A TIME SEQUENCE STUDY OF CHANGES IN LIPOGENESIS WITHIN THE ADIPOSE TISSUE OF RATS CONVERTED FROM AD LIBITUM FEEDING TO MEAL-EATING

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
MICHAEL K. ARMSTRONG
1974

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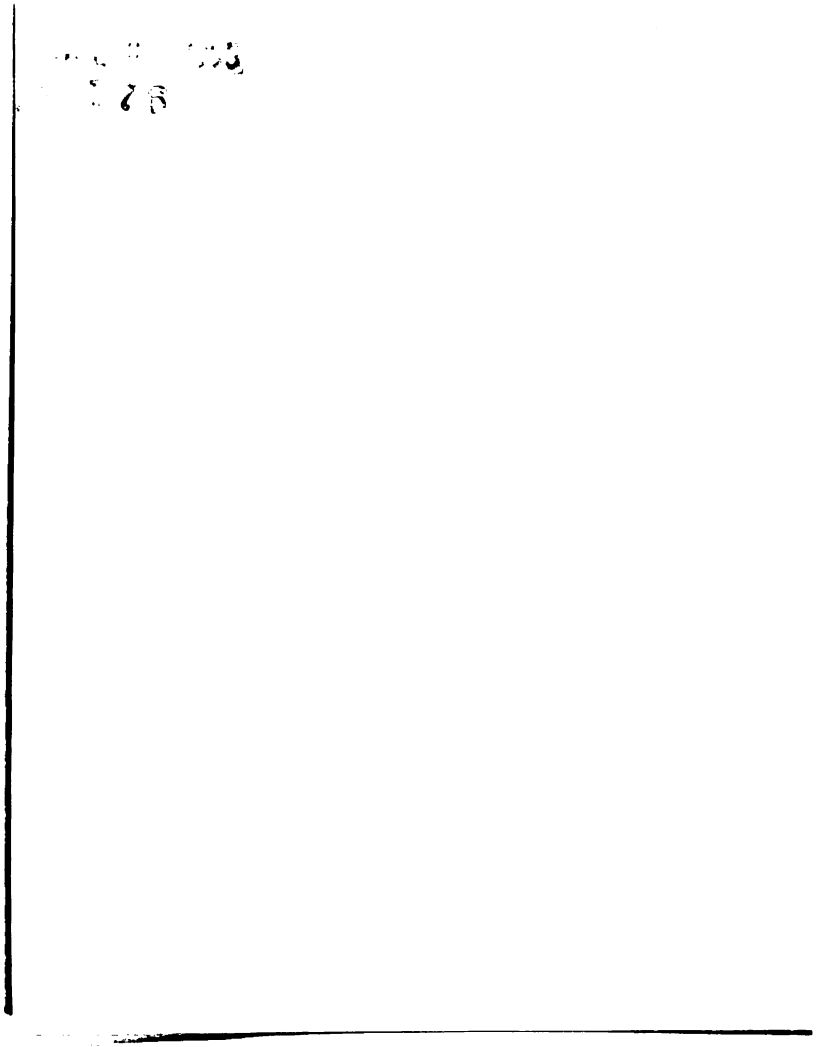

ABSTRACT

A TIME SEQUENCE STUDY OF CHANGES IN LIPOGENESIS WITHIN THE ADIPOSE TISSUE OF RATS CONVERTED FROM AD LIBITUM FEEDING TO MEAL-EATING

By

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Rats were given access to a high carbohydrate diet (75 percent glucose) once daily for two hours. Rats on this meal-eating regimen were sacrificed from 0 to 10 days after their conversion from ad libitum feeding. The high speed supernatant fraction from the epididymal fat pads was assayed for citrate cleavage enzyme, acetyl coenzyme A carboxylase, fatty acid synthetase, and malic enzyme activities.

The capacity of the adipose tissue to synthesize fatty acids was measured in vitro by incubating a portion of adipose tissue in the presence of glucose-U-^{14}C or in vivo by injecting the animals with tritiated water thirty minutes before sacrifice.

The enzyme activities declined steadily through the fourth day of meal-eating finally reaching a nadir 25 to 35 percent lower than the activities shown by the 0 day rats. In spite of the decreased enzyme activities, the in vitro rate of fatty acid synthesis continually increased through the eighth day of meal-eating--the

fourth day meal-eaters having a fatty acid synthesis rate 80 percent higher than their day 0 counterparts. The results of the in vivo analysis for fatty acid synthesis showed a twofold increase by the end of the fourth day of meal-eating.

In addition, the deposition of glycogen in the fat pad was also measured over the time course period. This experiment revealed that glycogen deposition steadily increased from the fifth through the tenth day of the time course. By the tenth day, glycogen levels were 200 percent above the 0 day level.

It is hypothesized that substrate concentration and increased metabolic flux are responsible for the initial hyperlipogenesis in meal-fed rats.

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Food Science and Human Nutrition

1974

ACKNOWLEDGMENTS

The author wishes to express his gratitude and appreciation to his major professor, Dr. Dale Romsos, for his patient advice and continuous encouragement through all aspects of the research that culminated in the writing of this thesis. Appreciation is also extended to Dr. Loren Bieber of the Department of Biochemistry for serving on the guidance committee and for critically reviewing this thesis.

Ineffable gratitude is extended to Dr. G. A. Leveille, Chairman of the Department of Food Science and Human Nutrition, for his efforts to secure a position and financial assistance on behalf of the author as well as serving on the guidance committee and critically reviewing this thesis.

Special acknowledgment goes to my wife, Sandy, for good-naturedly and zealously typing this thesis despite an overbearing and sometimes frenzied taskmaster.

The research performed in conjunction with this thesis was supported in part by NIH Training Grant Numbers GMO 1818-05 and AM-15847.

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LIST OF ABBREVIATIONS

ACC	Acetyl CoA carboxylase
ATP	Adenosine triphosphate
^{14}C-ACE	1-^{14}C-acetate
^{14}C-GLU	U-^{14}C-glucose
CCE	Citrate cleavage enzyme
CoA	Coenzyme A
FAS	Fatty acid synthetase
FAT SYN	Fatty acid synthesis
GLY	Glycogen
GSSG	Glutathione (oxidized)
ME	Malic enzyme

INTRODUCTION

Though the first reference to meal frequency which appears in the literature was written by Dr. von Seeland in 1887, the origin of modern day experimentation in meal feeding frequency begins in 1943 when Jay Tepperman, John Brobeck, and C.N.H. Long reported their findings on the effects of hypothalamic hyperphagia. Tepperman et al. (1943) observed that when animals with hypothalamic lesions were pair fed with control littermates, they would consume their entire ration in a matter of a few hours. Furthermore, all of the animals which had undergone surgery ate during daytime, which, according to Tepperman, "constitutes unusual eating behavior in our colony." In three pairs of animals, the rat with lesions gained weight more rapidly than the control animal fed the same amount of food. The greatest weight gain occurred in the animal which ate its daily ration of food in the shortest length of time (about one hour). This observation led Tepperman and his co-workers (1943) to perform an experiment specifically designed to ferret out the metabolic ramifications of quantity and time relative to food consumption.

A group of adult, female rats were permitted access to food and water three hours per day. These animals

exhibited the now classical response to meal-eating-- an initial decline in food consumption accompanied by weight loss and followed, after a period of several weeks, by a return of food intake to control levels and a corresponding weight gain (Tepperman et al., 1943). Once these normal animals had been trained to mimic the food eating habits of the pair fed animals with hypothalamic lesions, Tepperman et al., (1943) measured the respiratory quotients of trained versus untrained animals following an orally administered glucose load. The trained animals had significantly higher respiratory quotients which would be indicative of a greater rate of lipogenesis in trained animals. This experiment (Tepperman et al., 1943) clearly established that variations in the time required for the consumption of a ration of food can have a profound influence on the overall metabolism of the animal. Dickerson et al., (1943) incubated liver slices from meal-fed rats and ad libitum fed rats. They observed that the addition of glucose to the incubation media caused the respiratory quotients to rise sharply in the liver slices from the meal-fed rats while the respiratory quotients in the liver slices from the ad libitum fed animals remained virtually unchanged.

The years interceding 1942 and 1958 might be considered noteworthy by virtue of the dearth of reports in the literature relative to meal feeding. In 1958, Wakil and Lynen, working independently, reported the necessity

for bicarbonate ions in fat synthesis. Shortly thereafter, the scientific community was able to agree on the active pathway of lipogenesis. Consequently, there were many studies involved with finding ways to alter lipogenic activity. Most generally, these alterations took the form of reducing lipogenic activity by inducing diabetes, fat feeding, inanition, or fasting. Conversely, during this time, the isotope tracer technique was applied to determine if meal-eating results in an increased capacity to form fat from nonfat precursors (Tepperman, J. and Tepperman, H., 1958). Rats were trained to eat their daily food allowance in one hour. This training period lasted for at least six weeks before the actual experimentation was begun. In the first experiment, food was withdrawn from both the meal-fed animals and the controls. The Teppermans reported that the liver slices obtained from the meal-fed rats contained a significantly higher portion of labeled carbon derived from both acetate-1-^{14}C and glucose-U-^{14}C (Tepperman, J. and Tepperman, H., 1958). It was also noted that at the beginning of the liver incubation, the liver of the meal-fed rats contained about four times more glycogen than the controls (glycogen values were expressed as percent wet weight). The Teppermans further observed that while there were no significant differences in liver nitrogen content or liver weight, the stomachs of the meal-fed animals were extraordinarily distended immediately after feeding. In fact, when the volume of stomach contents were compared to

those of a meal-fed animal it was disclosed that the meal-fed animal had a volume of undigested food and water equal to about 22 ml while the control animals had a volume of undigested food equal to only 5 or 6 ml (Tepperman, J. and Tepperman, H., 1958). The Teppermans viewed their data on meal-feeding and lipogenesis cautiously because the large discrepancy in the volume of stomach contents implied that when food was removed from the control animals, their stomach contents would not sustain the absorptive state as long as those of the meal-fed rats. Thus, the apparently higher lipogenic activity of the liver slices from the meal-fed animals may simply have been due to the fact that the control rats had fasted longer. In order to resolve the problem brought about by this experiment, another experiment was performed in which the meal-eaters and the controls were fasted for 48 hours (Tepperman, J. and Tepperman, H., 1958). This prolonged fast brought about very low rates of lipogenesis in both groups; however, the lipogenic activity of the meal-fed animals was still significantly higher than the lipogenic rate of the control group. Under these fasting conditions, the meal-fed group had glycogen levels that had been depleted to the level of the control group. Now, convinced that the liver cell of the trained animals had a kind of "metabolic memory", the Teppermans embarked on still another experiment with an eye to view the effects of realimentation in two groups of animals treated as before (Tepperman, J. and Tepperman, H., 1958).

