight samples were incubated for 30 minutes at 30°C and 2°C for 16 hours while the 30 minutes reactions were incubated for 30 minutes at 30°C. The precipitates were removed by centrifugation at 50,000 x g for 30 minutes and malic enzyme was assayed in each supernatant.

Figure 14

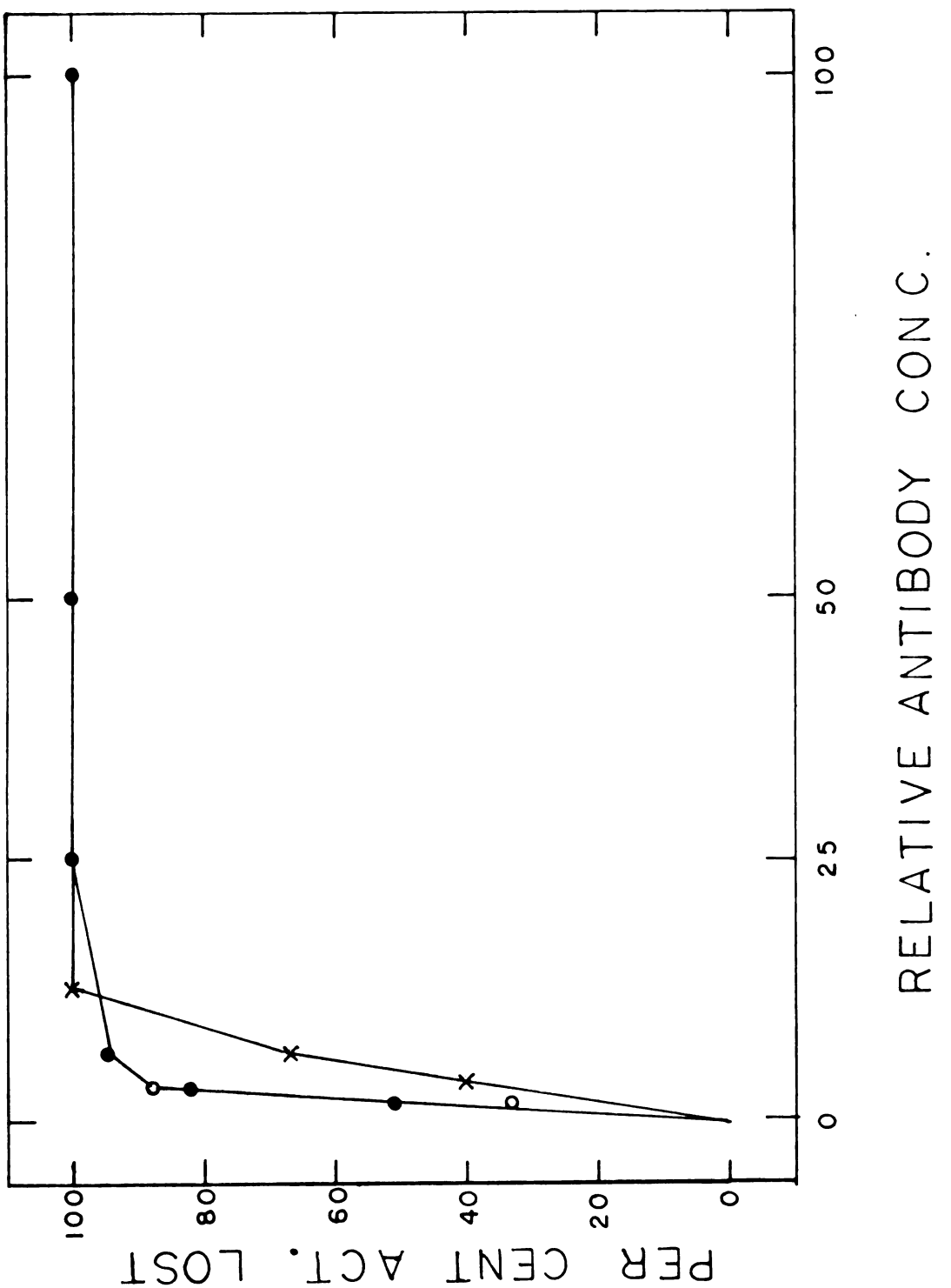

PER CENT ACT. LOST
100
80
60
40
20
0
0
25
50
100
RELATIVE ANTIBODY CONC.

Table 7

L-Arginine-guanidino-^{14}C or L-Lysine-U-^{14}C Incorporation into Tissue Proteins and Protein Precursor Pools

	Total Proteins (dpm/mg protein)	Soluble Proteins (dpm/mg protein)	Amino Acids (dpm/ml homogenate)
A) Liver			
a) L-arginine-guanidino-^{14}C (35 μ Ci/animal)	3	5	2481 ± 14
b) L-lysine-U-^{14}C (25 μ Ci/animal)	1238 ± 38	1320 ± 36	2161 ± 475
B) Fat Pad			
a) L-arginine-guanidino-^{14}C (35 μ Ci/animal)	3074 ± 78	4739 ± 263	843 ± 132
b) L-lysine-U-^{14}C (25 μ Ci/animal)	3489 ± 30	2527 ± 68	468 ± 89

the tissue sites of catabolism of the amino acids; that is, arginine undergoes catabolism in the liver while lysine is metabolized in the extra-hepatic tissues. Multiple doses did not increase, when compared to other experiments, the incorporation of L-arginine-guanidino-^{14}C into liver or epididymal fat pad malic enzyme (Table 8). Washing the malic enzyme-anti-body pellet with 1 M NaCl, instead of saline, had no effect on decreasing the non-specific precipitation of label. L-lysine-U-^{14}C resulted in a greater net incorporation of label into liver malic enzyme than L-arginine-guanidino-^{14}C. Net incorporation of label into the epididymal fat pad enzyme was similar for both amino acids. The questionable effectiveness of multiple doses, coupled with the inherent difficulties of administering them, caused their discontinuance in subsequent experiments.

L-Arginine-guanidino-^{14}C, L-Lysine-U-^{14}C and L-Leucine-U-^{14}C Incorporation

Table 9 shows a comparison of label incorporation from single IP doses of L-arginine-guanidino-^{14}C (25 μ Ci/animal), L-lysine-U-^{14}C (25 μ Ci/animal) and L-leucine-U-^{14}C (75 μ Ci/kg body weight) into the tissue protein and protein precursor pools of meal-fed and nibbling rats twenty-four hours after injection. No differences were observed in the levels of incorporation of any amino acid into any protein fraction or precursor pools between the meal-fed and nibbling rats. Of the three amino acids injected, L-leucine-U-^{14}C gave the highest level of incorporation into the total and soluble proteins and protein precursor pools of the liver and epididymal fat pad malic enzyme. An apparent difference was observed between the meal-fed and nibbling rats in the levels of label incorporation into epididymal fat

Table 8

L-Arginine-guanidino-^{14}C or L-Lysine-U-^{14}C Incorporation into Malic Enzyme

	ME-Ab Rx (dpm/unit enzyme)	Blank Rx (dpm/unit enzyme)	Δ (dpm/unit enzyme)
Liver			
a) L-arginine-guanidino-^{14}C (Saline Wash)[1] (35 μ Ci/animal)	15 ± 2	12 ± 1	3
b) L-arginine-guanidino-^{14}C (High Salt Wash)[2] (35 μ Ci/animal)	11 ± 1	9 ± 1	2
c) L-lysine-U-^{14}C (25 μ Ci/animal)	84 ± 1	22 ± 2	62
Fat Pad			
a) L-arginine-guanidino-^{14}C (Saline Wash) (35 μ Ci/animal)	253 ± 18	35 ± 7	218
b) L-arginine-guanidino-^{14}C (High Salt Wash) (35 μ Ci/animal)	227 ± 3	17 ± 3	210
c) L-lysine-U-^{14}C (25 μ Ci/animal)	323 ± 4	57	266

1. The term "Saline Wash" indicates that saline was used in the washing of the enzyme-antibody precipitate.

2. The term "High Salt Wash" indicates that 1 M NaCl was used in the washing of the enzyme-antibody precipitate.

Table 9

L-Arginine-guanidino-^{14}C, L-Lysine-U-^{14}C or L-Leucine-U-^{14}C

Incorporation into Tissue Protein and Protein Precursor Pools

	Total Proteins (dpm/ml protein)	Soluble Proteins (dpm/ml protein)	Amino Acids (dpm/ml homogenate)
Liver			
a) L-arginine-guanidino-^{14}C			
Meal-Eater	193 ± 5	164 ± 7	706 ± 70
Nibbler	211 ± 10	179 ± 8	729 ± 63
b) L-lysine-U-^{14}C			
Meal-Eater	1178 ± 36	1019 ± 21	1575 ± 72
Nibbler	1180 ± 29	1024 ± 45	2031 ± 53
c) L-leucine-U-^{14}C			
Meal-Eater	1550 ± 45	1441 ± 30	1249 ± 32
Nibbler	1784 ± 55	1555 ± 37	1382 ± 76
Epididymal Fat Pad			
a) L-arginine-guanidino-^{14}C			
Meal-Eater	2020 ± 27	1741 ± 66	112 ± 12
Nibbler	1852 ± 94	955 ± 84	100 ± 23
b) L-lysine-U-^{14}C			
Meal-Eater	1153 ± 61	779 ± 82	148 ± 33
Nibbler	1301 ± 40	785 ± 62	233 ± 29
c) L-leucine-U-^{14}C			
Meal-Eater	8130 ± 112	7656 ± 259	258 ± 30
Nibbler	11574 ± 232	8398 ± 318	389 ± 169

pad malic enzyme (Table 10). However, the high non-specific precipitation of label in the fat pad extract from nibblers was a probable cause of the lower net incorporation than in the extract from meal-eaters. This casts doubt on the validity of the differences observed.

Meal-Feeding Experiment II:

Incorporation of L-Leucine-U-^{14}C into Malic Enzyme and Protein of Meal-Fed and Nibbling Rats

Throughout the 10 week experimental period, the pair-fed meal-eaters and nibblers gained weight at the same rate (Figure 15). Daily food consumption of the meal-eaters (Figure 16) was low initially but rose steadily and stabilized by the end of the second week.

As in the previous meal-feeding experiment, the specific activities of malic enzyme, acetyl CoA carboxylase and glucose-6-phosphate dehydrogenase and the lipogenic capacity of the epididymal fat pads of the meal-fed rats were significantly greater ($P \leq 0.05$) than those of the nibbling rats (Table 11 and 12). There was no difference between the meal-fed and nibbling rats in so far as the hepatic enzyme activities and lipogenic capacities were concerned.

Incorporation of L-leucine-U-^{14}C into tissue proteins was maximal within one hour following IP injection and followed an exponential decay curve over the subsequent 72 hours. The half-lives ($T\frac{1}{2}$) of the liver and epididymal fat pad total and soluble protein were determined from the slope of a plot of dpm/mg protein (ordinate) against hours following injection (abscissa) (Figures 17 to 20). The slope was calculated from linear regression analysis of the data and was used to calculate the rate constants for degradation and synthesis of tissue protein (Tables 13 and 14). Label present in

Table 10

L-Arginine-guanidino-^{14}C, L-Lysine-U-^{14}C or L-Leucine-U-^{14}C

Incorporation into Malic Enzyme

	ME-Ab Rx (dpm/unit enzyme)	Blank Rx (dpm/unit enzyme)	Δ (dpm/unit enzyme)
Liver			
a) L-arginine-guanidino-^{14}C			
Meal-Eater	20 ± 4	9 ± 1	11
Nibbler	142 ± 69	132 ± 122	10
b) L-lysine-U-^{14}C			
Meal-Eater	117 ± 3	95 ± 1	22
Nibbler	319 ± 24	247 ± 29	72
c) L-leucine-U-^{14}C			
Meal-Eater	207 ± 24	236 ± 13	--
Nibbler	337 ± 98	472 ± 1	--
Epididymal Fat Pad			
a) L-arginine-guanidino-^{14}C			
Meal-Eater	285 ± 8	61 ± 17	224
Nibbler	242 ± 11	119	123
b) L-lysine-U-^{14}C			
Meal-Eater	118 ± 38	29 ± 3	89
Nibbler	153	77	76
c) L-leucine-U-^{14}C			
Meal-Eater	684 ± 15	87 ± 6	597
Nibbler	994 ± 74	635 ± 2	359

Figure 15

Body Weight of Meal-eating and Nibbling Rats

Body weight of meal-eating (o) and nibbling (x) rats. Each point represents the mean for 24 meal-eating or nibbling rats.