This time though, at the end of the 48 hour fast, the rats were given glucose by stomach tube and sacrificed five hours later. Once again, the meal-fed animals outperformed their nibbling counterparts by attaining significantly higher levels of hepatic glycogen, incorporating a greater amount of acetate-1-^{14}C label into the fat fraction of the liver, and by exhibiting a much greater ability to absorb glucose from the gastrointestinal tract. The supernormal rates of lipogenesis that were observed in this series of experiments led the Teppermans to perform an experiment which utilized normal rats that were fasted 48 hours and refed a high carbohydrate diet for 24 hours (Tepperman, J. and Tepperman, H., 1958). Liver slices from these animals showed an eight-fold higher capacity to synthesize fatty acids than either the prefasted control animals or the fasted but not refed animals. Concommitant with the increase in lipogenesis, the Teppermans observed a three-fold increase in hexosemonophosphate shunt activity (Tepperman, J. and Tepperman, H., 1958). The Teppermans coined the phrase "adaptive hyperlipogenesis" to describe the lipogenic events that occur when the fasted animals were refed (Tepperman, H. and Tepperman, J., 1958). They describe the hyperlipogenesis as adaptive "because the lipogenic activity of the liver in each circumstance seem to us to be teleologically appropriate to the nutritional state of the animal."

In another paper published in the same year, the Teppermans measured the activity of the pentose pathway

in rats that had been fasted 48 hours and refed either 0, 3, 6, 12, 24, or 48 hours (Tepperman, H. and Tepperman, J., 1958). When the curve of enzyme activity is plotted, one sees that the pentose pathway activity exponentially increases from 100 percent of the fasted value to 1400 percent of the fasted value.

While the Teppermans' experiments were doubtlessly the landmarks in the early studies of metabolic adaptation to a meal-feeding regimen, one must consider that these early experiments were all done with liver tissue. Since it has been shown that the liver is not the major organ for fatty acid synthesis in the rat, one must really look at the adaptations that are occurring in the adipose tissue before the full impact of feeding patterns and adaptation can be realized (Chakrabarty and Leveille, 1969).

A comparison of adaptations in the liver versus the adipose tissue was made in 1962 by Hollifield and Parson. They reported that rats fed ad libitum and then fasted for 24 hours had little liver glycogen and that the adipose tissue contained large amounts of free fatty acids but incorporated little acetate-1-^{14}C into lipids in vitro. In the animals fed 2 hours per day and studied immediately at the end of the feeding period on days one through seven, in vitro acetate-1-^{14}C incorporation into lipids by epididymal fat rose each day and by the fifth day was about 25 times that of the animals which were fed ad libitum, then fasted 22 hours and refed 2 hours on Day 1 (Hollifield

and Parson, 1962). Incorporation of acetate-1-^{14}C into fatty acids in liver slices of animals trained to meal eat rose only four-fold from day 1 through day 7.

Glucose-6-phosphate and 6-phospho-gluconate dehydrogenase activities (expressed as change in optical density/minute per mg nitrogen) rose in the adipose tissue and on the fifth day was over 200 percent of that on the first day of the feeding program. Glucose-6-phosphate and 6-phospho-gluconate dehydrogenase activities in the liver of these animals was much lower than in adipose tissue and rose only 25 and 40 percent respectively during the five day period (Hollifield and Parson, 1962).

Further studies on the effect of meal-eating on the adipose tissue were reported by Leveille and Hanson in 1965. A number of new facts relative to meal-eating and adipose tissue metabolism were revealed as a result of those studies. Leveille and Hanson established that: (1) the increased rate of lipogenesis is not due to a physiological response caused by a visceral reaction to a large bolus of food, (2) de novo enzyme synthesis stimulated by the sudden presence of substrate is not responsible for the hyperlipogenesis observed in the refed meal-eater since the intraperitoneal injection of puromycin failed to alter the refeeding response, (3) Though the meal-eating group ate less and weighed less, they had a significantly higher feed efficiency (11.1 percent) as compared to the nibbling animals (8.6 percent), (4) adipose tissue from meal-eaters

converted more glucose to CO_2, fatty acids, nonsaponifiable lipids, and glycogen than did tissue from nibbling animals, (5) the rate of β-oxidation in the liver was greatly increased while the rate in the muscle was not significantly different between meal-eaters and nibblers (Leveille and Hanson, 1965).

Leveille and Hanson made a second major contribution germane to metabolic adaptation in the epididymal fat of meal-fed rats in the following year (Leveille and Hanson, 1966). This paper contained information concerning adaptive changes in enzyme activity and metabolic pathways as well as differences in the ways that a high fat versus a high carbohydrate diet affected the adaptive process.

Briefly summarized, it was shown that a high fat diet markedly depressed rates of lipogenesis and the level of lipogenic activity in the meal-eater was not significantly different from the lipogenic activity of the nibbler. This study also revealed that the animals which had been fed once daily for two hours throughout a three week period had greatly increased levels of glucose-6-phosphate dehydrogenase (218 percent above the nibbling control) and NADP-malic dehydrogenase (409 percent above control). The other enzymes studied (NAD-malic dehydrogenase, isocitrate dehydrogenase, and 6-phosphogluconate dehydrogenase) showed no significant (P greater than 0.10) increases in the total activity as an effect of the meal-eating (Leveille and Hanson, 1966). Table 1 summarizes the influence of

meal-eating on a number of different enzymes in adipose tissue. It should be noted that the enzymes are grouped according to the function they perform.

The effects that can be wrought on various adaptive enzymes by feeding pattern alterations is undisputed. The literature is replete with data showing increased rates of lipogenesis, increases in enzyme activities and descriptions of alterations in the rates of glycogen depletion and accumulation. However, hitherto 1966, there had been no concerted effort to establish the order of events as they occur sequentially during the adaptive period. Then, at the end of 1966, a report concerning the time sequence of adaptive enzyme changes and changes in the rate of lipogenesis appeared (Leveille, 1966). This paper involved the experiments performed by G. A. Leveille in which he measured the activities of malic enzyme, glucose-6-phosphate dehydrogenase, 6-phospho-gluconate dehydrogenase, and the rate of acetate-1-^{14}C incorporation into fatty acids in meal-fed rats and chickens. Hepatic changes were examined in meal-fed chickens (Figure 1) and the adipose tissue changes were explored in the meal-fed rat (Figure 2). This paper constitutes an extremely important contribution to the area of meal-feeding studies by virtue of its content and especially its concept. The sum of the work done in this area previously could be considered as an interesting repository of factual, but somewhat discontinuous observations. If the exact mechanism of adaptation is to ever be elucidated, the

Table 1. Activity of various enzymes in adipose tissue of meal-fed and nibbling rats (Leveille, 1970).

Metabolic Process and Enzyme Studied	Feeding Regimen		% Difference[b]
	Ad libitum	Meal-fed	
	units/mg protein[a]		
Pyruvate and α-glycerophosphate formation			
Hexokinase	6	23	283
Phosphofructokinase	5	6	20*
Pyruvate kinase	73	102	40
α-Glycerophosphate dehydrogenase	704	1,190	69
Fatty acid synthesis			
Citrate cleavage enzyme	3	33	1,000
Acetyl-CoA carboxylase	7	16	129
Fatty acid synthetase	3	8	167
NADPH production			
Glucose 6-phosphate dehydrogenase	41	130	217
6-Phosphogluconate dehydrogenase	23	38	65
Pyruvate carboxylase	32	142	344
Malic dehydrogenase	2,114	3,085	46*
Malic enzyme	38	199	424
Isocitrate dehydrogenase	43	56	30

[a]A unit is defined as the transformation of 1 nanomole of substrate per minute at 25°. [b]Percentage increase due to meal ingestion; an asterisk indicates that the difference is not significant statistically.

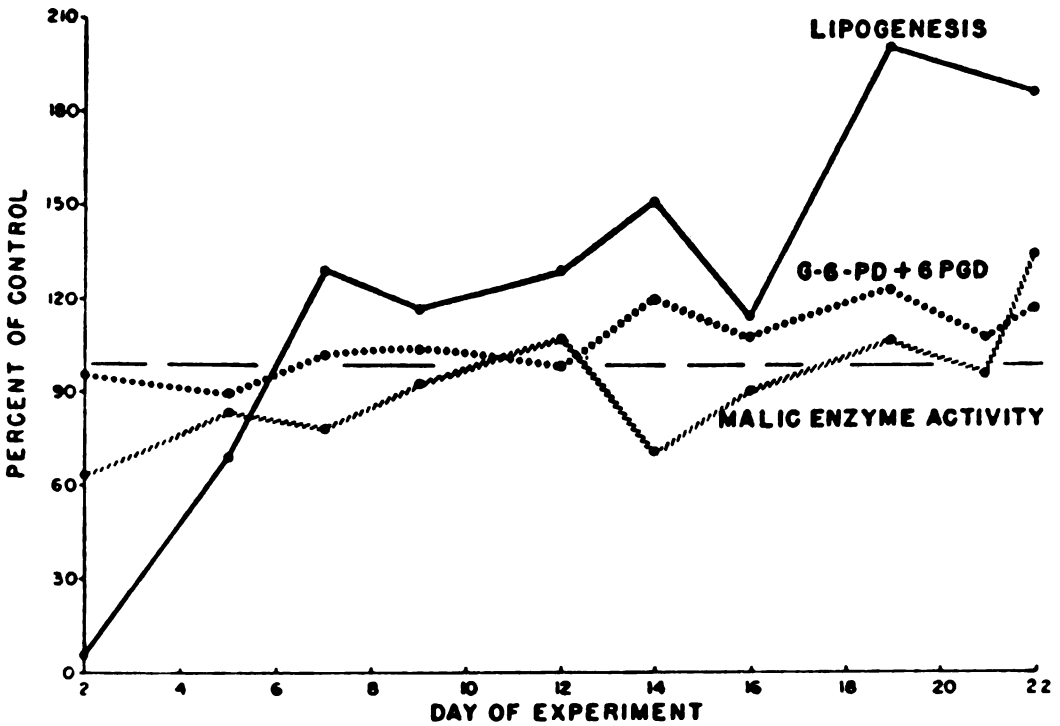


Fig. 1.--A time sequence study of adaptation to meal-eating in chick liver (Leveille, 1966).

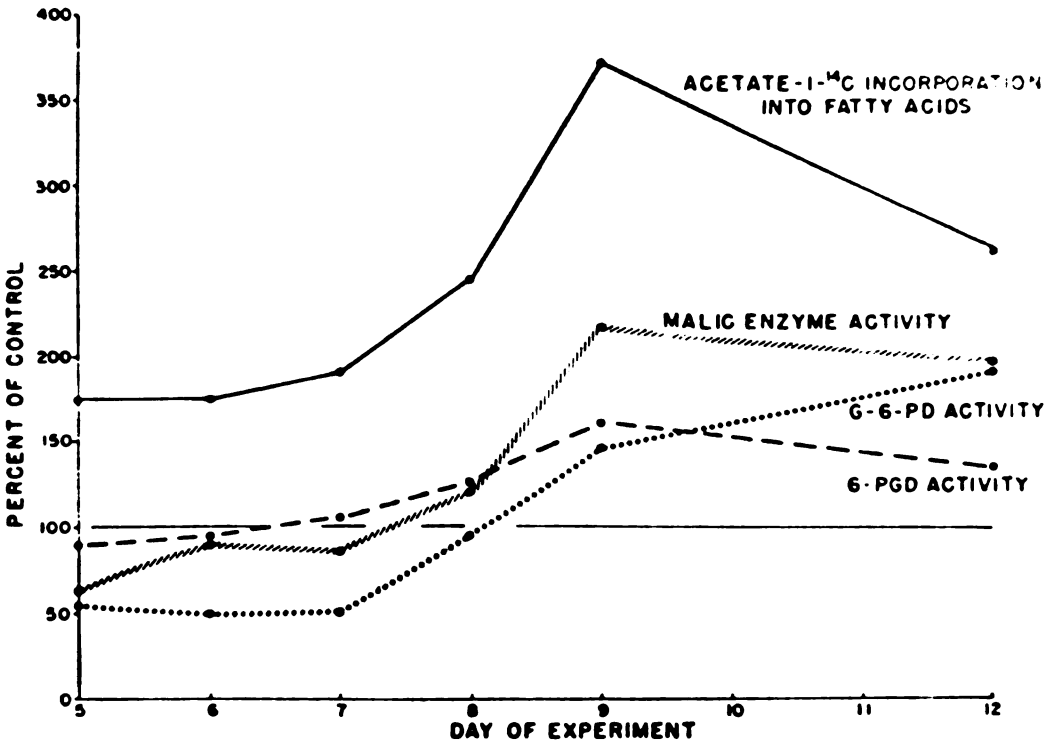


Fig. 2.--A time sequence study of adaptation to meal-eating in rat adipose tissue (Leveille, 1966).

sequence of changes must be ordered as a first step in separating causes from effects. It is the purpose of this thesis to order some of the metabolic adaptations that are observed when rats are forced to change their pattern of food intake.

EXPERIMENTAL

Materials

Animals

The rats used in this study were a Sprague-Dawley strain obtained from Spartan Research Animals Inc., Haslett, Michigan. All rats used in this study were males weighing 200 to 240 g. Males rather than females were used preferentially because they possess epididymal fat pads which offer a large and quickly obtainable source of adipose tissue. Rats within the weight range chosen adapt more quickly and dramatically than younger rats. Younger rats, weighing less than 200 g are not able to consume a sufficient quantity of food within a two hour eating period to maintain a body weight gain.

Diets

Wayne Lablox, a standard laboratory chow tailored to meet the needs of laboratory rats, was fed to all of the animals pending the day when they were placed on a meal-feeding regimen. A semi-purified diet was fed to the rats on the day (Day 0) that they were switched from ad libitum feeding to a time restricted eating pattern. The composition of this diet is listed in Tables 2 through 4.

Table 2. Rat vitamin mix, supplied in mg/kg of diet when fed at the rate of 0.4% of the diet (Yeh and Leveille, 1969).

Vitamin	Mg/Kg diet
Ascorbic acid	200.0
p-Amino benzoic acid	110.0
Inositol	100.0
Niacin	100.0
Calcium pantothenate	66.0
Menadione	50.0
Pyridoxine	22.0
Riboflavin	22.0
Thiamine HC1	22.0
Folic acid	4.0
Biotin	0.6
Vitamin B_{12}	0.3
Vitamin A	20,000 IU
Vitamin D_3	2,200 IU
Alpha tocopherol acetate	100 IU

Table 3. Percentage composition of rat mineral mix (Leveille and O'Hea, 1967).

Mineral	Percent
Calcium Phosphate (Dibasic)	35.510
Potassium Citrate	23.650
Calcium Carbonate	16.360
Sodium Chloride	10.810
Potassium Phosphate (Dibasic)	7.730
Magnesium Carbonate	4.090
Ferric Citrate	1.600
Manganese Sulfate	0.140
Zinc Carbonate	0.040
Cuprous Sulfate	0.020
Potassium Iodide	0.004

Table 4. Percentage composition of the semi-purified diet fed to meal-eating rats.

Ingredient	Percent
Glucose	56.0
Casein	30.0
Corn Oil	5.0
Mineral Mix	4.0
Solka Floc	4.0
Vitamin Mix	0.4
Methionine	0.3
Choline Chloride	0.2

Chemicals and Reagents

Buffers and solutions.--All reagents used in the preparation of buffers and solutions used for chemical analysis met the purity standards for, and were labeled as, A.C.S. Reagent Grade.

Biochemicals.--The cofactors NADPH, and NADH as well as CoA and ATP were ordered from Sigma Chemical Co., St. Louis, Missouri. Acetyl CoA and malonyl CoA were ordered from P.L. Biochemicals, Milwaukee, Wisconsin. Malate dehydrogenase was obtained from Boehringer Mannheim.

Carbon-labeled isotopes.--Glucose-U-^{14}C and acetate-1-^{14}C were ordered from New England Nuclear, Boston, Massachusetts; while, the $NaH^{14}CO_3$ was ordered from Amersham/Searle, Arlington Heights, Illinois.

Hydrogen-labeled isotopes.--Tritiated water was procured from New England Nuclear, Boston, Massachusetts.

Scintillators.--Omnifluor, a premixed scintillor, was ordered from New England Nuclear, Boston, Massachusetts.

Methods

Animal Environment

The rats were housed singly in metal cages having raised wire floors. The ambient temperature was mechanically controlled and maintained at $21^{\circ} \pm 1^{\circ}$. Room lights

were turned on at 7:00 A.M. and turned off at 7:00 P.M. daily by a motor driven timer. Water was available to all animals at all times. Food was available to some rats continuously while other rats had access to food for a two hour period (8:00 A.M. to 10:00 A.M.).

Body weight.--Body weights of the animals were measured on the day the animals were received. Animals not within the weight range specified in the materials section were excluded from the experiment. Body weights of experimental animals were measured again on the day they were switched to a meal-eating regimen and finally on the day they were to be sacrificed. All body weights were measured prior to presenting the meals to the rats.

Food intake.--Food intake was measured only in the animals with the restricted access to food. The food cups were weighed prior to being placed in the animals' cages. The food cups were weighed again after the animals had been sacrificed. The difference between the two weights represented the total amount of food the rat had eaten from its first meal through the last meal it had eaten prior to sacrifice. When this total food intake was divided by the number of meals the rat had been given, the average weight of food consumed per meal was determined. The amount of food spilled from the cups by the rats was negligible.

Experimental Design

The experimental design used in initiating the changes in feeding patterns is illustrated in Figure 3. The rats were given two days of ad lib feeding to adjust to the new environment before any experimental changes were begun. An experimental group consisted of six animals. When that group started the meal-eating regimen, all of the "chow" type biscuits were removed from the cage at 8:00 A.M. Next, the animals were weighed and a cup of the semi-purified diet was fed to the rats and removed from the cages at the same time daily until the animals were sacrificed.

The day of these dietary manipulations is Day 0 of the meal-feeding program. The next day, a second group of six animals would be started on the meal-feeding regimen. By staggering the starting times in this way, it was possible to sacrifice all of the animals on the same day; thus, blocking a number of experimental variables that could occur had the animals been sacrificed on a day-by-day basis.

Sacrifice.--Two animals from each experimental group were serially killed by decapitation. Immediately after an animal was sacrificed, both epididymal fat pads were excised and weighed. Approximately 1 g of the fat pad was placed in a buffer for homogenization and a 150 to 200 mg sample was placed in a buffer for the determination of the rate of fatty acid synthesis. This procedure was repeated until all of the animals in an experiment had been processed.

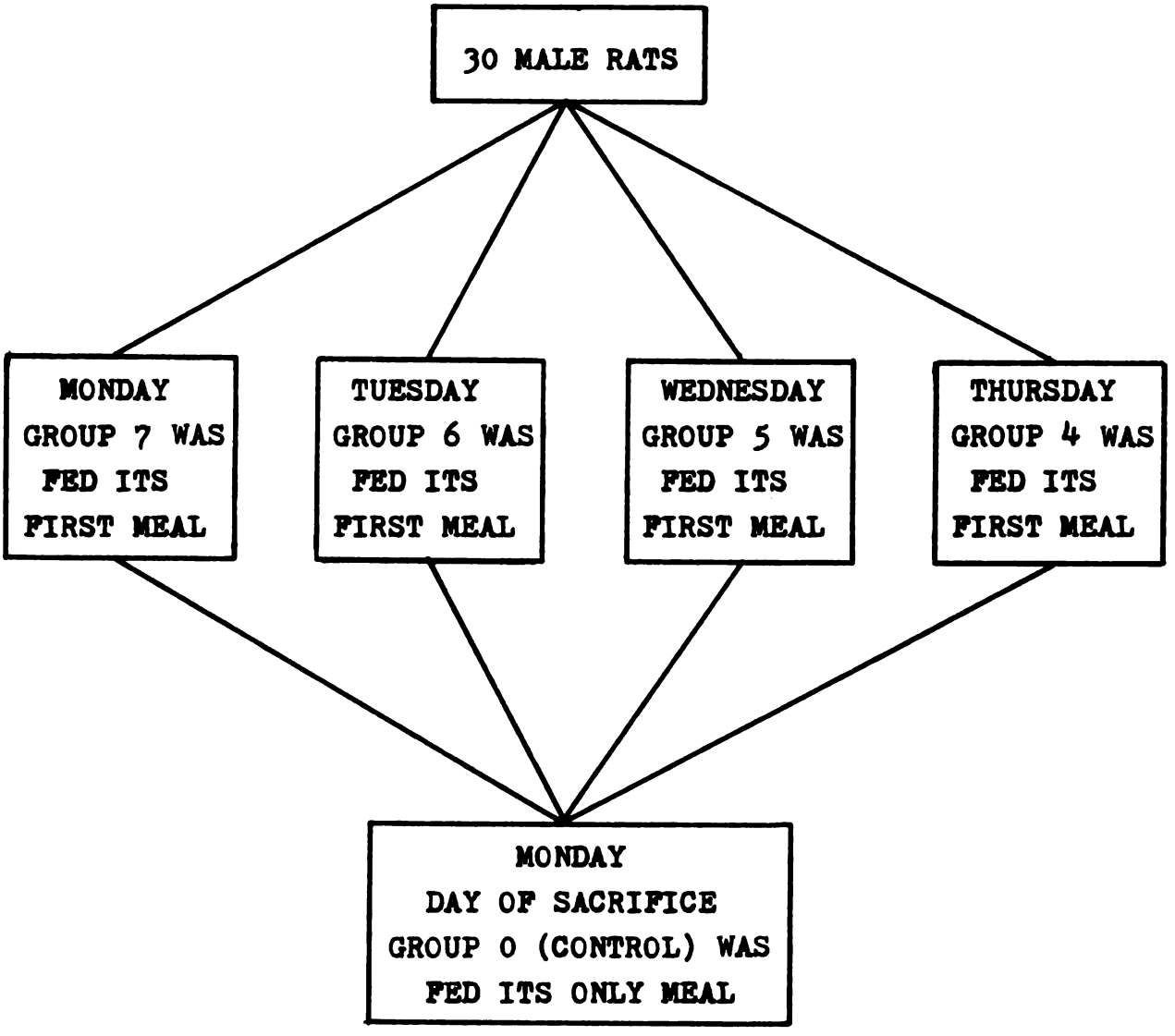


Fig. 3.--Diagramatic representation of the experimental design used throughout this study. The group number is equal to the number of days that a particular group of rats has been meal-fed.