Figure 15

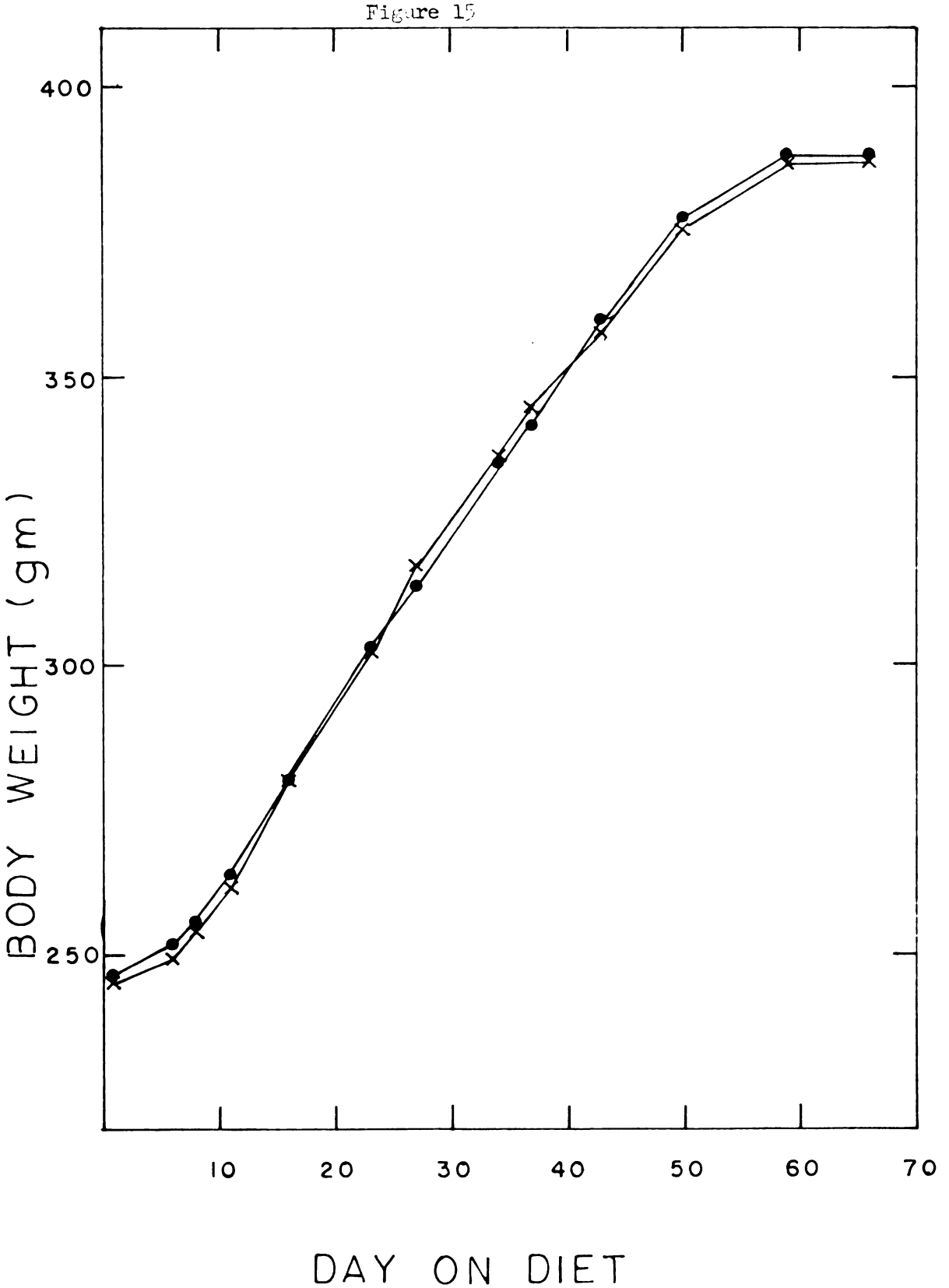


Figure 16

Food Consumption of Meal-eating Rats

Each point represents the mean for 24 meal-eating rats.

Figure 16

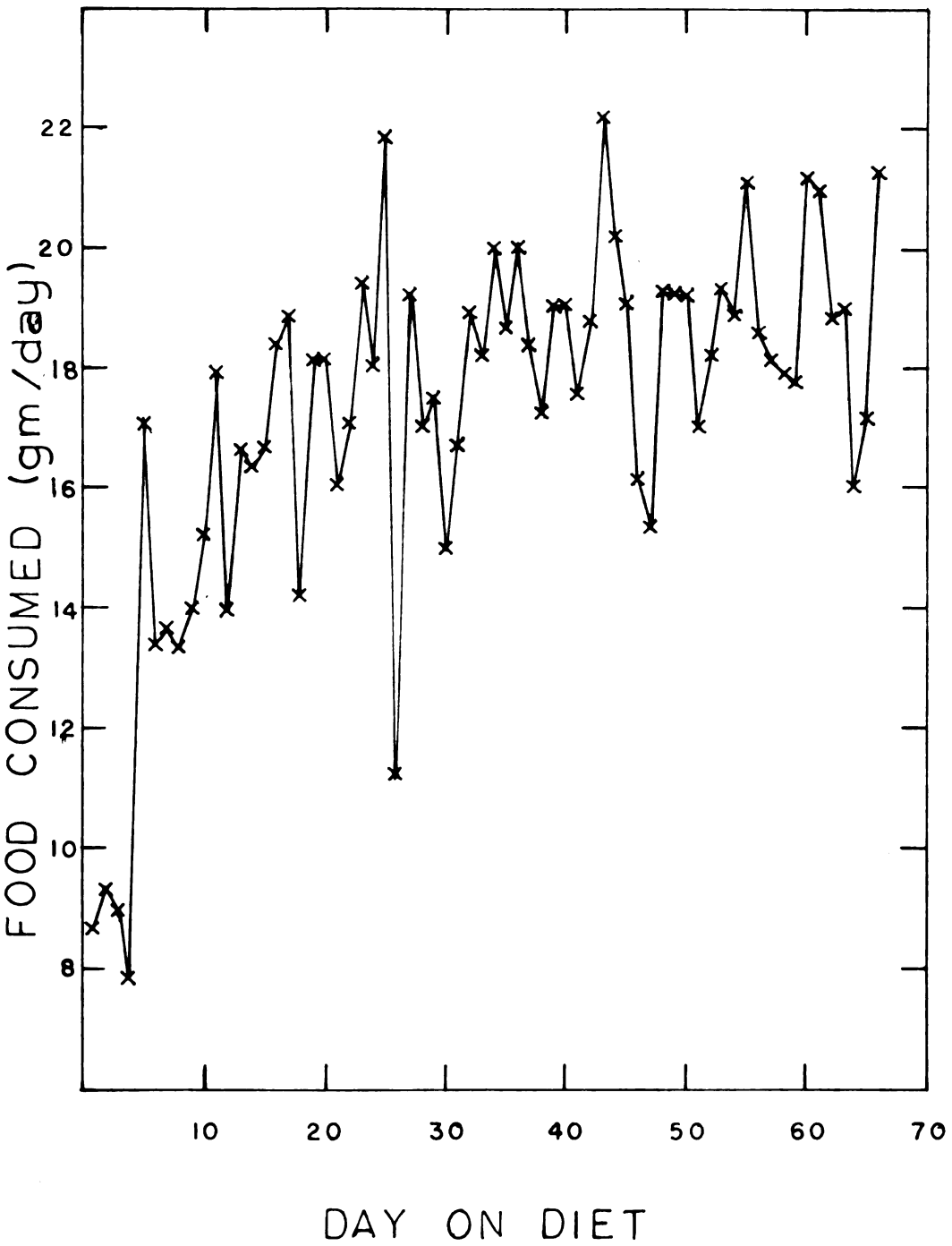

FOOD CONSUMED (gm/day)
8
10
12
14
16
18
20
22
10
20
30
40
50
60
70
DAY ON DIET

Table 11

Hepatic and Epididymal Fat Pad Malic Enzyme Activities of Meal-Fed and Nibbling Rats.[1,2]

	Liver		Epididymal Fat Pad	
	Meal-Eaters	Nibblers	Meal-Eaters	Nibblers
Group A	12.8 ± 2.8	10.0 ± 4.9	163.7 ± 6.2*	54.8 ± 3.13*
Group B	6.9 ± 2.1	6.4 ± 3.1	122.8 ± 27.9*	32.5 ± 14.3*

1. Enzyme activities are expressed as nanomoles of substrate utilized per minute per milligram of soluble protein at 25°C.

2. Mean ± SD for 8 rats.

* Asterisk indicates that the difference between meal-fed and nibbling values is significant ($P \leq 0.05$).

Table 12

Hepatic and Epididymal Fat Pad Enzyme Activities and Lipogenic Capacities of Meal-Fed and Nibbling Rats

	Liver		Epididymal Fat Pad	
	Meal-Eaters	Nibblers	Meal-Eaters	Nibblers
Malic Enzyme[1,3]	9.2 ± 1.5	7.1 ± 3.2	167.1 ± 45.2*	37.8 ± 11.2*
G-6-PD[1,3]	22.2 ± 6.2	13.0 ± 2.5	168.3 ± 30.7*	39.5 ± 10.2*
Acetyl CoA Carboxylase[1,3]	2.5 ± 1.3	1.2 ± 0.3	7.3 ± 1.2* (3)	1.3 ± 0.5* (3)
Fatty Acid Synthesis[2,3]	813 ± 374	969 ± 220	12283 ± 2900*	2814 ± 1817*

1. Enzyme activities are expressed as nanomoles of substrate utilized per minute per milligram of soluble protein at 25°C.

2. Fatty acid synthesis is expressed as nanomoles of glucose-U-^{14}C converted to fatty acids per g wet tissue per 2 hours.

3. Mean ± SD for 4 rats unless indicated otherwise in parentheses.

* Asterisk indicates that the difference between meal-fed and nibbling values is significant ($P \leq 0.05$)

Figure 17

Turnover of the Total Protein in Livers from Meal-fed and Nibbling Rats

Turnover of the total protein fraction in livers of meal-fed (A) and nibbling (B) rats. Two separate groups (o and x) of meal-fed and nibbling rats were injected with L-leucine-U-^{14}C and killed after the specified intervals.

Figure 17

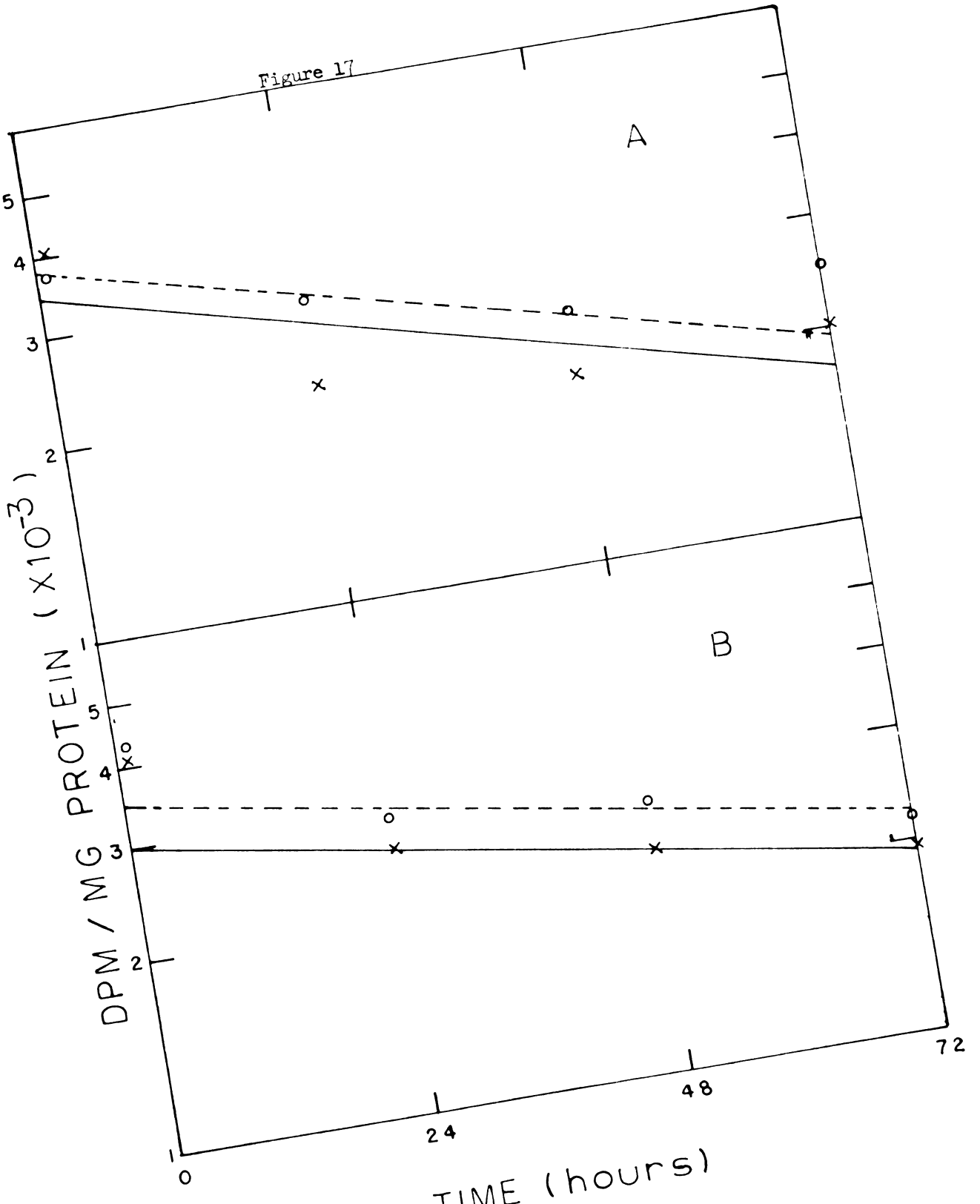


Figure 18

Turnover of the Total Protein in Epididymal Fat Pad from Meal-fed and Nibbling Rats

Turnover of the total protein fraction of the epididymal fat pads of meal-fed (A) and nibbling (B) rats. Two separate groups (o and x) of meal-fed and nibbling rats were injected with L-leucine-U-^{14}C and killed after the specified intervals.