Tissue Preparation

Homogenization.--A piece of adipose tissue was weighed and placed in a 18 mm X 150 mm test tube containing cold buffer. The ratio of tissue to buffer was fixed at 1 g of fat pad per 10 ml of buffer. The composition of the buffer is outlined in Table 5. The adipose tissue was homogenized for approximately 30 seconds with a Polytron tissue homogenizer. After homogenization the samples were kept on ice pending centrifugation.

Table 5. Constituency of the homogenization buffer (pH 7.0).

Sucrose	250 mM
Tris (hydroxymethyl) nitromethane	30 mM
β-mercaptoethanol	2 mM
(Ethylenedinitrilo)-tetraacetic acid	1 mM

Centrifugation.--Homogenized fat samples were centrifuged in a Spinco Model L3-40 ultracentrifuge at 100,000 X gravity for 45 minutes. The centrifuge chamber was maintained at 5°. After centrifugation, the supernatant fluid containing the soluble portion of the tissue homogenate was transferred from the centrifuge tubes to test tubes and placed on ice.

Fatty Acid Assay Techniques

In vitro rate of fatty acid synthesis.--A Krebs-Ringer-Bicarbonate solution (DeLuca and Cohen, 1964) was used as the

incubation medium after it had been slightly modified to make it more suitable for use with adipose tissue. The modification consisted of: (1) the addition of 10 units (24 mg per unit) of porcine insulin per 100 ml of buffer, (2) the addition of a quantity of glucose to the solution sufficient to make it 10 mM, (3) the addition of either glucose-U-^{14}C or acetate-1-^{14}C in sufficient quantity to yield 0.1 uCi of radioactivity per ml of buffer. The buffer was gassed with a 5% CO_2-95% O_2 mixture for 10 minutes. The pH of the completed buffer was 7.4. A 3 ml aliquot of the buffer was pipetted into 25 ml Erlenmeyer flasks. A 150 to 200 mg piece of the fat pad was taken from the distal portion of the excised epididymal fat and added to a flask containing the buffer. The flask was placed in a 37° Dubnoff Metabolic Shaker for a 2 hour incubation. An atmosphere of 5% CO_2-95% O_2 was maintained. All of the samples were incubated in duplicate. At the end of the incubation period, the flask was removed from the incubator and the tissue was removed from the flask, blotted lightly on filter paper, successively dipped into three beakers of saline, and lightly blotted again to remove any of the radioactive buffer. Finally, the tissue was dropped into a 20 X 150 mm screw top culture tube containing 3 ml of 30% potassium hydroxide. The culture tubes were set aside for saponification and extraction on the following day.

In vivo rate of fatty acid synthesis.--In one experiment, the rats were subjected to the in vivo measurement of the rate of fatty acid synthesis. The in vivo technique

consisted of injecting 1 uCi of tritiated water diluted to 1.0 ml with 0.9% saline per animal. Each rat was injected exactly one-half hour before sacrifice. After sacrificing the rat, a portion of the excised fat pad was weighed and placed directly into a screw top culture tube containing 3 ml of 30% potassium hydroxide. The culture tubes were set aside for saponification and extraction on the following day.

Saponification.--Saponification, the process of breaking triglycerides into free fatty acids and glycerol, was achieved by adding 10 ml of absolute ethyl alcohol to the culture tubes containing the portion of the epididymal fat pad and the potassium hydroxide. Marbles were placed on the tops of the tubes to reduce evaporation and the tubes were placed in an 85° water bath for 2 hours.

Extraction.--After the samples had been saponified, the tubes were cooled to room temperature and 10 ml of distilled water was added to each of them. Approximately 2 ml of 6 N HCl was then added to each of the tubes to acidify the medium. The efficacy of acidification was checked in every tube with Congo Red test paper. Extraction was accomplished by adding 5 ml of petroleum ether to the tubes, capping them, and mixing each tube on a Vortex Mixer. After mixing, the tubes were set aside until the aqueous and organic phases in the tubes had separated. The organic phase (containing the radioactive fatty acids as well as a negligible amount of nonsaponifiable lipids) was removed with a Pasteur Pipette and placed into plastic scintillation vials.

The extraction process was repeated for each culture tube two more times. The scintillation vials were left uncovered and placed in a forced draft hood to allow all of the ether to evaporate. Evaporation to dryness usually required 12 to 15 hours.

Scintillation Counting.--A scintillation fluid consisting of 4.0 g of Omnifluor, 230 ml of ethyl alcohol, and diluted to 1,000 ml with tolulene was prepared. Each of the scintillation vials received a 10 ml portion of this scintillation fluid. The β radiation emitted by the samples was counted in a Packard Tri-Carb Scintillation Spectrometer, Model 3310. A set of progressively quenched standards were also counted and efficiency was plotted against the channels ratio; thus, it was possible to calculate the counting efficiency of the fatty acids dissolved in the scintillation fluid.

Enzyme Assay Techniques

Acetyl CoA carboxylase.--The total activity of acetyl CoA carboxylase (EC 6.4.1.2) was assayed by modifying the method of Numa, 1969. The modification consisted of adding $NaH^{14}CO_3$ to the reaction mix. The samples were assayed in test tubes measuring 10 X 75 mm containing 500 ul of reaction mix and 20, 50, or 100 ul of tissue homogenate. The tubes were pre-incubated for one-half hour to allow the citrate in the reaction mix to fully activate the acetyl CoA carboxylase.

At the end of the pre-incubation period, 20 ul of 0.1 M ATP was added to the sample tubes and 200 ul of the sample milieu was immediately withdrawn and placed in a scintillation vial containing 200 ul of 6 N HCl. This vial was labeled as "time 0" for that sample. The sample left in the test tube with the reaction mix was allowed to continue incubating at 37° for an additional eight minutes. At the end of the incubation period, 200 ul of sample were withdrawn from the reaction tube and placed in a second scintillation vial containing 200 ul of 6 N HCl and labeled as "time 8". All scintillation vials were set aside until they had evaporated to dryness. When completely dry, 10 ml of a scintillation fluid was added to the vials and the β-emmisions were counted in a Packard Spectrometer.

Citrate cleavage enzyme.--The total activity of citrate cleavage enzyme (EC 4.1.3.8) was assayed by the method of Srere, 1959. A 20 ul sample aliquot was added to the reaction mix and placed in a Gilford Spectrophotometer Model 240 to establish the amount of nonspecific background activity present. The reaction was started by the addition of 100 ul of 2 mM CoA.

Fatty acid synthetase.--The total activity of the multienzyme fatty acid synthetase complex was measured by the method of Hsu et al., 1969. A sample aliquot of 20 ul was added to the 0.8 ml of reaction mix followed by 0.1 ml of a 10 mM NADPH solution. This mixture was placed in a Gilford Spectrophotometer Model 240 to establish the amount of

nonspecific background present. After recording the background activity, the reaction was started by adding 0.1 ml of a 1 mM malonyl CoA solution.

Malic enzyme.--The total activity of malic enzyme (EC 1.1.1.40) was measured by the method of Ochoa et al., 1948. A 50 ul sample aliquot was added to the reaction mix and placed in a Gilford Spectrophotometer Model 240 to establish the amount of nonspecific background activity present. The reaction was started by the addition of 50 ul of L-malate.

Methods for Chemical Determinations

Protein.--The protein concentrations of the sample homogenates were determined by reacting the aromatic amino acids with phenol and measuring the optical density of the resulting color in the reaction mixture (Lowry, et al., 1959).

Glycogen.--Adipose tissue samples excised for glycogen determination were first placed in a 2:1 chloroform:methyl alcohol mixture and shaken overnight to extract as much of the lipid material as possible. The adipose tissue "ghost" was removed from the extraction solvent, dried overnight at room temperature, and weighed. The dried tissue was placed in a 18 X 125 mm test tube containing 3 ml of a 30% KOH solution. The tubes were covered with marbles and placed in a boiling water bath for one hour with occasional mixing. The glycogen was precipitated by the addition of ethyl alcohol and sodium sulfate according to the method of Van Handel,

1965. The glycogen precipitate was washed in ethyl alcohol and sodium sulfate twice and finally dissolved in water. The quantitative analysis of the dissolved glycogen was determined colorimetrically by the anthrone method as described by Van Handel, 1965.

RESULTS

General Signs of Adaptation

Weight Changes

Figure 4 shows the amount of weight lost or gained by the various groups as a function of meal-eating. Animals that have been meal-fed 4, 5, and 6 days show the greatest weight loss--approximately 25 g or nearly 10 percent of the total body weight they registered at the beginning of the meal-feeding regimen. Starting with the seventh day, the meal-fed rats begin to gain weight and by Day 10, the animals weigh about 8 g more than they did at the beginning of the meal-feeding period. Meanwhile, the control group or ad libitum fed animals gained weight steadily. During the series of seven day experiments, the control group gained nearly 60 g and during the ten day experiment, the control group gained a total of 85 g. These weight changes are characteristic of adaptation to meal-eating (Leveille, 1970).

Food Consumption

Figure 5 defines the pattern of food consumption during the adaptation to meal-eating. Typically, the first time the meal cups were placed in the cages (Day 0)

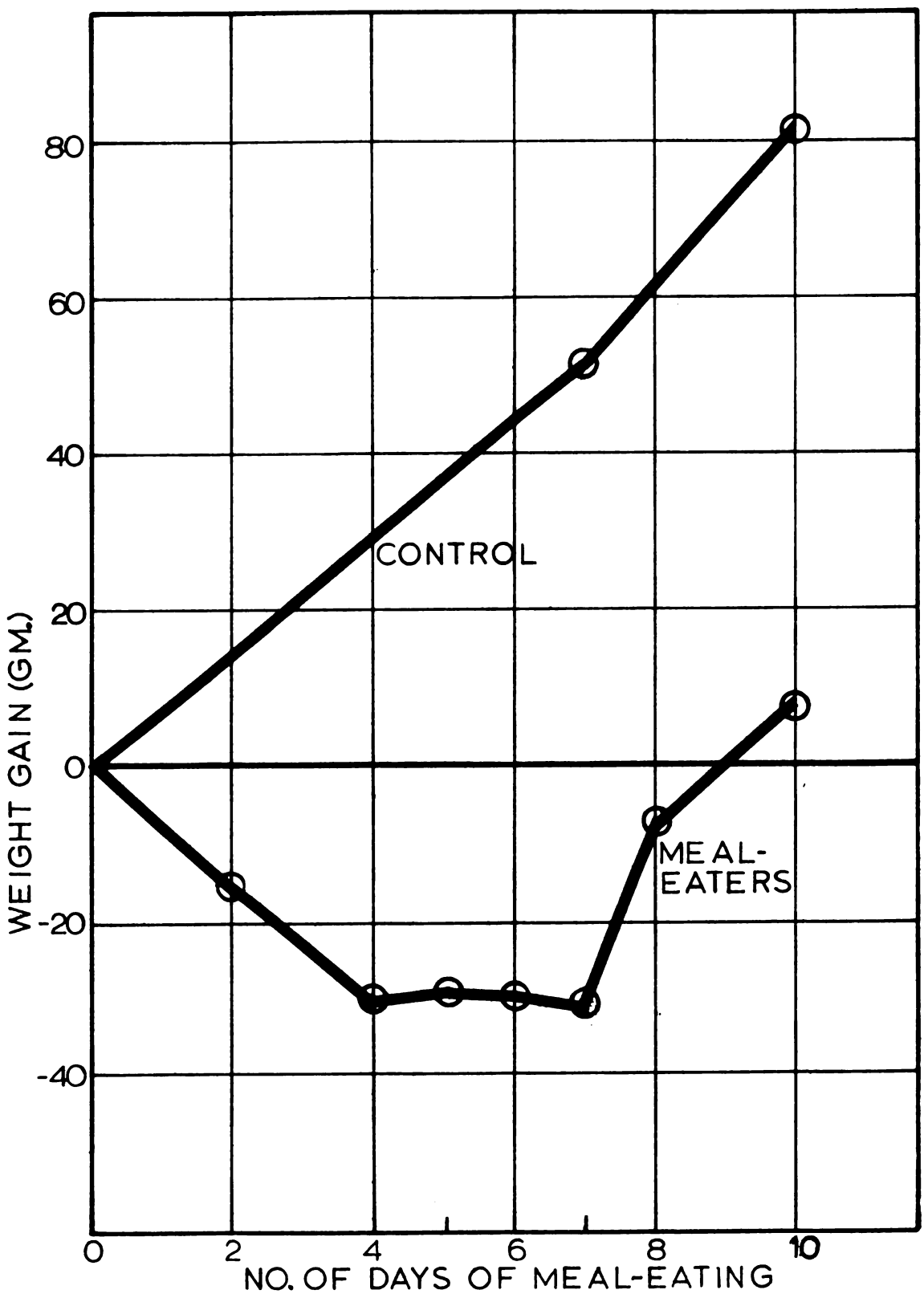


Fig. 4.--Body weight changes of control and meal-eating rats throughout the time course (each point represents the mean for 6 rats).

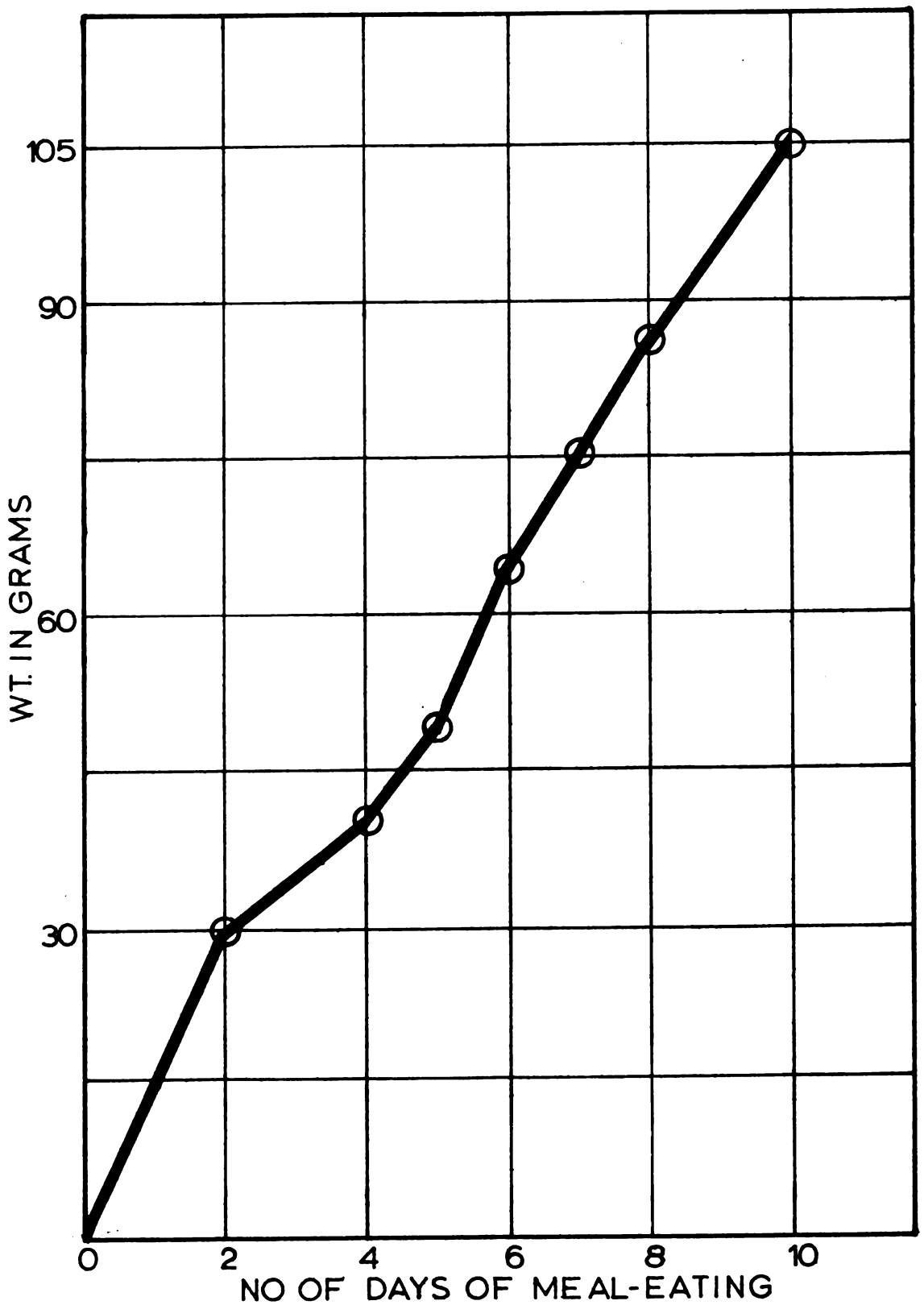


Fig. 5.--Total food consumption of the various groups during adaptation (each point represents the mean for 6 rats).

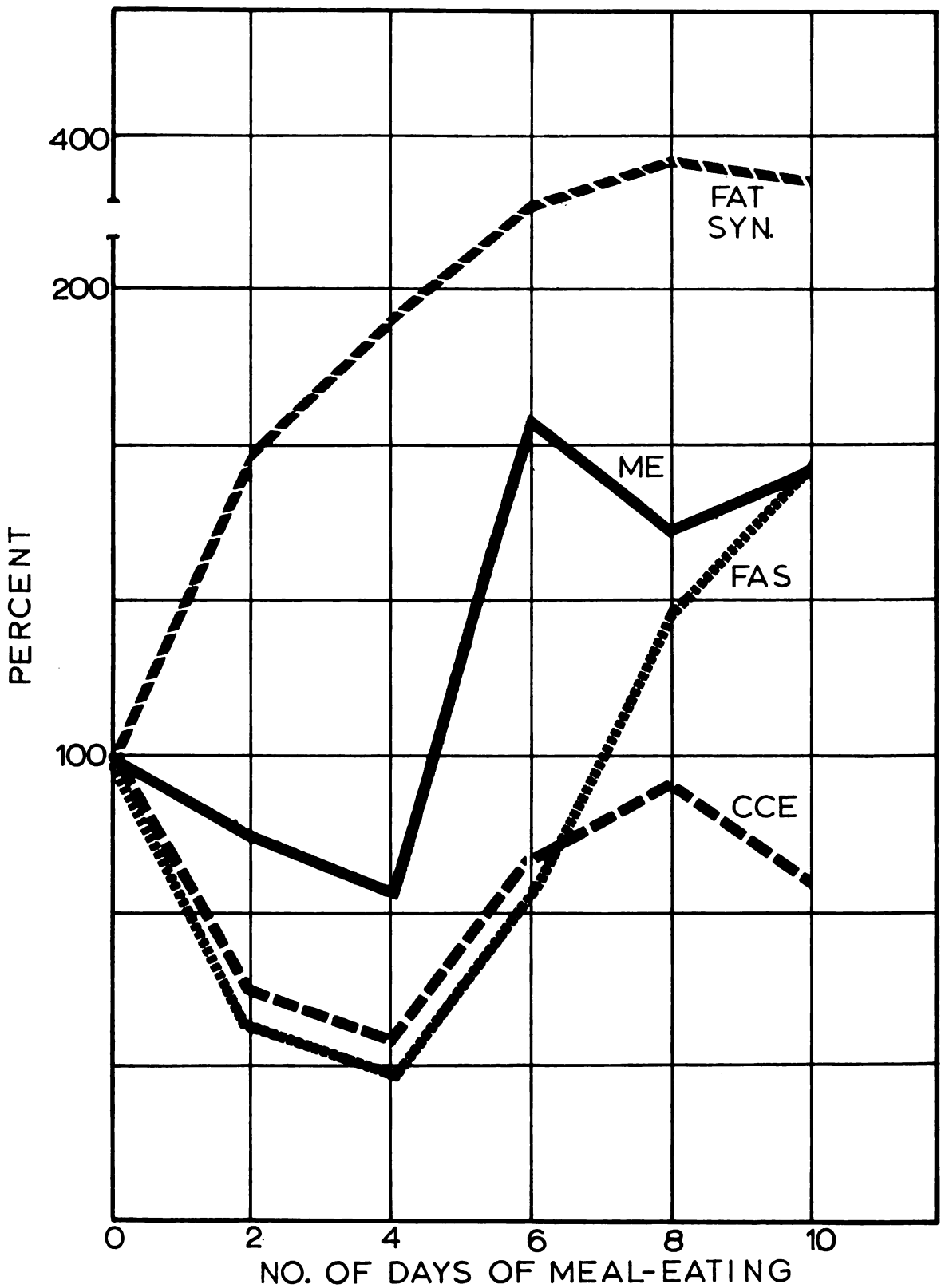
the rats declined to eat any of the diet. The rest of the points on the graph represent the total amount of food the animals ate during all of the days that they were on the meal-eating regimen.

Enzyme Adaptation

Experiment I.--In order to avoid a large, unwieldly experiment, six groups of five rats were used and their response to meal-eating was measured by sacrificing a group of rats on the second, fourth, sixth, eighth, and tenth day after starting the meal-eating regimen. An additional group of rats was used as a control group and labeled as the group sacrificed on Day 0.

The changes in enzyme activities and the rate of fatty acid synthesis in adipose tissue are shown graphically as percentages of the control group (Figure 6) and tabulated as absolute values (Table 6). Initially, enzyme activities are calculated on the basis of micromole of substrate converted to product per minute per mg of protein (Table 6). Then, using the Day 0 or control value as representative of 100% or normal activity, the activities of FAS, CCE, and ME as well as the rate of fatty acid synthesis were plotted as percentages of normal activity (Figure 6). The enzyme curves show a decrease in total activity ranging from 35% (ME) to 33% (FAS) by the end of the fourth day of meal-eating. By the end of the sixth

Fig. 6.--Influence of adaptation to meal-eating on in vitro rates of fatty acid synthesis and lipogenic enzyme activities in adipose tissue from rats meal-fed for 0, 2, 4, 6, 8, or 10 days (each point represents the mean for six rats).


400
200
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FAT
SYN.
ME
FAS
CCE
0
2
4
6
8
10
NO. OF DAYS OF MEAL-EATING

Fig. 6.