Figure 18

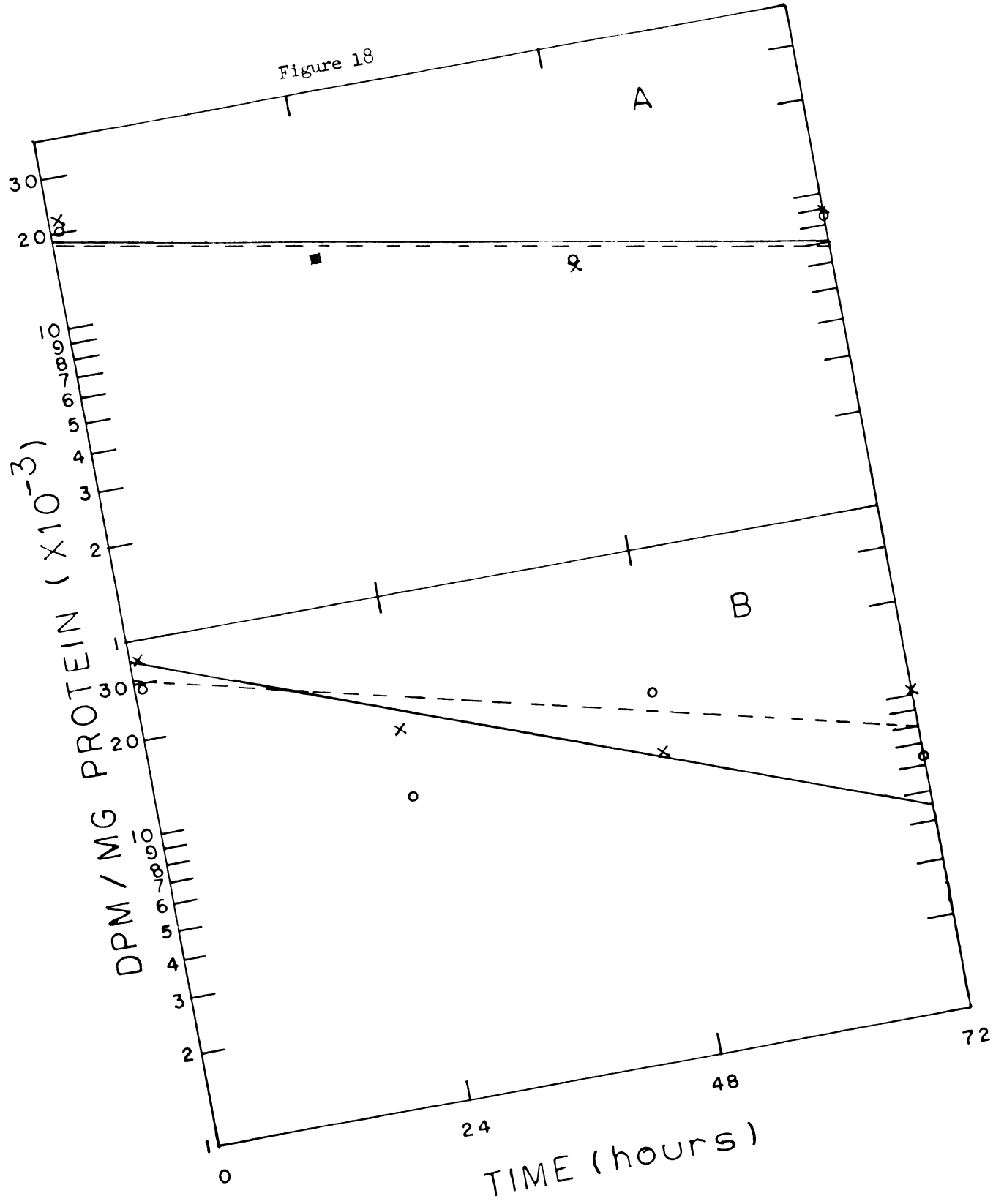


Figure 19

Turnover of the Soluble Protein Fraction in Livers from Meal-fed and Nibbling Rats

Turnover of the soluble protein fraction in livers of meal-fed (A) and nibbling (B) rats. Two separate groups (o and x) of meal-fed and nibbling rats were injected with L-leucine-U-^{14}C and killed after the specified intervals.

Figure 19

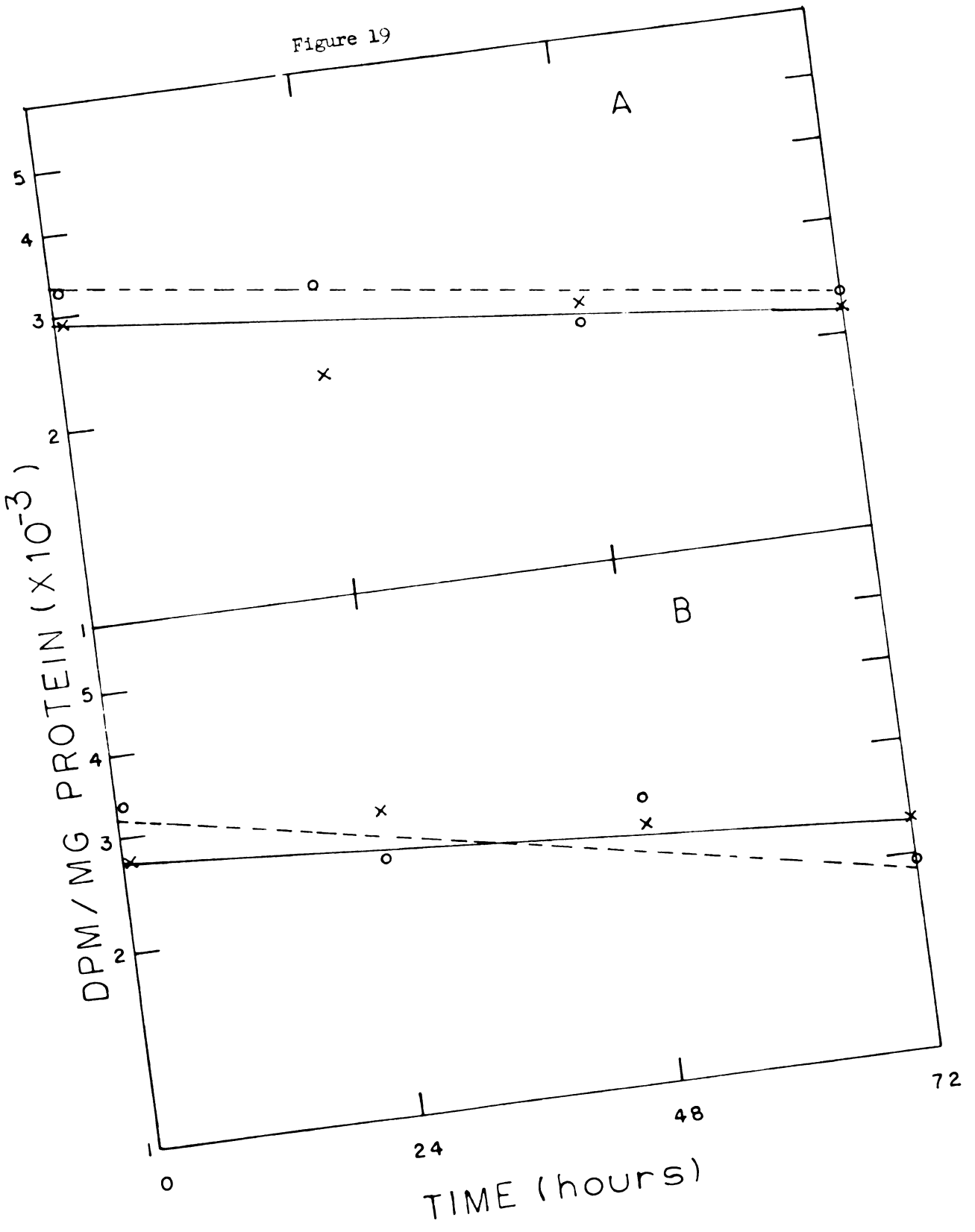


Figure 20

Turnover of the Soluble Protein Fraction in Epididymal Fat Pads from Meal-fed and Nibbling Rats

Turnover of the soluble protein fraction of the epididymal fat pads of meal-fed (A) and nibbling (B) rats. Two separate groups (o and x) of meal-fed and nibbling rats were injected with L-leucine-U-^{14}C and killed after the specified intervals.

Figure 20

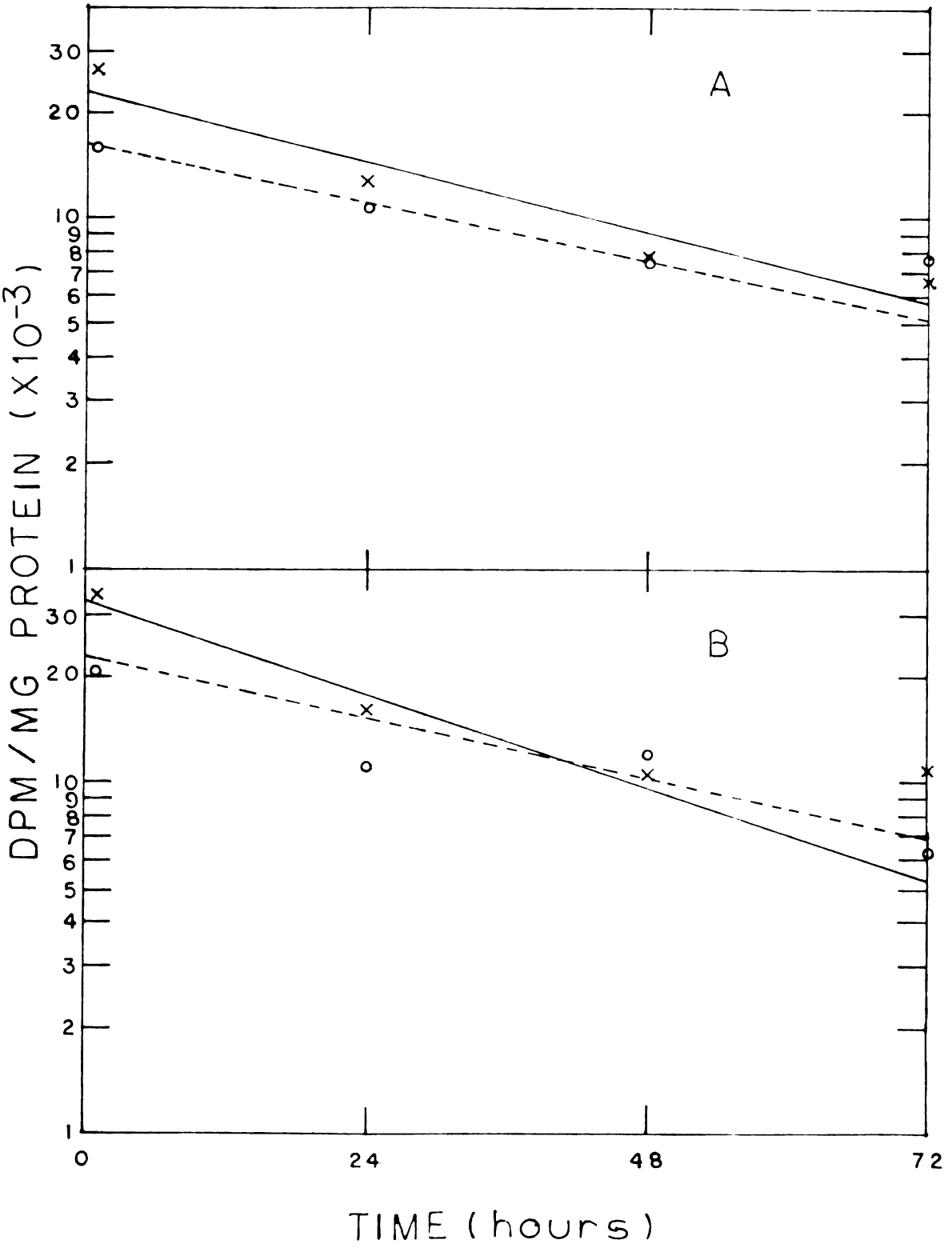


Table 13

Rates of Degradation and Synthesis: L-Leucine-U-^{14}C Labelled Liver Total and Soluble Proteins

		Protein Conc. mg/ml	r^1	m^2	$b^3_{y.x}$	$T_{\frac{1}{2}}^4$	K^5_D (est.)	K^6_S (est.)
Meal Eaters								
Total Protein	A	14.87	-0.8304	-0.0040	3435	75.31	0.0092	0.1368
	B	12.40	-0.9986	-0.0038	3749	78.75	0.0088	0.1085
Soluble Protein	A	6.48	-0.9988	-0.0016	2931	187.30	0.0037	0.0239
	B	6.48	-0.9980	-0.0021	3321	144.38	0.0048	0.0314
Nibblers								
Total Protein	A	13.33	-0.9991	-0.0025	3002	119.48	0.0058	0.0768
	B	13.41	-0.9823	-0.0027	3477	111.77	0.0062	0.0834
Soluble Protein	A	6.76	-0.9833	-0.0011	2770	277.20	0.0025	0.0171
	B	6.94	-0.9676	-0.0030	3206	100.44	0.0069	0.0480

1. coefficient of correlation

2. slope

3. intercept (dpm/mg protein·0 hour)

4. half-life: calculated by formula: $T_{\frac{1}{2}} = 0.693/K$

5. first-order rate constant for degradation: calculated by formula: $K_D = -2.303$ (slope) (time^{-1})

6. zero-order rate constant of synthesis: calculated by formula: $K_S = K_D$ P (mass time^{-1})

Table 14

Rates of Degradation and Synthesis: L-Leucine-U-^{14}C Labelled Epididymal Fat Pad Total and Soluble Proteins

		Protein Conc. mg/ml	r^1	m^2	b^3 y.x	$T_{\frac{1}{2}}^4$	K_D^5	K_S^6
Meal Eaters								
Total Protein	A	0.30	-0.9015	-0.0059	19,167	50.96	0.0136	0.0041
	B	0.88	-0.9876	-0.0079	20,159	38.08	0.0182	0.0160
Soluble Protein	A	0.30	-0.9572	-0.0083	23,333	36.28	0.0191	0.0057
	B	0.52	-0.9996	-0.0068	16,127	44.14	0.0157	0.0081
Nibblers								
Total Protein	A	0.26	-0.9889	-0.0123	34,417	24.49	0.0283	0.0074
	B	0.51	-0.9862	-0.0081	30,727	37.06	0.0187	0.0095
Soluble Protein	A	0.18	-0.9835	-0.0109	32,689	27.61	0.0251	0.0045
	B	0.36	-0.9740	-0.0069	22,468	43.59	0.0159	0.0057

1. coefficient of correlation

2. slope

3. intercept (dpm/mg protein·0 Hour)

4. half-life: calculated by formula: $T_{\frac{1}{2}} = 0.693/K$

5. first-order rate constant for degradation: calculated by formula: $K_D = -2.303$ (slope) (time^{-1})

6. zero-order rate constant of synthesis: calculated by formula: $K_S = K_D$ P (mass time^{-1})

the protein precursor pool was maximal within one hour following IP injection and rapidly decreased to minimal levels within 24 hours after injection.