Table 6. The effects of adaptation to meal-eating on in vivo synthesis and lipogenic enzyme activities.

	Days of adaptation to meal-eating					
	0	2	4	6	8	10
Citrate cleavage enzyme[2]	98±15	49±6[4]	38±6[4]	74±14	90±17	71±10
Fatty acid synthetase[2]	34±4	13±2	11±2[4]	23±4	45±14	55±8
Malic enzyme[2]	141±22	114±19	99±13	255±13	206±54	221±42
Fatty acid synthesis[3]	253±15	408±84	464±65	743±225	925±193[4]	892±186[4]

[1]Values are means ±SEM for 6 rats. [2]Nanomoles of substrate converted to product/min per mg soluble protein. [3]Nanomoles of U-^{14}C glucose incorporated into fatty acids per 100 mg of fat pad weight during a 2 hr incubation. [4]Value significantly different from Day 0 as determined by Dunnett's T test $P<.05$.

day of meal-eating the total activity of the enzymes has risen to within 33% of the control activity (FAS and CCE) or more (ME).

Contrarily, the rate of fatty acid synthesis rises sharply within two days of meal-eating (160%). After eight days of meal-eating, the rate of fatty acid synthesis is nearly 370% of the control value.

Experiment II.--The most pronounced changes in enzyme activity in the first experiment took place between the fourth and eighth day of meal-eating. With this in mind, the second experiment was performed with animals that had been meal-fed for 0,4,5,6,7, or 8 days. The results of this experiment are shown in Figure 7 and Table 7. Once again, average enzyme activity is decreased by more than 80% while the rate of fatty acid synthesis as measured in vitro steadily increases--reaching a level 180% above the control value by Day 8. Based on the observations made in this experiment, it was decided to carry out future experiments for a time period consisting of Days 0,4,5,6, and 7.

Experiment III.--This experiment was performed to ascertain whether or not the rate of fatty acid synthesis is limited by the rate of flow of glucose through the glycolytic pathway. This problem was attacked by performing side-by-side incubations utilizing either glucose-U-^{14}C or acetate-1-^{14}C. Figure 8 and Table 8 show the results of this experiment. Once again, a 30% (ME) to 70% (FAS) decrease in

Fig. 7.--Influence of adaptation to meal-eating on in vitro rates of fatty acid synthesis and lipogenic enzyme activities in adipose tissue from rats meal-fed for 0, 4, 5, 6, 7, or 8 days (each point represents the mean for six rats.

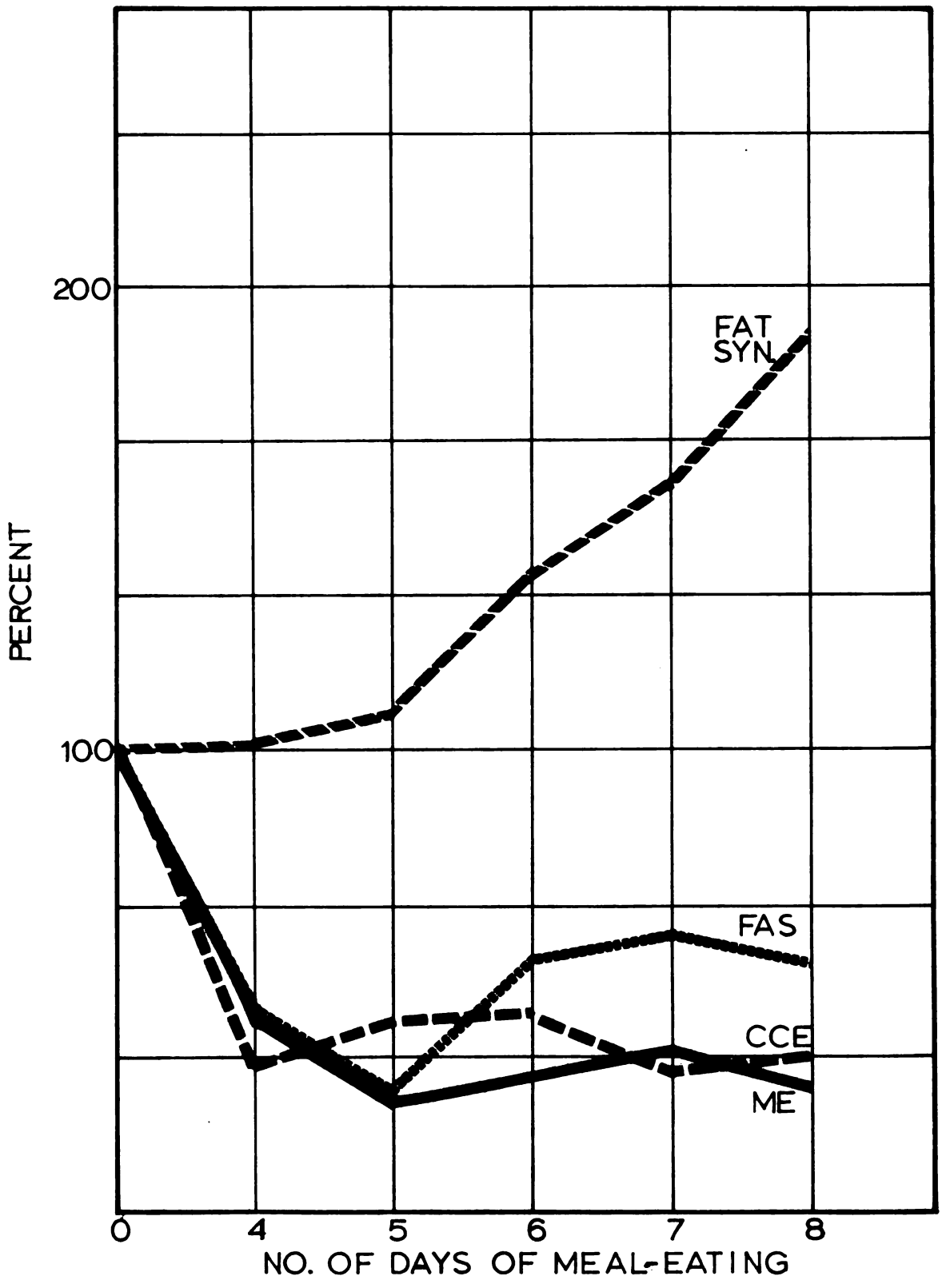
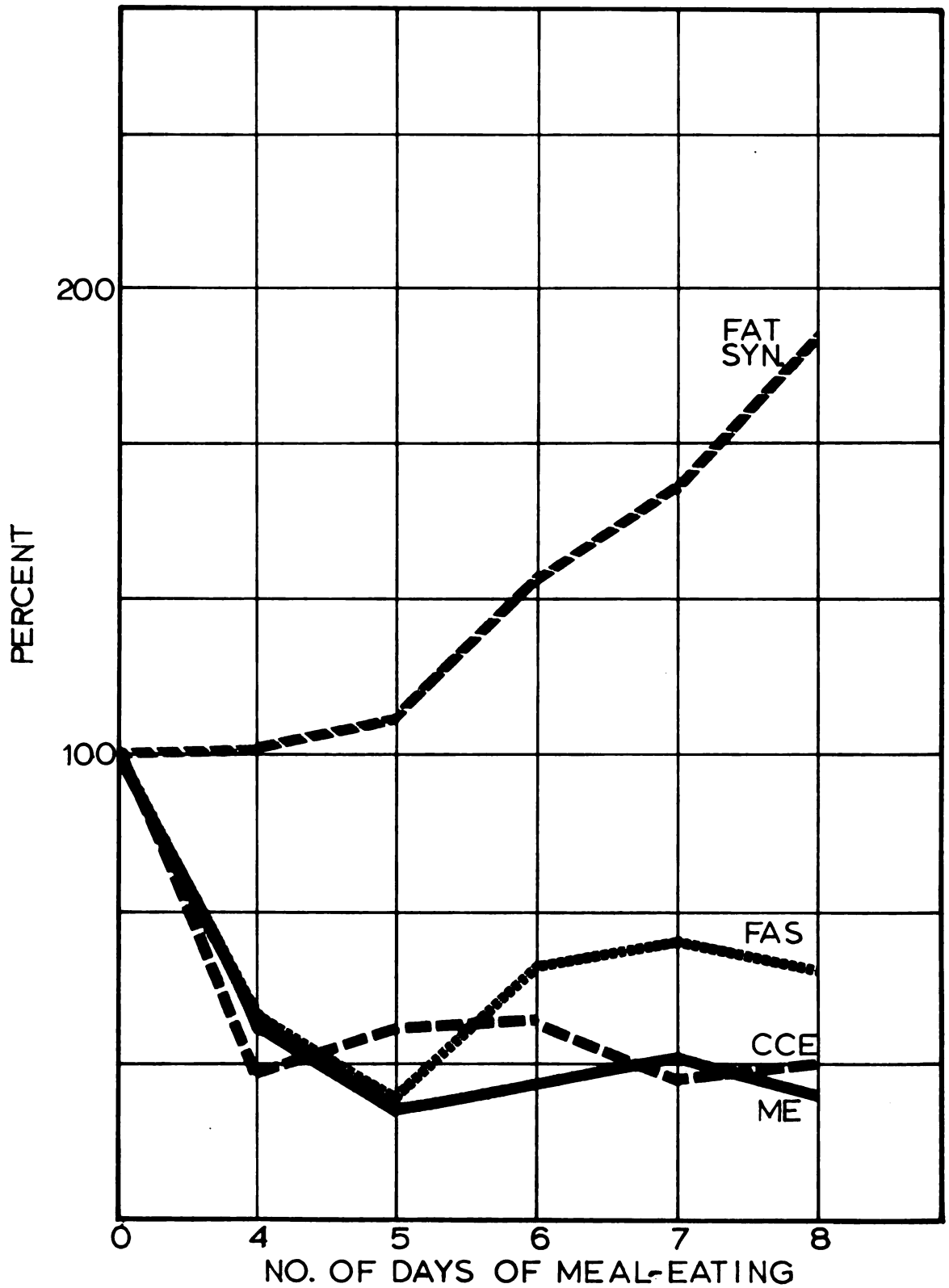

200
100
PERCENT
FAT SYN.
FAS
CCE
ME
0
4
5
6
7
8
NO. OF DAYS OF MEAL-EATING

Fig. 7.

Fig. 7.--Influence of adaptation to meal-eating on in vitro rates of fatty acid synthesis and lipogenic enzyme activities in adipose tissue from rats meal-fed for 0, 4, 5, 6, 7, or 8 days (each point represents the mean for six rats.


200
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0
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8
NO. OF DAYS OF MEAL-EATING

Fig. 7.

Table 7. Influence of adaptation to meal-eating on in vitro rates of fatty acid synthesis and lipogenic enzyme activities in adipose tissue from rats meal-fed for 4, 5, 6, 7, or 8 days.

	Days of adaptation to meal-eating					
	0	4	5	6	7	8
Citrate cleavage enzyme[2]	55±22	17±4[4]	21±5[4]	21±4[4]	16±3[4]	18±4[4]
Fatty acid synthetase[2]	12±4	4±2	3±0.5[4]	6±2	6±2	4±1
Malic enzyme[2]	283±107	101±31	63±9[4]	85±8	95±15[4]	75±20[4]
Fatty acid synthesis[3]	400±68	387±41	422±53	564±61	590±99	729±60[4]

[1]Values are means ±SEM for 6 rats. [2]Nanomoles of substrate converted to product/min per mg soluble protein. [3]Nanomoles of U-^{14}C glucose incorporated into fatty acids per 100 mg of fat pad weight during a 2 hr incubation. [4]Value significantly different from Day 0 as determined by Dunnett's T test $P<.05$.

Fig. 8.--Influence of adaptation to meal-eating on lipogenic enzyme activities and in vitro rates of fatty acid synthesis using either 1-^{14}C-acetate or U-^{14}C-glucose as substrate for reactions occurring in the adipose tissue from rats meal-fed for 0, 4, 5, 6, or 7 days (each point represents the mean for six rats).

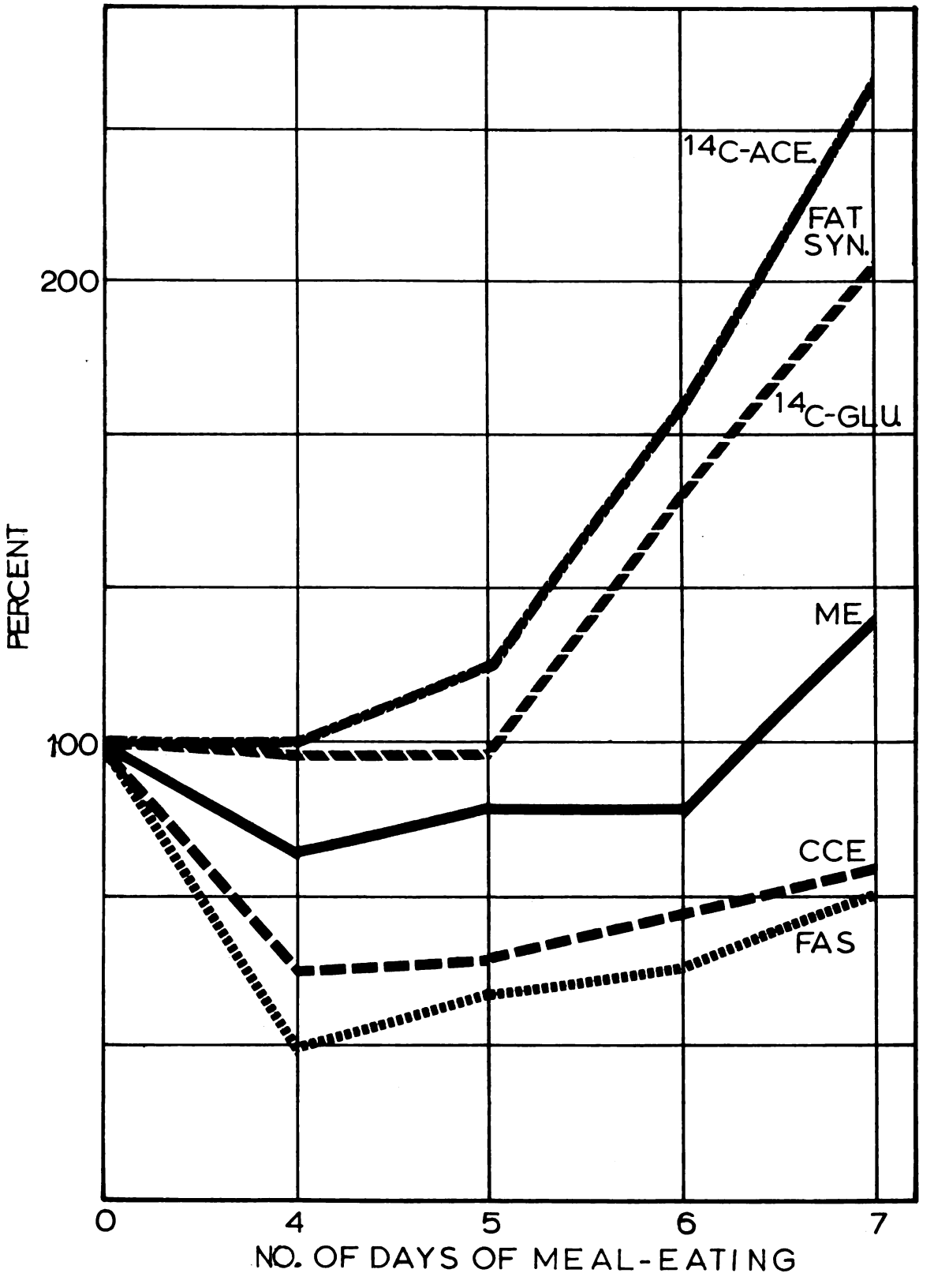

14C-ACE.
FAT SYN.
14C-GLU.
ME
CCE
FAS
200
100
PERCENT
0
4
5
6
7
NO. OF DAYS OF MEAL-EATING

Fig. 8.

Table 8. Influence of adaptation to meal-eating on lipogenic enzyme activities and in vitro rates of fatty acid synthesis using either 1-^{14}C acetate or U-^{14}C glucose as substrate for fatty acid synthesis occurring in the adipose tissue from rats meal-fed for 0, 4, 5, 6, or 7 days.[1]

	Days of adaptation to meal-eating				
	0	4	5	6	7
Citrate cleavage enzyme[2]	39±11	18±11	20±6	28±8	28±8
Fatty acid synthetase[2]	20±2	6±4[4]	8±2[4]	8±2[4]	14±3
Malic enzyme[2]	72±9	57±4	65±8	70±18	95±17
Fatty acid synthesis[3]					
1-^{14}C-acetate	383±55	357±73	384±53	617±101	856±119
U-^{14}C-glucose	400±68	365±73	440±36	648±68	992±116

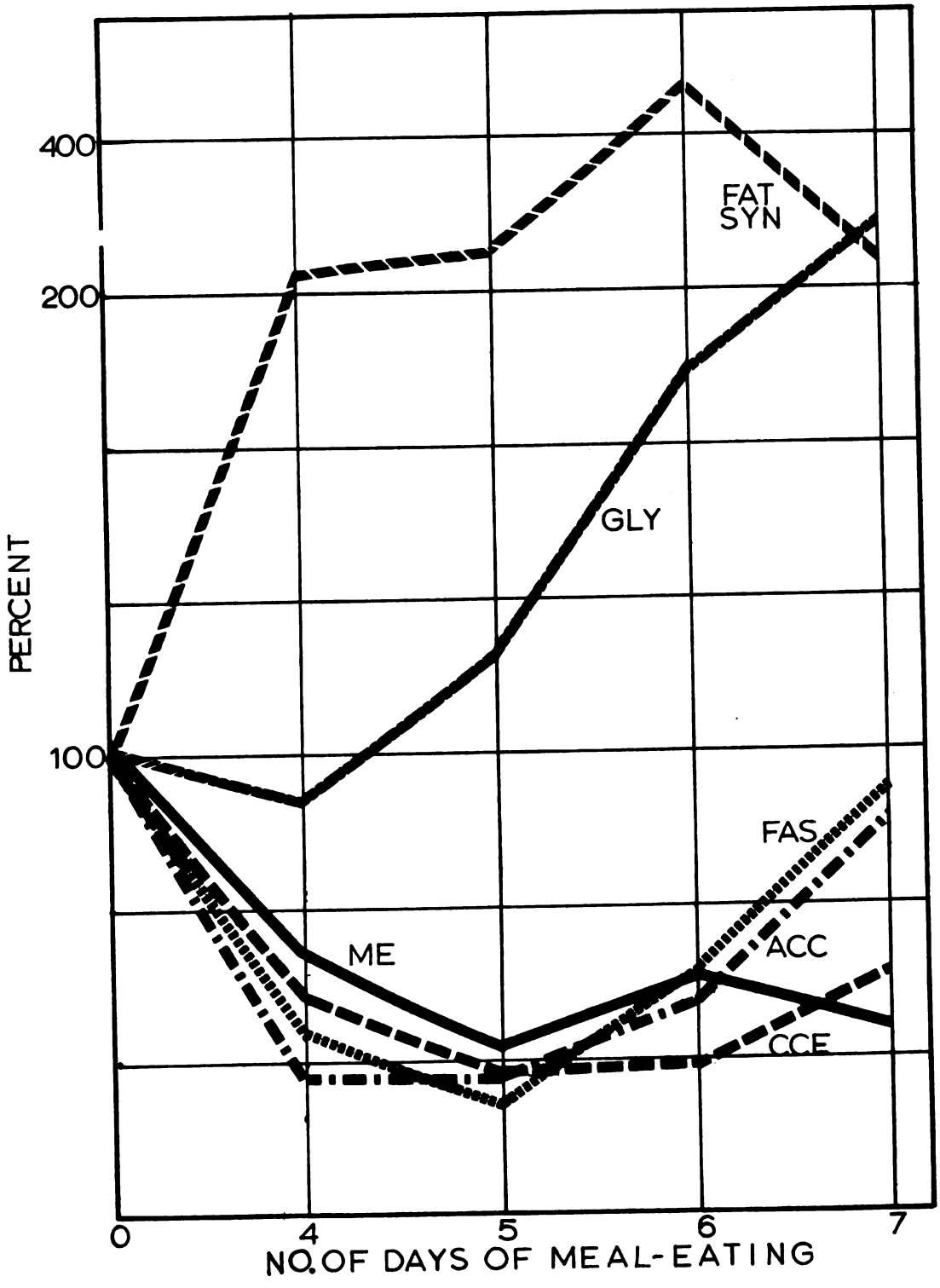
[1]Values are means ±SEM for 6 rats. [2]Nanomoles of substrate converted to product/min per mg soluble protein. [3]Nanomoles of 1-^{14}C-acetate or U-^{14}C-glucose incorporated into fatty acids per 100 mg of fat pad weight during a 2 hr incubation. [4]Value significantly different from Day 0 as determined by Dunnett's T test $P<.05$.

enzyme activity takes place while the rate of fatty acid synthesis increases to approximately 150% of the control value by the end of the sixth day. Furthermore, there are very small differences in the percent increase of the rate of fatty acid synthesis whether it be measured by an incubation in acetate-1-^{14}C or glucose-U-^{14}C.

Experiment IV.--The next question that arose is whether or not the results concerning the rates of fatty acid synthesis in vitro are a true reflection of what is really happening in vivo. The fourth experiment was designed in an attempt to answer this question. In vivo rates of fatty acid synthesis were measured as described in the materials and methods. The total enzyme activities were measured in vitro as in the previous experiments. Results from the fourth experiment are shown in Figure 9 and Table 9. It was discovered that the trace describing the in vivo rate of fatty acid synthesis reached a point more than 200% above the control value by the end of the fourth day of meal-eating. By the end of the sixth day of meal-eating, the rate of fatty acid synthesis (in vivo) reached an acme of 440% above the synthesis for ad libitum fed rats.