L-leucine-U-^{14}C incorporation into liver and epididymal fat pad malic enzyme was maximal within one hour following IP injection. Plotting a curve of the loss of label from liver malic enzyme with time proved impossible due to a high degree of variability in dpm/unit enzyme precipitated after correction for non-specific precipitation of label. Loss of label from epididymal fat pad malic enzyme with time approximated an exponential curve (Figure 21) and half-lives and rate constants for degradation and synthesis were calculated from linear regression analysis of the data (Table 15). Estimated half-lives of the malic enzyme of meal-fed rat epididymal fat pad are 59 and 61 hours for Groups A and B respectively. Estimated half-lives of the malic enzyme of nibbling rat epididymal fat pad are 44 and 42 hours for Groups A and B respectively.

Meal-Feeding Experiment III:
Incorporation of L-Leucine-U-^{14}C into Malic Enzyme and Protein of Meal-Fed and Nibbling Rats

During the 4 week feeding period, the pair-fed meal-eaters and nibblers gained weight at the same rate (Figure 22). Daily food consumption of the meal-eaters (Figure 23) was low initially but rose steadily and stabilized by the end of the second week.

As in the previous meal-feeding experiments, the specific activity of malic enzyme of the epididymal fat pads of the meal-fed rats was significantly greater ($P \leq 0.05$) than that of the nibbling rats (Table 16).

Figure 21

Turnover of Malic Enzyme of the Epididymal Fat Pads from Meal-fed and Nibbling Rats

Turnover of malic enzyme of the epididymal fat pads of meal-fed (A) and nibbling (B) rats. Two separate groups (o and x) of meal-fed and nibbling rats were injected with L-leucine-U-^{14}C and killed after the specified intervals.

Figure 21

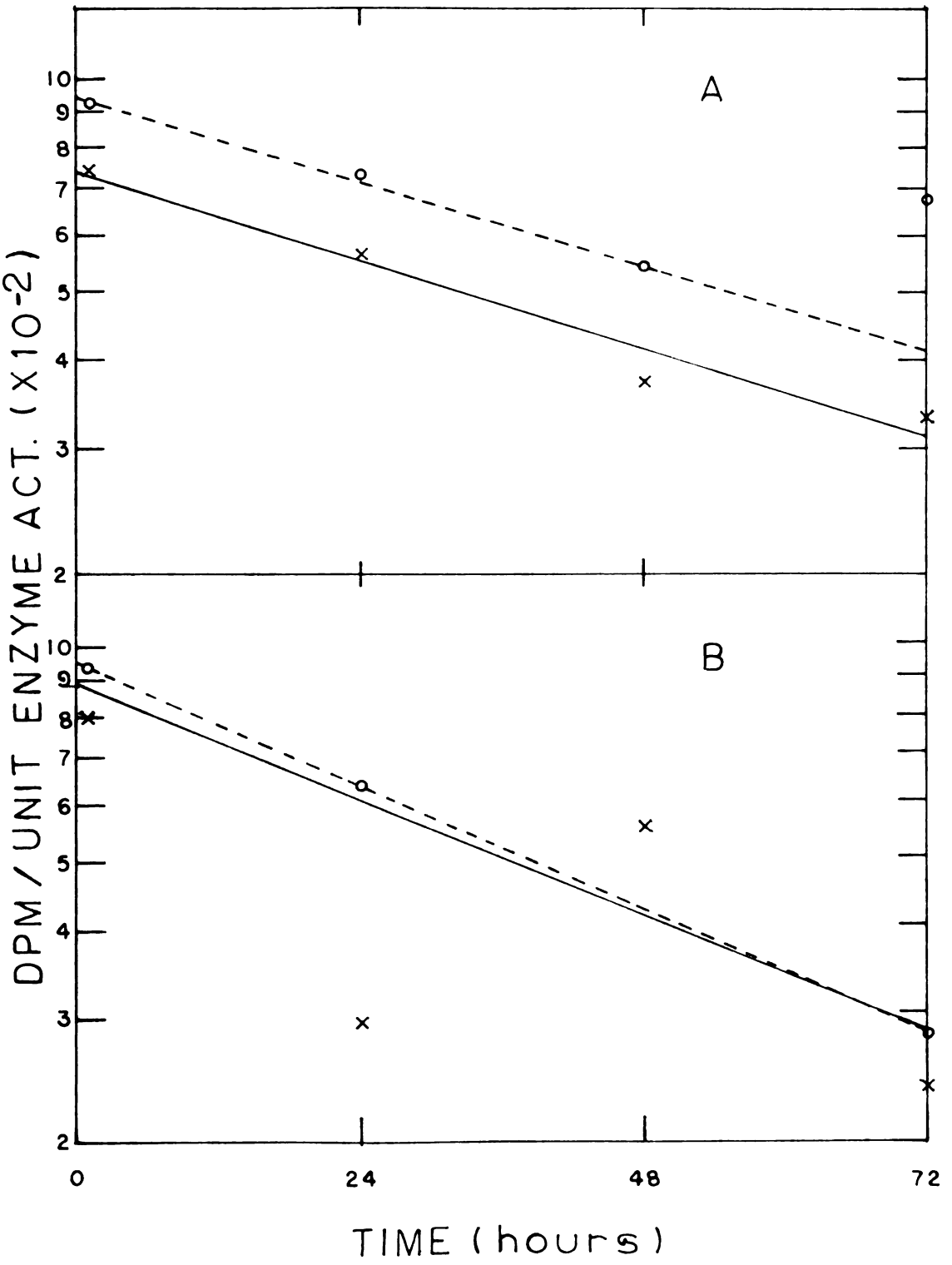


Table 15

Rates of Degradation and Synthesis: L-Leucine-U-^{14}C Labelled Epididymal Fat Pad Malic Enzyme of Meal-Fed and Nibbling Rats

	E[1]	r[2]	m[3]	b[4] y.x	$T_{\frac{1}{2}}$[5]	K[6] D (est.)	K[7] S (est.)
Meal Eaters							
Group A	163.7	-0.9785	-0.0051	738	59	0.0118	1.9
Group B	122.8	-0.9971	-0.0049	942	61	0.0113	1.4
Nibblers							
Group A	54.8	-0.9149	-0 0068	894	44	0.0157	0.9
Group B	32.5	-0.9999	-0.0072	957	42	0.0166	0.5

1. enzyme concentration: units per mg tissue protein
2. coefficient of correlation
3. slope
4. intercept (dpm/unit enzyme 0 hour)
5. half-life: calculated by formula: $T_{\frac{1}{2}} = 0.693/K$
6. first-order rate constant for degradation: calculated by formula: $K_D = -2.303$ (slope) (time^{-1})
7. zero-order rate constant of synthesis: calculated by formula: $K_S = K_D$ E (units time^{-1} mass^{-1})

Figure 22

Body Weight of Meal-eating and Nibbling Rats

Body weight of meal-eating (x) and nibbling (o) rats. Each point represents the mean for 24 meal-eating or nibbling rats.

Figure 22

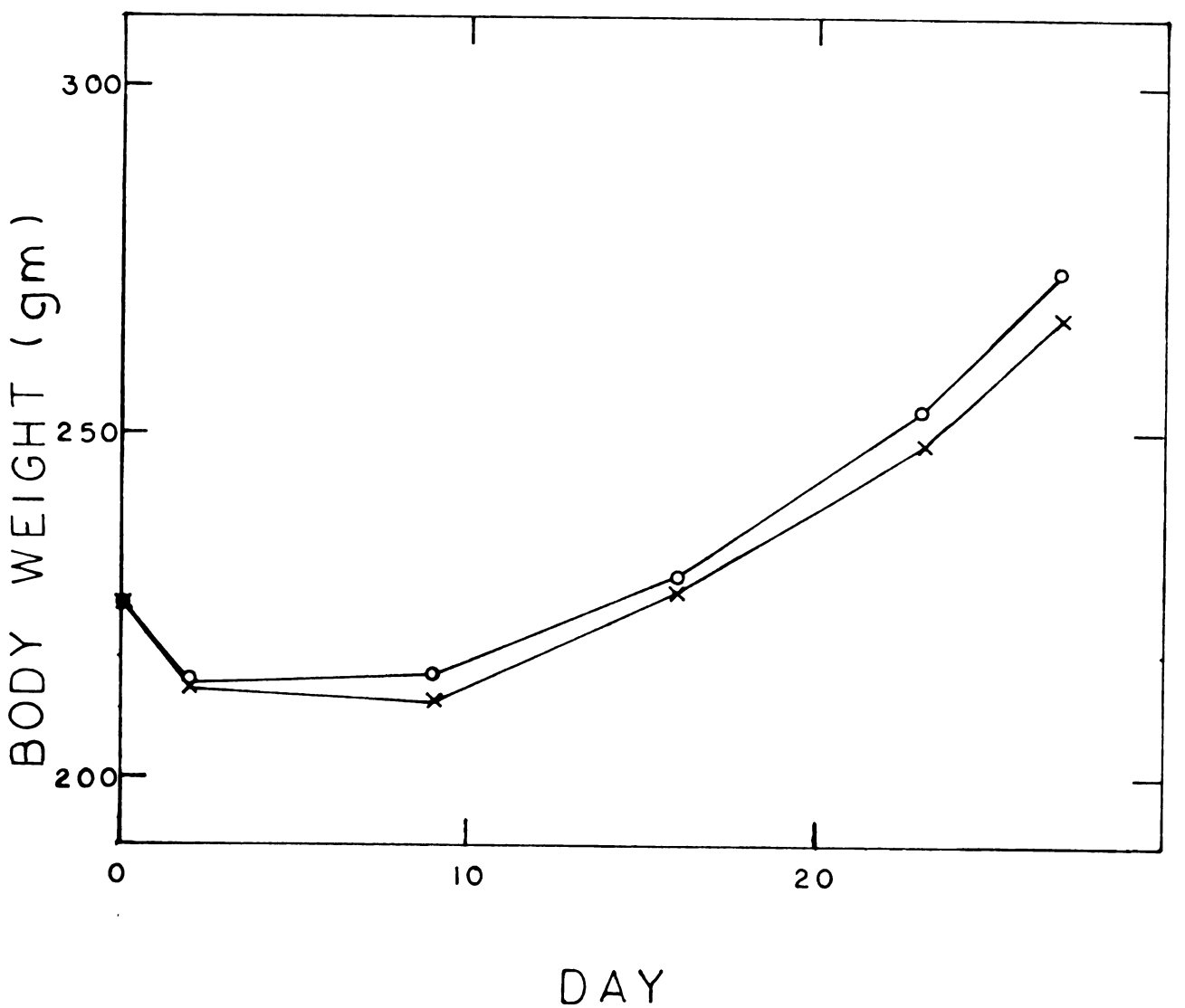


Figure 23

Food Consumption of Meal-eating Rats

Each point represents the mean for 24 meal-eating rats.

113

Figure 23

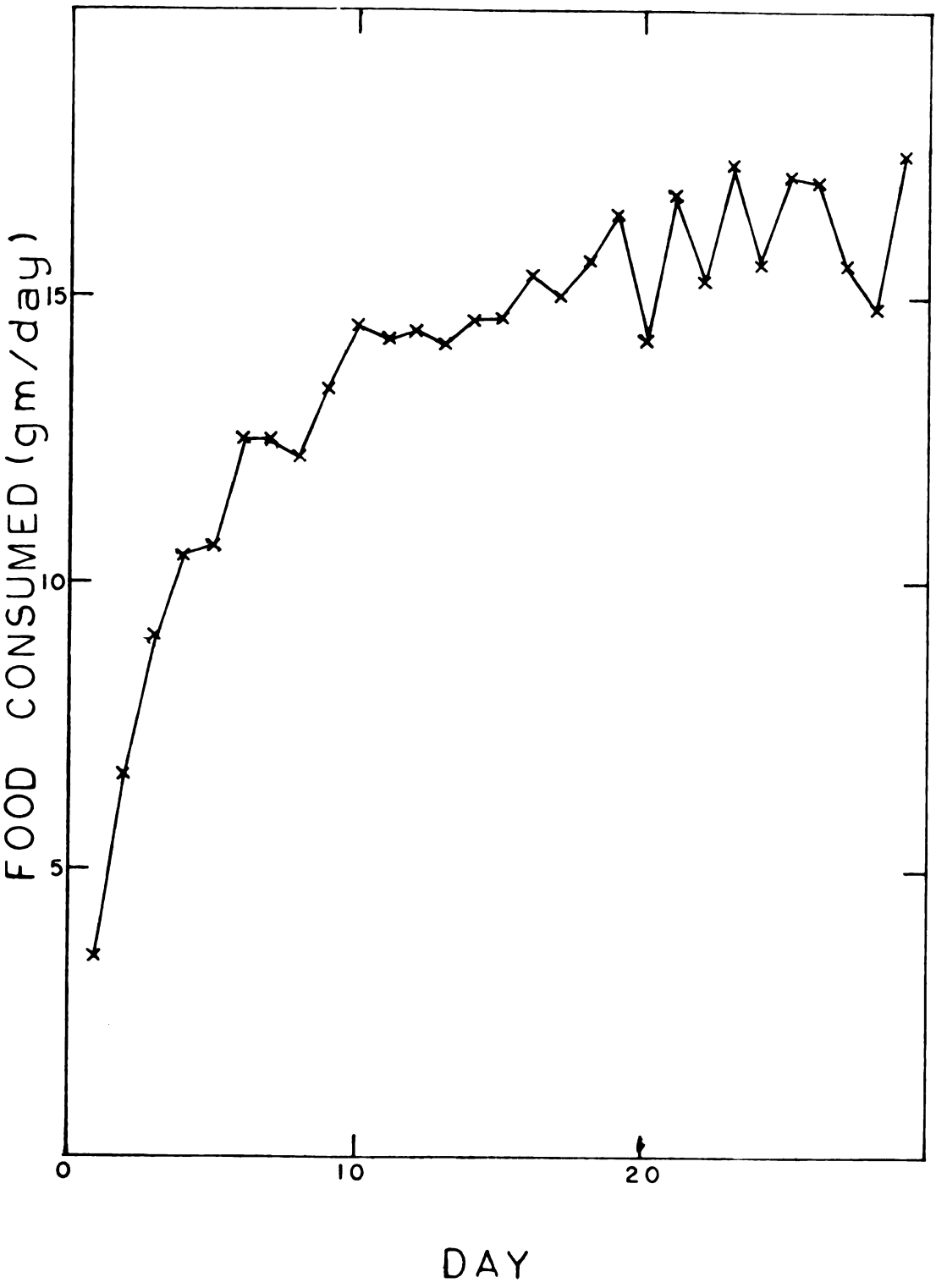


Table 16

Epididymal Fat Pad Malic Enzyme Activity of Meal-Fed and Nibbling Rats[1,2]

	Malic Enzyme Activity
Meal-Eaters	120.6 ± 22.3*
Nibblers	57.4 ± 14.1*

1. Enzyme activities are expressed as nanomoles of substrate utilized per minute per milligram of soluble protein at 25°C.

2. Mean ± SD for 24 rats.

* Asterisk indicates that the difference between meal-fed and nibbling values is significant ($P \leq 0.05$).

Incorporation of L-leucine-U-^{14}C into tissue protein was maximal within one hour following IP injection and followed an exponential decay curve over the subsequent 72 hours. The half-lives ($T\frac{1}{2}$) of the liver and epididymal fat pad total and soluble protein fractions were determined from the slope of a plot of dpm/mg protein (ordinate) against hours following injection (abscissa) (Figures 24 to 27). The slope was calculated from linear regression analysis of the data and was used to calculate the rate constants for degradation and synthesis of tissue protein (Tables 17 and 18). Label present in the protein precursor pool was maximal within one hour following IP injection and rapidly decreased to minimal levels within 24 hours after injection.

L-leucine-U-^{14}C incorporation into epididymal fat pad malic enzyme was maximal within one hour following IP injection. Loss of label from malic enzyme with time approximated an exponential curve (Figure 28) and half-lives and rate constants for degradation and synthesis were calculated from linear regression analysis of the data (Table 19). Estimated half-lives of the malic enzyme of epididymal fat pad are 198 and 75 hours for meal-fed and nibbling rats, respectively.

Figure 24

Turnover of the Total Protein in Livers from Meal-fed and Nibbling Rats

Turnover of the total protein fraction of livers from meal-fed (A) and nibbling (B) rats. The rats were injected with L-leucine-U-^{14}C and killed after the specified intervals. Vertical bars indicate standard error of the mean.

Figure 24

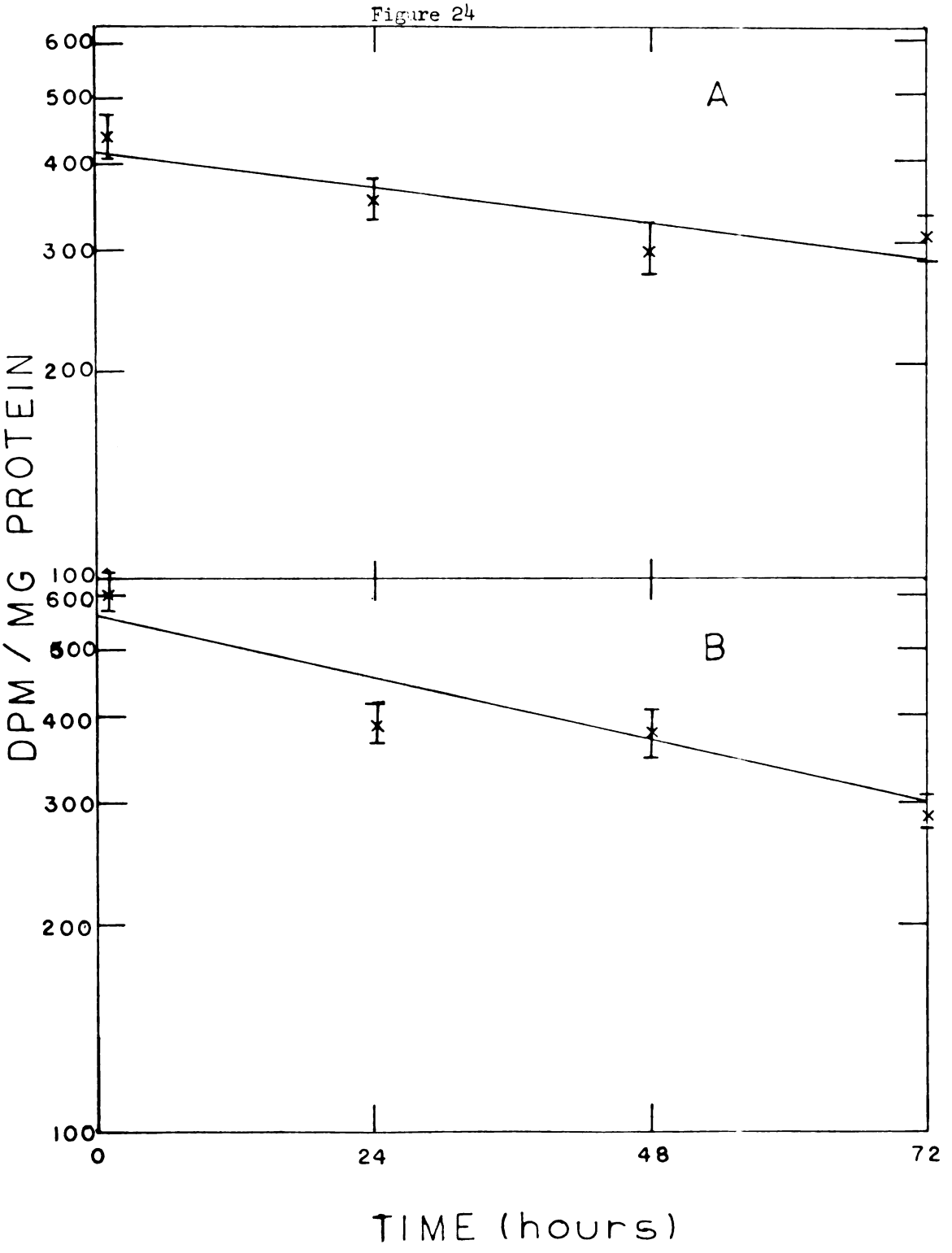


Figure 25

Turnover of the Total Protein in Epididymal Fat Pads from Meal-fed and Nibbling Rats

Turnover of the total protein fraction of the epididymal fat pads from meal-fed (A) and nibbling (B) rats. The rats were injected with L-leucine-U-^{14}C and killed after the specified intervals. Vertical bars indicate standard error of the mean.

Figure 25

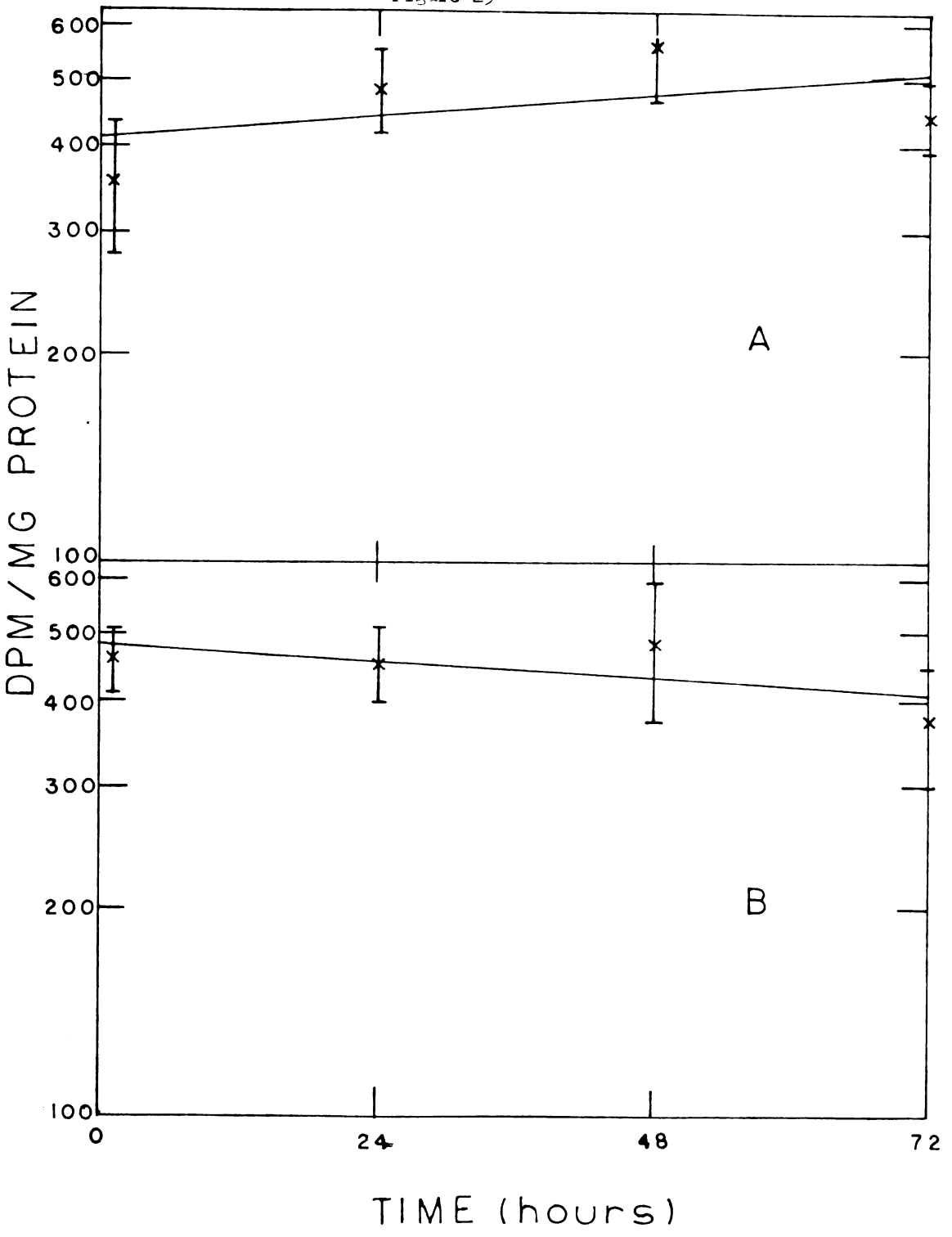

600
500
400
300
200
100
DPM / MG PROTEIN
A
600
500
400
300
200
100
B
0
24
48
72
TIME (hours)

Figure 26

Turnover of the Soluble Protein Fraction in Livers from Meal-fed and Nibbling Rats

Turnover of the soluble protein fraction of livers from meal-fed (A) and nibbling (B) rats. The rats were injected with L-leucine-U-^{14}C and killed after the specified intervals. Vertical bars indicate standard error of the mean.

Figure 26

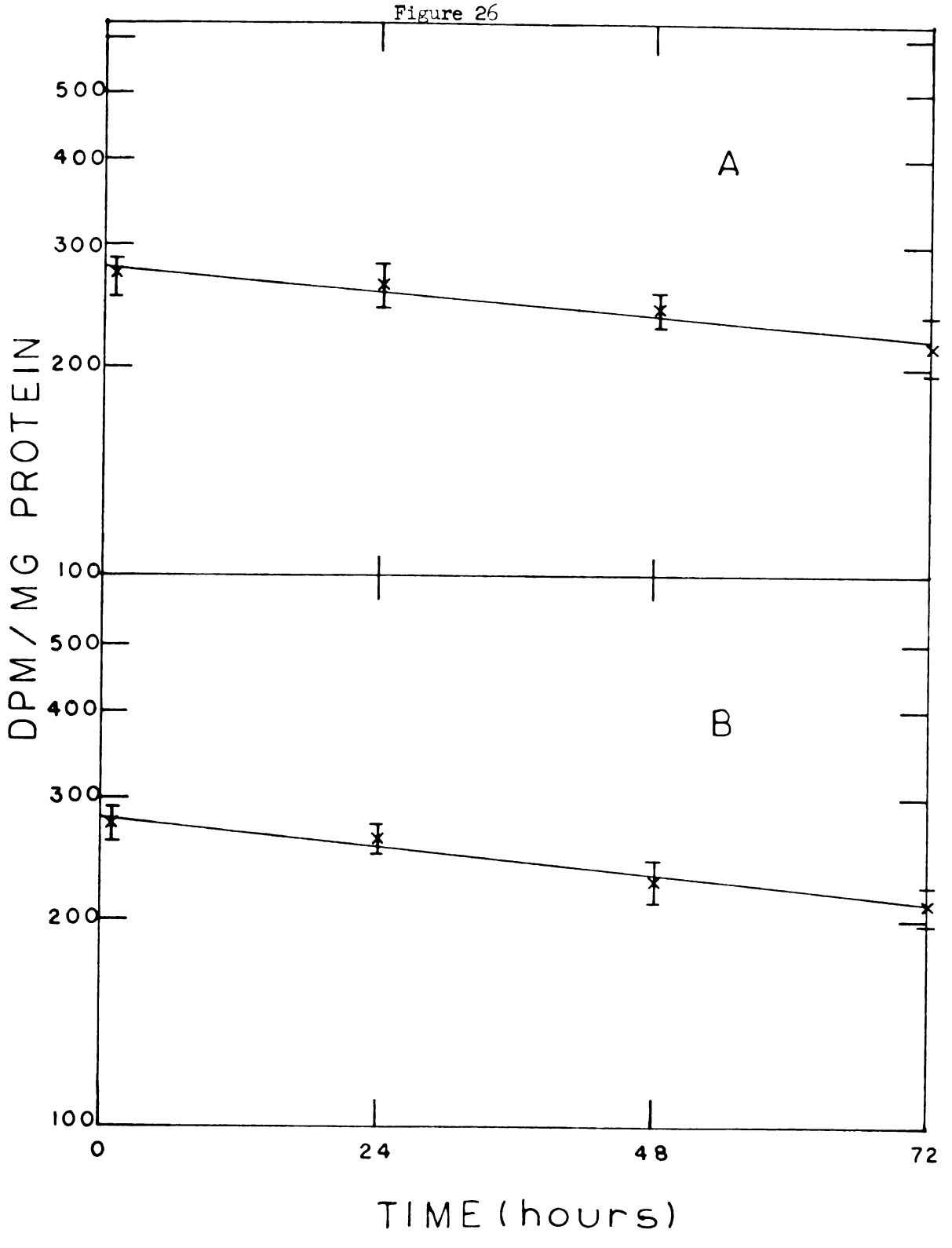

500
400
300
200
100
A
DPM / MG PROTEIN
500
400
300
200
100
B
0
24
48
72
TIME (hours)

Figure 27

Turnover of the Soluble Protein Fraction in Epididymal Fat Pads from Meal-fed and Nibbling Rats

Turnover of the soluble protein fraction of the epididymal fat pads from meal-fed (A) and nibbling (B) rats. The rats were injected with L-leucine-U-^{14}C and killed after the specified intervals. Vertical bars indicate standard error of the mean.

Figure 27

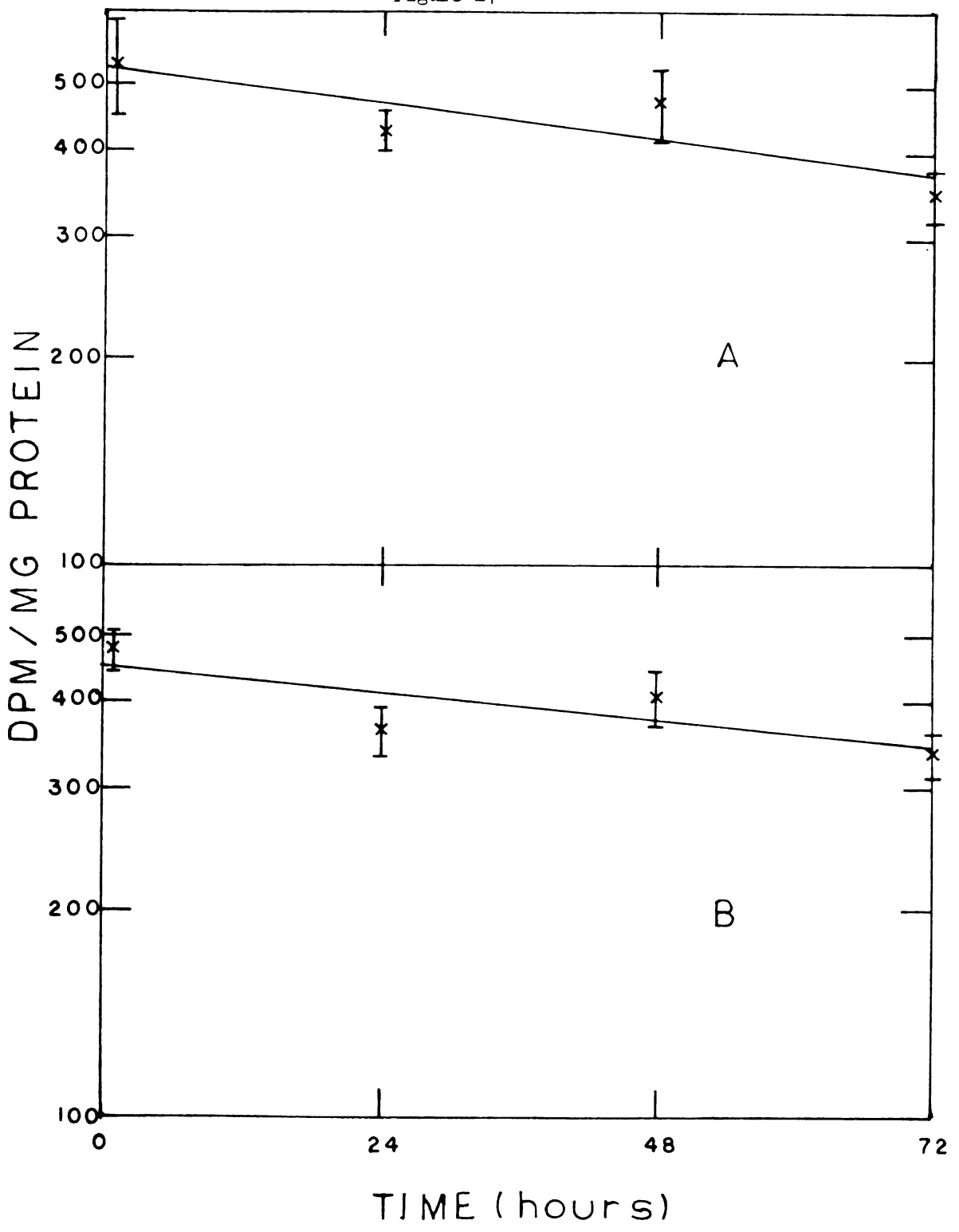

DPM / MG PROTEIN
500
400
300
200
100
A
500
400
300
200
100
B
0
24
48
72
TIME (hours)

Table 17

Rates of Degradation and Synthesis: L-Leucine-U-^{14}C Labelled Liver Total and Soluble Proteins

	Protein Conc. mg/ml	r^1	m^2	$b^3_{y.x}$	$T_{\frac{1}{2}}^4$	K^5_D (est.)	K^6_S (est.)
Meal-Eaters							
Total Protein	76.82	-0.8909	-0.0020	414	151	0.0046	0.353
Soluble Protein	37.63	-0.9678	-0.0014	279	216	0.0032	0.121
Nibblers							
Total Protein	75.15	-0.9221	-0.0040	561	75	0.0092	0.691
Soluble Protein	40.65	-0.9831	-0.0017	280	178	0.0039	0.159

1. coefficient of correlation

2. slope

3. intercept (dpm/mg protein· 0 hour)

4. half-life: calculated by formula: $T_{\frac{1}{2}} = 0.693/K$

5. first-order rate constant for degradation: calculated by formula: $K_D = -2.303$ (slope) (time^{-1})

6. zero-order rate constant of synthesis: calculated by formula $K_S = K_D$ P(mass time^{-1})

Table 18

Rates of Degradation and Synthesis: L-Leucine-U-^{14}C Labelled Epididymal Fat Pad Total and Soluble Proteins

	Protein Conc. mg/ml	r^1	m^2	b^3 y.x	$T^4_{\frac{1}{2}}$	K^5_D (est.)	K^6_S (est.)
Meal-Eaters							
Total Protein	9.25	0.5064	0.0014	413	--	--	--
Soluble Protein	6.87	-0.8515	-0.0021	525	141	0.0049	0.034
Nibblers							
Total Protein	7.50	-0.6471	-0.0010	483	300	0.0023	0.017
Soluble Protein	5.40	-0.7963	-0.0016	455	193	0.0036	0.019

1. coefficient of correlation
2. slope
3. intercept (dpm/mg protein 0 hour)
4. half-life: calculated by formula: $T_{\frac{1}{2}} = 0.693/K$
5. first-order rate constant for degradation: calculated by formula: $K_D = -2.303$ (slope) (time^{-1})
6. zero-order rate constant of synthesis: calculated by formula: $K_S = K_D$ P (mass time^{-1})

Figure 28

Turnover of Malic Enzyme of the Epididymal Fat Pads from Meal-fed and Nibbling Rats

Turnover of malic enzyme of the epididymal fat pads from meal-fed (A) and nibbling (B) rats. The rats were injected with L-leucine-U-^{14}C and killed after the specified intervals. Vertical bars indicate standard error of the mean.

Figure 28

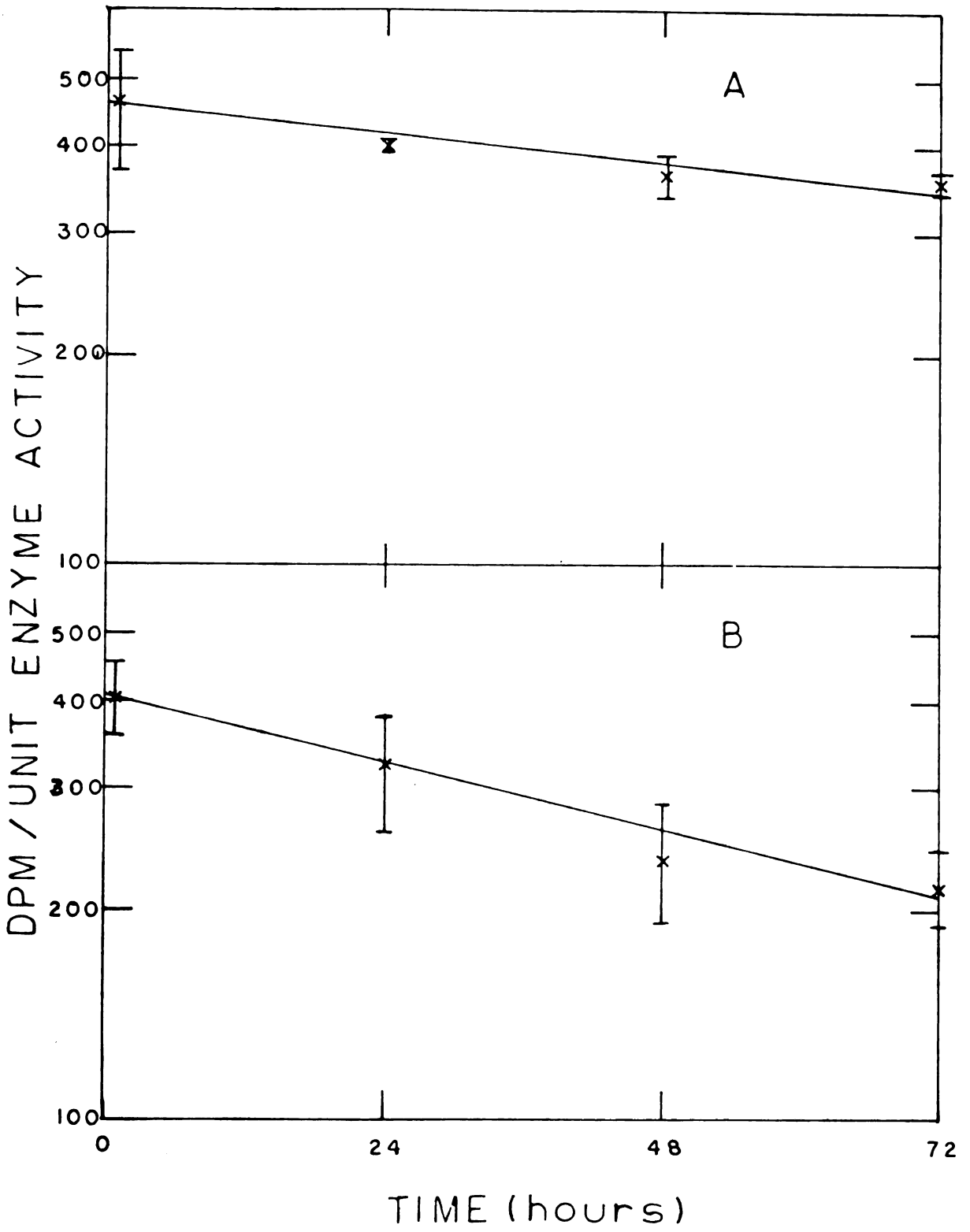

A
B
500
400
300
200
100
500
400
300
200
100
DPM / UNIT ENZYME ACTIVITY
0
24
48
72
TIME (hours)

Table 19

Rates of Degradation and Synthesis: L-Leucine-U-^{14}C Labelled Epididymal Fat Pad Malic Enzyme of Meal-Fed and Nibbling Rats

	E^1	r^2	m^3	$b^4_{y.x}$	$T^5_{\frac{1}{2}}$	K^6_D (est.)	K^7_S (est.)
Meal-Eaters	120.6	-0.9325	-0.0015	450.2	198	0.0035	0.4
Nibblers	57.4	-0.9696	-0.0040	395.2	75	0.0092	0.5

1. enzyme concentration: units per mg tissue protein
2. coefficient of correlation
3. slope
4. intercept (dpm/unit enzyme· 0 hour)
5. half-life: calculated by formula: $T_{\frac{1}{2}} = 0.693/K$
6. first-order rate constant for degradation: calculated by formula: $K_D = -2.303$ (slope) (time^{-1})
7. zero-order rate constant of synthesis: calculated by formula: $K_S = K_D$ E (units time^{-1} mass^{-1})

Discussion

The ingestion of a single, daily meal (meal-eating), as compared to ad libitum feeding (nibbling), by rats has many physiological and biochemical effects (7). One such effect is an increased activity of malic enzyme in the epididymal fat pad of the meal-fed rat as compared to that in the adipose tissue of nibbling rats (7). The purpose of the present study was to determine whether the rise in adipose tissue malic enzyme activity in the meal-fed rat was due to an altered turnover rate of the enzyme. To facillitate the study, rat liver malic enzyme was purified, an antibody to it was generated and an analysis of the degradation rate of adipose tissue malic enzyme in meal-fed and nibbling rats was conducted. Turnover of the total and soluble protein fractions of the liver and epididymal fat pad of meal-fed and nibbling rats was also determined.

Malic enzyme has been purified from rat liver and the preparation was shown to be pure by the following criteria: (i) a constant specific activity of 12,500 nanomoles of substrate cleaved per minute at 25°C per milligram of protein (Table 1); (ii) repeated crystallization; (iii) single, coincident protein-and enzyme activity-stained bands on polyacrylamide gel electrophoresis (Figure 3); and (iv) the occurrence of a single precipitin line in the double-diffusion analysis of the anti-rat liver malic enzyme and the purified enzyme reaction (Figure 1).

SDS- polyacrylamide gel electrophoresis gave single protein bands (Figure 4). This suggests that rat liver malic enzyme consists of several identical subunits. Li *et al.* (18) reported that rat liver malic enzyme is an isologous tetramer.

Several immunological studies were carried out with the anti-rat liver malic enzyme that was generated in rabbits with the purified enzyme. Double-diffusion analysis of the anti-serum yielded a single precipitin band for the purified enzyme (Figure 1). This band fused with the single precipitin line that developed with a rat liver extract. However, there was no observable precipitin line formed for a rat epididymal fat pad extract. This could be due to the malic enzyme concentration in the extract being too low to yield a noticable precipitate. Li et al. (18) reported the formation of a precipitin line for rat epididymal fat pad malic enzyme. Also, Flatt (17) reported that anti-rat liver malic enzyme reacted with rat adipose malic enzyme. Quantitative precipitin tests showed that the extent of cross-reactivity of the anti-serum with the liver and adipose enzyme was the same (Figure 2). This suggests that the malic enzyme present in each of the two tissues is antigenically similar. The equal cross-reactivity supports the suggestion that the absence of a precipitin band with the double-diffusion analysis of the adipose extract could be due to a low enzyme concentration rather than immunological differences.

Dietary regimen did not alter the antigenicity of rat malic enzyme. Precipitin analyses of the liver and adipose tissue malic enzyme of meal-fed and nibbling rats did not affect the extent of cross-reactivity of the enzyme with the anti-serum (Figure 5).

The specificity of the anti-rat liver malic enzyme for the liver and adipose tissue malic enzyme of several species was also studied. Double diffusion analysis and quantitative precipitin tests showed that the liver malic enzymes of genetically lean (Ob/+ or +/+)

and obese (Ob/Ob) mice were antigenically similar to each other and to rat liver malic enzyme (Figures 6 and 7). The immunological similarity of the liver enzymes of lean and obese mice suggests that the observed differences in the activity of the enzyme (47) are due to different concentrations of the same enzyme rather than the presence of two allelic forms of the enzyme with different specific activities. Similar cross-reactivity of the anti-rat liver malic enzyme with lean and obese mouse adipose tissue malic enzyme, as shown by the precipitin test, suggests that different levels of the same enzyme are present in the mouse adipose tissues. The extent of cross-reactivity of pig liver and adipose tissue malic enzyme with anti-rat liver malic enzyme as measured by precipitin test, was lower than that for rat liver malic enzyme (Figure 8). Cross-reaction between the anti-serum and chicken liver and adipose tissue malic enzyme was least. The precipitin test for the malic enzymes of dog liver and cow and lamb liver and adipose tissues were inconclusive due to the low tissue enzyme activities and the instability of the enzyme during incubation. The pig and chicken tissue malic enzymes, at least, could be said to be antigenically different from rat liver malic enzyme but appear to share at least some structural or sequential features with which the anti-rat liver malic enzyme reacts.

Three meal-feeding experiments were conducted. In each experiment, the meal-eating and nibbling rats were pair-fed. The 8:00 to 10:00 a.m. consumption of the meal-fed rat was measured and this amount was placed in a mechanical feeder which distributed the food evenlythroughout the day to the nibbling rat. By this means the meal-eating and nibbling rats consumed the same amount and the

nibbling rat did not experience peak periods of consumption. Consumption by the meal-eaters rose steadily as they adjusted to the dietary regimen and leveled off within two weeks (Figures 10,16 and 23). Body weights and rates of gain of the meal-eating and nibbling rats were identical in every experiment (Figures 9,15 and 22). Thus the discrepencies in body weights and consumption observed in earlier meal-feeding experiments (7) were eliminated.

A comparison of the enzyme activities and lipogenic capacities of the epididymal fat pads of the meal-fed and nibbling rats was made in the first two experiments. In the first experiment, the activities of adipose tissue malic enzyme and glucose-6-phosphate dehydrogenase and the rate of fatty acid synthesis was significantly greater in the meal-fed rats (Table 3). Acetyl CoA carboxylase activity was not significantly different between the rats. This may be due to insufficient replication of the enzyme assay or too few animals were used in the estimation of enzyme activity. In the second experiment, adipose tissue malic enzyme, glucose-6-phosphate dehydrogenase and acetyl CoA carboxylase activities and the rate of lipogenesis were significantly greater in the meal-fed rats (Tables 11 and 12). Hence, the induction of adipose tissue malic enzyme in meal-fed rats parallels the increases in the activities of two other enzymes involved in lipogenesis and parallels the increase in the lipogenic capacity of the tissue. Although the adipose tissue enzyme activity and lipogenic rates observed in the meal-fed rats were only about 2- to 3- fold greater than those in the nibbling rats, the changes were comparable, but slightly lower, to those reported for earlier meal-feeding experiments. By eliminating body size and consumption differences, a more valid

meal-feeding effect may have been observed. In both experiments no differences were observed in the hepatic enzyme activities and lipogenic rates between the meal-fed and nibbling rats (Tables 2,3,11 and 12). In all three experiments, the levels of epididymal fat pad malic enzyme were significantly greater in the meal-fed rats (Tables 2,11 and 16).

An analysis of the rate of degradation of the epididymal fat pad malic enzyme of the meal-fed and nibbling rats was carried out in each meal-feeding experiment. In the first experiment, the half-lives of adipose tissue malic enzyme were estimated to be 30 and 32 hours for the meal-fed and nibbling rats, respectively. The low level of incorporation of L-arginine-guanidino-^{14}C into malic enzyme and inadequate replication made accurate determination of the degradation rate impossible. The preliminary experiments conducted after this experiment helped solve procedural problems and indicated that L-leucine-U-^{14}C would give better incorporation into adipose tissue malic enzyme than would L-arginine-guanidino-^{14}C.

In the second experiment, the half-lives of adipose tissue malic enzyme were found to be 59 and 61 hours for the meal-eating rats and 44 and 42 hours for the nibbling rats. The slope of the decay curve was used to calculate the rate constants of degradation and synthesis. As the animals had been adjusted to the meal-feeding regimen, they could be assumed to be in a steady state condition and the rate of synthesis of malic enzyme could be estimated from the rate of degradation of the enzyme. The rate constants for degradation of adipose tissue malic enzyme were similar for the meal-fed and nibbling rats. The rate of synthesis of the adipose malic enzyme for the meal-fed rats was twice that for nibbling rats; being 1.9 and 1.4 units of

enzyme activity synthesized per milligram of tissue protein per hour for the meal-fed rats and 0.9 and 0.5 units of enzyme activity synthesized per milligram of tissue protein per hour for the nibbling rats. This would suggest that the level of adipose tissue malic enzyme is more greatly affected by the rate of synthesis of the enzyme than by its rate of degradation.

A third meal-feeding experiment was conducted to substantiate the findings of the second experiment. The half-lives of adipose tissue malic enzyme were found to be 198 and 75 hours for the meal-fed and nibbling rats respectively. However, the rate of degradation of the adipose tissue enzyme for the meal-fed rat was only about one-third that for the nibbling rat, being 0.0035 and 0.0092 per hour for the meal-fed and nibbling rats, respectively. The rates of synthesis of the adipose tissue malic enzyme were similar for the meal-fed and nibbling rats. The reasons for the longer half-lives of the malic enzyme in this as compared to the earlier experiment and the apparent importance of degradation rather than synthesis in controlling the adipose tissue enzyme levels are not clear.

The half-life of rat liver malic enzyme has been reported by Gibson *et al.* (4) to be 51 hours. Silpananta and Goodridge (3) reported a half-life of 55 hours for chick liver malic enzyme. Gibson *et al.* (4), Murphy and Walker (6), Li *et al.* (18) and Silpananta and Goodridge (3) report that the rate of synthesis of liver malic enzyme is more important than the rate of degradation in regulating the tissue level of the enzyme. Flatt (17) reports that adipose tissue malic enzyme has a half-life of 59 to 89 hours, depending on the labelled amino acid used. The above reports are in accord with the findings of the second turnover experiment in which adipose tissue malic enzyme of meal-fed

and nibbling rats had half-lives of 60 and 43 hours respectively and in which the rate of synthesis appeared to affect the tissue level of the enzyme. Schimke (81) also suggested that, for enzymes with long half-lives, the rate of synthesis is important in controlling the tissue level of the enzyme. The longer half-lives and the importance of the rate of degradation in controlling the enzyme level found in the third experiment are at variance with the above findings and are unexplainable.

Recalculation of the rate constant of synthesis of malic enzyme by the method of Millward (98,99) was considered. He proposed that the synthesis of tissue protein could be treated as a first-order reaction and that the rate constant of synthesis could be obtained from the slope of the exponential plot of the decrease of label in the protein fraction with time. He acknowledged that protein synthesis follows zero-order reaction kinetics but stated that treatment of the data by the above method would not seriously affect the results. The rate of synthesis of the muscle protein fraction of weanling rats, after a single IP injection, was calculated from the decay curve of counts per milligram of tissue protein versus time (days) while the rate of degradation of muscle protein was calculated from the decay curve of total counts versus time (days). No explanation of why a decay curve based on protein content should represent protein synthesis while one based on counts should represent protein degradation was given. Perry (100) used the same techniques to measure protein synthesis in the liver and skeletal muscle protein fractions of suckling pigs. Although he based his calculations on the specific activity of aspartate plus glutamate in the protein fractions, the loss of isotope from a

protein fraction over time can not be construed to represent protein synthesis. Plotting the data on the turnover of the protein fractions of liver and epididymal fat pad of the second and third meal-feeding experiments based on dpm (to represent degradation) or dpm per milligram protein (to represent synthesis) produced identical decay curves. Plotting the decay curves of malic enzyme on basis of dpm per milligram of protein precipitated or dpm per unit of enzyme activity also gave similar results. The data are not shown as no differences between the "degradation" and "synthesis" curves were observed. Garlick et al. (101), using the same theoretical basis, calculated the rate constant of synthesis of muscle and liver protein of meal-fed and ad libitum-fed rats. In their experiments a 6-hour IV infusion of the labelled amino acid was used rather than the single IP injection used in the experiments reported by Millward (98,99) and Perry (100). The time course required for the specific activity of the label to plateau in the tissue proteins could then be followed and the rate of protein synthesis, based on the rate of incorporation into tissue protein, could then be calculated. The data from the meal-feeding experiments reported here could not be treated in the same manner as labelled amino acid was administered as a single IP injection and the period of time needed for the level of label in tissue protein to plateau was not followed.

The rate of loss of isotope from the protein fractions of the liver and adipose tissue of the meal-fed and nibbling rats was measured in all of the meal-feeding experiments. In the first experiment, using L-arginine-guanidino-^{14}C, the half-lives of liver proteins were estimated to be about 100 hours for the total and soluble

protein fractions of both meal-fed and nibbling rats (Table 4). Those for epididymal fat pads were about 60 hours. Inadequate replication and the poor linearity of the decay curves (Figures 11 and 12) decreased the accuracy of measurement of the protein half-lives. Hence, the values obtained can only be considered as estimates. No differences in the half-lives of the tissue protein fractions of meal-fed and nibbling rats were observed. Similar values were obtained whether the estimation of half-lives of the protein fractions were based on the protein content of the TCA-precipitated pellets or of the tissue extracts.

In the second meal-feeding experiment, using L-leucine-U-^{14}C, the half-lives of the liver total protein fraction were 77-and 116 hours for the meal-fed and nibbling rats, respectively (Table 13). The corresponding values for the liver soluble protein fraction were 166- and 188 hours (Table 13). Although there were no apparent differences between the half-lives of the liver protein fractions of the meal-fed and nibbling rats, the total protein fraction of the liver had a much shorter half-life (96 hours) than the liver soluble protein fraction (177 hours). The observed half-lives of the epididymal fat pad total protein fraction were 45-and 31 hours for the meal-fed and nibbling rats, respectively (Table 14). The corresponding values for the epididymal fat pad soluble protein fraction were 40-and 36 hours (Table 14). No apparent differences in half-life values were observed between the epididymal fat pad protein fractions (total or soluble) or between those of meal-fed and nibbling rats. Improved replication and linearity of the decay curves (Figures 17 to 20) allowed linear regression analysis; thereby increasing the reliability of the values obtained.

In the last meal-feeding experiment, the half-lives of the

liver total protein fraction were 151-and 75 hours for the meal-fed and nibbling rats, respectively (Table 17). The corresponding values for the soluble protein fraction were 216-and 178 hours (Table 17). As in the previous experiment, the total protein fraction of the liver had a much shorter half-life (113 hours) than the liver soluble protein fraction (197 hours). The half-life of the epididymal fat pad total protein fraction was 300 hours for the nibbling rat (Table 18). The positive slope for the total protein fraction of the epididymal fat pad of the meal-eaters (Figure 25) is surprising but may be due to animal variation or experimental error. As a consequence, there was no available half-life value for this particular protein fraction. The half-lives of the epididymal fat pad soluble protein fraction were 141-and 193 hours for the meal-fed and nibbling rats, respectively (Table 18). No apparent differences were observed for the half-lives of the protein fractions of the epididymal fat pad of meal-fed and nibbling rats. Good linearity and replication were obtained for most of the curves (Figures 24 to 27).

The half-life values obtained for the liver protein fractions (about 100 hours) are comparable to those reported by Schimke (84) who reported half-life values of 3.2 days for rat liver homogenate protein and 5.1 days for rat liver supernatant protein when L-arginine-guanidino-^{14}C was used. Uniformly labelled L-arginine gave values of 4.5 and 6.2 days respectively. Kuehl and Sumsion (102) reported a half-life value of about 6.6 days for total liver protein of the rat when L-leucine-4,5-^{3}H was used. The above reports are in accord with the findings of the second and third turnover experiments in which the total protein fraction of the liver had half-life values of 96 hours (4.0 days) and 113 hours

(4.7 days), respectively. The corresponding values for the soluble protein fraction of the liver were 177 hours (7.4 days) and 197 hours (8.2 days). No values have been reported for the turnover of adipose tissue protein fractions. The half-life value obtained for the protein fractions (total and soluble) of the epididymal fat pad was 38 hours in the second experiment while that observed in the third experiment was 211 hours.

There is no ready explanation for the extended half-lives obtained for the malic enzyme and the protein fractions of the epididymal fat pad in the last meal-feeding experiment as compared to the observations in the preceeding experiment. One reason may be that, due to the younger age, lighter body weight and a greater rate of weight gain of the animals at the time of isotope administration in the third meal-feeding experiment, they were probably more metabolically active than the animals of the prior experiment. This increased metabolic rate could lead to an increased rate of recycling of L-leucine-U-^{14}C in the tissue proteins of the younger animals. This would be reflected in a longer apparent half-life for adipose tissue malic enzyme and the adipose tissue protein fractions.

A curvilinear tendency in the decay curves of the tissue protein fractions has been noted. The slope is initially steep and then tends to plateau after 48 hours (Figures 11,12,17,18,19,20,24,25, 26 and 27). The curvilinear nature of the decay curves may be a reflection of different turnover rates of the various proteins comprising the tissue protein fractions. Silpananta and Goodridge (3) reported that they had measured label incorporation into proteins of chick liver but they had not calculated half-life values due to the

non-linearity of the decay curves obtained. Kuehl and Sumison (102) also reported this non-linearity in the decay curves of rat liver total protein. They suggested that the initial steepness of the slope of the decay curve may be due to contributions of proteins that have rapid turnover rates.

Summary and Conclusions

Malic enzyme (L-Malate: NADP oxidoreductase (decarboxylating), EC 1.1.1.40) was purified from rat liver. The enzyme was found to be homogeneous according to several criteria of purity.

Immunological analysis by double diffusion on agar and quantitative precipitin tests with anti-serum to rat liver malic enzyme indicated that the liver and epididymal fat pad malic enzymes were immunologically similar. The liver and adipose tissue malic enzymes of genetically lean and obese mice were shown to be immunologically similar to each other and to rat liver malic enzyme. Pig and chicken liver and adipose tissue malic enzymes showed a decreased cross-reactivity with anti-rat liver malic enzyme.

L-leucine-U-^{14}C incorporation analysis of the epididymal fat pad malic enzyme of meal-fed (one 2 hour meal per day) and nibbling (ad libitum) rats showed half-lives of 60- and 43 hours respectively in one experiment. The rate of synthesis of the enzyme was found to be more important in controlling the tissue level of the enzyme than the rate of degradation. A second meal-feeding experiment indicated half-lives of 198- and 75 hours for the adipose tissue malic enzyme of meal-fed and nibbling rats, respectively. In the latter experiment, the rate constant of degradation of epididymal fat pad malic enzyme was found to be less in the meal-fed rat than in the nibbling rat. No differences were observed in the rate constants of synthesis of epididymal fat pad malic enzyme.

Turnover analysis of protein fractions of the liver and

adipose tissue of the meal-fed and nibbling rats was conducted, using L-leucine-U-^{14}C. The half-lives of the protein fractions of liver and epididymal fat pad were the same in both meal-fed and nibbling rats. The values for the total protein fraction were found to be 96- and 113 hours in the first and second meal-feeding experiments, respectively. The corresponding half-life values of the soluble protein fraction of liver were 177- and 197 hours. The half-life values of both total and soluble protein fractions of the epididymal fat pad were 38 hours in the first experiment while that observed in the second experiment was 211 hours.

In conclusion, meal-feeding causes an increase in the activity of several enzymes involved in lipogenesis and in the lipogenic capacity of rat epididymal fat pad. The observed 2-fold increase in adipose tissue malic enzyme in the feal-fed rat, as compared to the nibbling rat, is due to an increased half-life. The rate of synthesis of the adipose tissue enzyme was increased in the meal-fed rat and may be instrumental in controlling the tissue level of the enzyme.

APPENDICES

Appendix I

Diet Composition

A. Composition

	g/100 g	g/10 Kg
Casein	20.0	2,000
DL-Methionine	0.3	30
Mineral Mix (87)	4.0	400
Vitamin Mix	0.4	40
Choline Cl	0.2	20
Solka Flok	4.0	400
Corn Oil	5.0	500
CHO (cerelose)	66.1	6,610
	100.0	10,000

B. Vitamin Mix for Rats*

	mg/Kg diet
Thiamine HCL	22
Pyridoxine HCL	22
Riboflavin	22
Ca pantothenate	66
p-Amino benzoic acid	110
Menadione	50
Inositol	100
Ascorbic Acid	200
Niacin	100
Vitamin B 12	0.3 mg
Biotin	0.6
Folic Acid	4.0
Subtotal	696.9 mg
Vitamin A	20,000 IU
Alpha tocopherol acetate	100 IU
Vitamin D_3	2,200 IU
Cerelose to	400 g

* Add at rate of 0.4% of diet

Appendix II

Modified Lowry Protein Procedure

This is a modification of the standard Lowry protein procedure to allow estimation of the protein content of NaOH-solubilized samples. Every sample and standard is brought to a volume of 1.0 ml with a final NaOH concentration of 0.5 N NaOH.

A) Standard Curve

i) the standard is bovine serum albumin, fraction V, at a concentration of 500 μg/ml with 0.1% sodium azide as a preservative.

ii) standard curve is established from samples containing 0, 25, 50, 100, and 150 μg protein in a volume of 1.0 ml containing 0.5 N NaOH.

	BSA (500 μg/ml)	H_2O	1 N NaOH
0 μg	- ml	0.5 ml	0.5 ml
25 μg	0.05 ml	0.45 ml	0.5 ml
50 μg	0.10 ml	0.40 ml	0.5 ml
100 μg	0.20 ml	0.30 ml	0.5 ml
150 μg	0.30 ml	0.20 ml	0.5 ml

B) Sample

i) dilute solubilized sample to 1.0 ml to a final NaOH concentration of 0.5 N NaOH.

eg. if sample is solubilized in 0.5 ml of 1 N NaOH, add 0.5 ml H_2O

eg. if sample is solubilized in 1 ml of 1 N NaOH but has a high protein concentration, mix an 0.05 or 0.10 ml aliquot with 0.45 or 0.40 ml of 1 N NaOH and 0.50 ml H_2O

C) Lowry Protein Determination

i) to each sample, add 5.0 ml of Lowry copper reagent:

2.00% Na_2CO_3	(20 gm/liter)
-0.01% $CuSO_4$ 5 H_2O	(.1 gm/liter)
-0.02% potassium tartate	(.2 gm/liter)

ii) mix and allow to stand for 15 minutes

iii) add 0.5 ml of 1 M (1:2 diluted) Folin reagent

iv) mix immediately and allow to stand for 30 minutes

v) read absorbance at 500 nm

Appendix III

Fatty Acid Synthesis

A. Procedure for In vitro Incorporation of ^{14}C into Fatty Acids from D-glucose-U-^{14}C

i) Tissue Preparation

a) after killing, quickly remove and weigh the liver and epididymal fat pads

b) slicing

(1) liver--obtain 75-150 mg slices of the liver from the central lobe using a microtome blade and Stally-Riggs microtone

(2) fat pad-- using sharp scissors, cut 75-150 mg pieces from the thin distal portion of the fat pad

ii) Incubation

a) place the tissue slices into incubation flasks containing 3.0 ml of incubation media (Krebs-Ringer buffer with D-glucose-U-^{14}C and porcine insulin (0.1 unit/ml))

b) record the time

c) incubate for 2 hours at 37°C under oxygen (5% CO_2-95% O_2)

d) at the end of 2 hours, remove the tissue slices from the incubation media, rinse in saline and blot dry

e) place tissue slices into stoppered tubes containing 10.0 ml of 5% KOH- 95% ethanol

iii) Saponification

a) carry out saponification for 3 hours at 80-85°C, using glass marbles on tube opening

b) after saponification, add 6.0 ml H_2O and cool

iv) Fatty Acid Extraction

a) remove the non-saponifiable fraction with three 5.0 ml petroleum ether extractions

b) acidify the saponification tubes with 5-6 ml of 12 N HCL, checking each tube with Congo Red paper

c) extract 3 times with 5 ml portions of petroleum ether, transferring each portion to a scintillation vial

d) evaporate petroleum ether and add 10 ml of toluene scintillation cocktail

e) blank is 10 ml of toluene scintillation cocktail

v) Toluene Scintillation Cocktail

200 ml ethanol
4 gm Omnifluor

Toluene to 1000 ml

B. Krebs-Ringer Bicarbonate Buffer

i) Solutions (prepare fresh monthly)

1X	5X	5X g
1. 0.90% NaCl (0.154 M)	4.5 %	4.5 g/liter
2. 1.15% KCl (0.154 M)	5.75%	5.75g/100 ml
3. 2.11% KH_2PO_4(0.154 M)	10.55%	10.55g/100 ml
4. 1.85% $MgSO_4$ (0.154 M)	9.25%	9.25g/100 ml
5. 1.30% $NaHCO_3$ (0.154 M) (gas with CO_2 for 1 hour)	6.5 %	65 g/liter

ii) Proportions (for 5X solution)

Solution	A	B	C	D
A. NaCl	100.0	50.0	25.0	12.5
B. KCl	4.0	2.0	1.0	0.5
C. KH_2PO_4	1.0	0.5	0.25	0.125
D. $MgSO_4$	1.0	0.5	0.25	0.125
E. $NaHCO_3$	21.0	10.5	5.25	2.625
F. H_2O	3.0	1.5	0.75	0.375
Total	130.0	65.0	32.5	16.25

After all ingredients are added, gas the buffer for 10 minutes with 5% CO_2- 95% O_2 mixture. Keep stoppered and in cold.

C. Preparation of Incubation Media

i) Liver

a) Krebs-Ringer buffer
- for 100 ml media, use 20 ml of 5 x Krebs-Ringer buffer
- for 50 ml media, use 10 ml of 5 x Krebs-Ringer buffer

b) add 100 mM glucose
- 1.80 gm for 100 ml media
- 0.90 gm for 50 ml media

c) add 0.3 μ Ci D-glucose-U-^{14}C/ml for 100 ml media
- 3 ml of 10 μ Ci D-glucose-U-^{14}C/ml for 100 ml media
- 1.5 ml of 10 μ Ci D-glucose-U-^{14}C/ml for 50 ml media

d) add 0.1 unit porcine insulin/ml
- 1.0 ml of 10 units insulin/ml for 100 ml media
- 0.5 ml of 10 units insulin/ml for 50 ml media

e) bring to volume (100 or 50 ml) with H_2O

f) gas the buffer for 10 minutes with 5% CO_2- 95% O_2 and check pH (pH 7.4)

ii) Adipose Tissue

a) Krebs-Ringer buffer
- for 100 ml media, use 20 ml of 5 x Krebs-Ringer buffer
- for 50 ml media, use 10 ml of 5 x Krebs-Ringer buffer

b) add 25 mM glucose
- 0.450 gm for 100 ml media
- 0.225 gm for 50 ml media

c) add 0.2 μ Ci D-glucose-U-^{14}C/ml
- 2.0 of 10 μ Ci D-glucose-U-^{14}C/ml for 100 ml media
- 1.0 of 10 μ Ci D-glucose-U-^{14}C/ml for 50 ml media

d) add 0.1 unit porcine insulin/ml
- 1.0 of 10 units insulin/ml for 100 ml media
- 0.5 of 10 units insulin/ml for 50 ml media

e) bring up to volume (100 or 50 ml) with H_2O

f) gas the buffer for 10 minutes with 5% CO_2- 95% O_2 and check pH (pH 7.4)

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