In addition to the enzymes measured in the previous experiments, acetyl CoA carboxylase was also measured during this experiment. Figure 9 shows the time sequence curve for acetyl CoA carboxylase. Note that the total enzyme activity of ACC has decreased by more than 75% by the end of the fourth day and has regained about 80% of the control activity

Fig. 9.--Influence of adaptation to meal-eating on in vivo rates of fatty acid synthesis, glycogen stores, and lipogenic enzyme activities in adipose tissue from rats meal-fed for 0, 4, 5, 6, or 7 days (each point represents the mean for six rats).


400
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PERCENT
FAT SYN
GLY
FAS
ACC
ME
CCE
0
4
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6
7
NO. OF DAYS OF MEAL-EATING

Fig. 9.

Table 9. Influence of adaptation to meal-eating on in vitro rates of fatty acid synthesis, lipogenic enzyme activities, and glycogen accumulation in adipose tissue of rats meal-fed for 0, 4, 5, 6, or 7 days.[1]

	Days of adaptation to meal-eating				
	0	4	5	6	7
Citrate cleavage enzyme[2]	71±15	32±5[4]	22±4[4]	23±7[4]	36±10[4]
Fatty acid synthetase[2]	12±2	4±1[4]	4±1[4]	6±1	10±2
Malic enzyme[2]	52±13	28±4[4]	18±2[4]	25±7[4]	19±2[4]
Acetyl CoA carboxylase[2]	7.4±1.4	2.3±0.5[4]	2.3±0.6[4]	3.3±0.9[4]	5.7±1.0
Fatty acid synthesis[3]	99±17	235±100	251±89	438±116[4]	239±44
Glycogen					
ug/g wet wt	323±26	283±38	391±69	593±142	888±149[4]
ug/mg fat free	20±3	14±1	20±3	23±6	33±5[4]

[1]Values are means ±SEM for 6 rats. [2]Nanomoles of substrate converted to product/min per mg soluble protein. [3]Determined in vivo by injecting 1 μCi of ^3H_2O IP 30 min before killings (expressed as dpm/100 mg of adipose tissue [4]Value significantly different from Day 0 as determined by Dunnett's T test $P<.05$.

by the end of the seventh day. The time course of the activity of ACC is in general agreement with the pattern of adaptation that the other enzymes previously mentioned follow.

The final portion of this experiment consisted of investigating the time sequence changes in the rate of glycogen accretion. The results of this experiment are shown in Figure 9. The amount of glycogen in the adipose tissue initially decreases by about 30% (based on ug of glycogen per mg of "ghost") and then rebounds to 100% on the fifth day. By the end of the seventh day, the amount of glycogen in the adipose tissue of meal-fed rats is 165% of the amount found in the control rats. Figure 10 expresses the percent of changes in the glycogen per g of wet weight as compared to the number of ug of glycogen found in the adipose tissue per mg of solvent-extracted adipose tissue ("ghost").

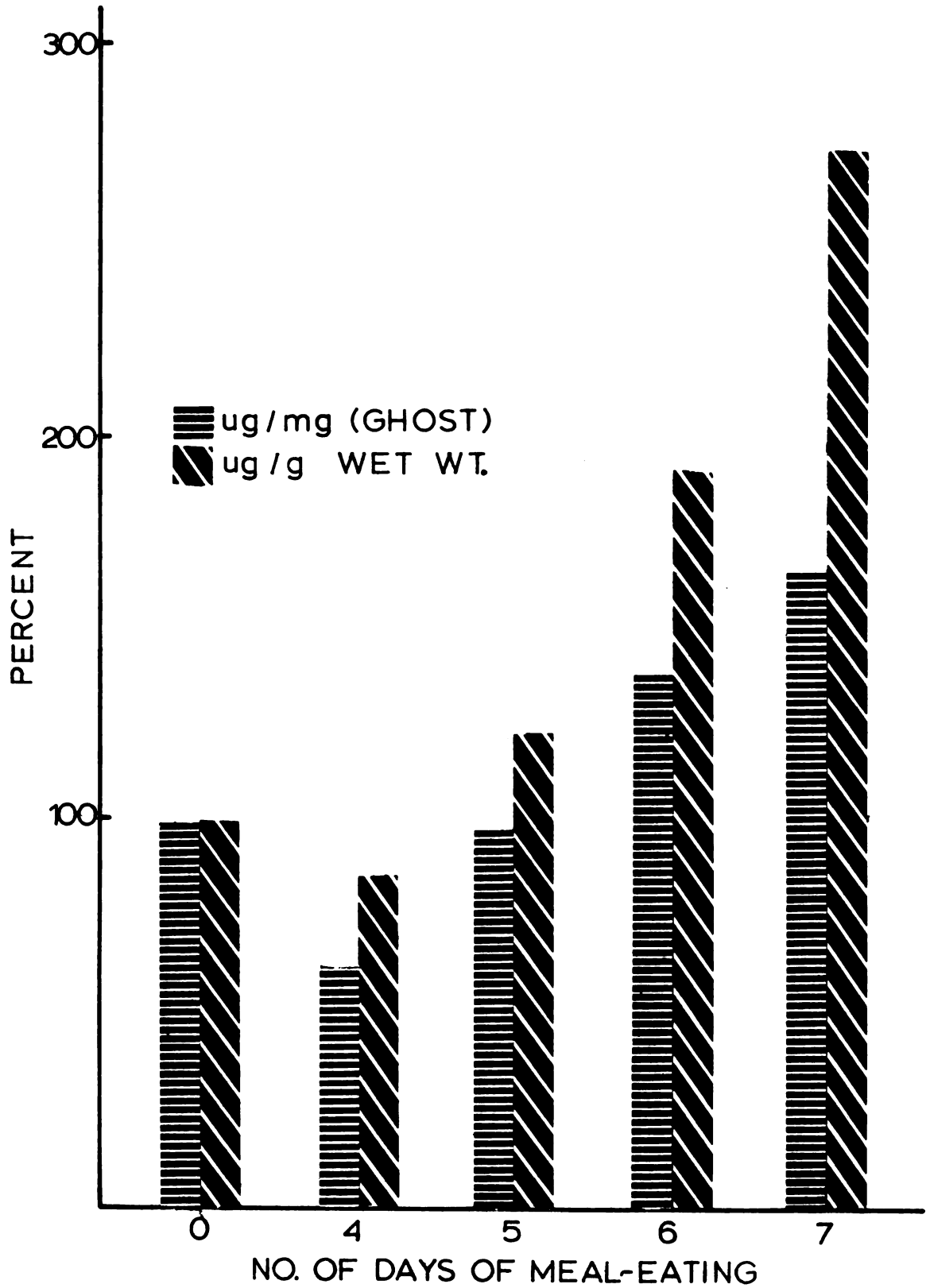

Fig. 10.--Glycogen values in terms of lipid free, dried weight and wet weight.

DISCUSSION

Quite naturally, Tepperman's description of adaptive hyperlipogenesis in meal-fed rats (Tepperman, J. and Tepperman, H., 1958) was subsequently followed by efforts to reveal the underlying mechanism which would cause lipogenesis to accelerate so markedly.

Since it was known that there is an absolute requirement for NADPH in the lipogenic pathway, Tepperman reasoned that the hexosemonophosphate shunt could play an extremely important role in promoting hyperlipogenesis as a response to meal-eating (Tepperman, J. and Tepperman, H., 1958). In the 1958 paper published by the Teppermans, it was shown that enzyme activity of the HMP shunt increased fourfold in the livers of rats fasted for 48 hours and refed a high carbohydrate diet for 24 hours. Tepperman concluded, "When the cell must dispose of extremely large amounts of glucose the shunt pathway becomes very prominent, NADPH is produced in very large amounts and the rates of the reactions involved in fatty acid synthesis are then permitted accelerate." Actually, this conclusion was rather tenuous and Tepperman himself later stated that it is not certain whether the increase in HMP shunt activity precedes the increased rate of lipogenesis. In fact it is possible that an increased rate

of lipogenesis would create a demand for NADPH which would, in turn, cause an increase in the direct oxidative pathway (Tepperman, J. and Tepperman, H., 1961).

A paper published by the Teppermans shows that when rats were fasted 48 hours and refed, the animals that had been refed for 12 hours had a rate of lipogenesis in the liver that was more than 300 percent above the control level. The liver HMP shunt enzymes had only recovered 100 percent of the control activity (Tepperman, H. and Tepperman, J., 1958). It is clear that the accelerated rates of lipogenesis are not initially dependent on an increased activity in the HMP pathway in the liver. There is further evidence to indicate that an increased rate of lipogenesis is not necessarily preceded by an increase in total HMP shunt activity. Leveille (1966) performed a time sequence study with isolated rat adipose tissue which clearly shows an increased rate of lipogenesis (130 percent of the control value or more) within 7 days after the animals had been switched to meal-eating. In comparison, the activities of glucose-6-phosphate dehydrogenase did not apparently increase above the control value before the ninth day after the alteration of feeding patterns.

The current consensus of some investigators is that glucose-6-phosphate dehydrogenase is the rate-limiting enzyme in the HMP shunt and is subject to allosteric modulation (Hizi and Yagil, 1974; Kather et al., 1972a, b; Eggleston

and Krebs, 1974). The point of contention materializes when the nature of the allosteric effector is discussed. Irrespective of whether GSSG counteracts the inhibition of glucose-6-phosphate dehydrogenase by NADPH (Eggleston and Krebs, 1974), or the actual utilization of NADPH during lipogenesis serves to de-inhibit the glucose-6-phosphate dehydrogenase (Kather et al., 1972a, b), the recurring conclusion is that changes in lipogenesis bring about changes in the substrate flux of the HMP shunt.

With the realization that the HMP shunt activity does not necessarily exert an initial regulatory control on the rate of fatty acid synthesis, some investigators began to consider citrate cleavage enzyme as a regulator of fatty acid synthesis. The rationale for such a hypothesis was based on the fact that citrate cleavage enzyme is localized in the cellular cytoplasm as are the enzymes for fatty acid synthesis (Srere, 1959). Furthermore, it was shown that citrate serves as precursor for fatty acids (Spencer and Lowenstein, 1962), and variations in citrate cleavage enzyme activity coincide with variations in the rate of fatty acid synthesis (Kornacker and Lowenstein, 1965; Kornacker and Ball, 1965).

There are at least as many reasons to reject the hypothesis as there are to accept it. Numa et al. (1961) and Wieland et al. (1963) demonstrated that fatty acid synthesis remains depressed when acetyl CoA is added to an

incubation mixture containing tissue from a rat with depressed lipogenic activity. This indicates that the lipogenic pathway is probably blocked someplace beyond the step where acetyl CoA is produced. In addition, it has been shown that in the fasting state (a time when both CCE activity and fatty acid synthesis are depressed) the liver contains a greater amount of acetyl CoA. It has not been determined whether the increased amounts of acetyl CoA are compartmentalized in the cytosol, or the mitochondria. Positive identification of the source of the increased levels of acetyl CoA must await the intracellular determination of acetyl CoA levels within the various "compartments". In any event, increased levels of acetyl CoA within the cytosol would certainly not be consistent with a hypothesis that demands the production of acetyl CoA to be rate-limiting. As a final argument directed against the hypothesis stating that CCE plays a primary regulatory role in lipogenesis, Srere and Foster (1967) and Foster and Srere (1968) performed some time sequence studies comparing the total CCE activity with the rate of fatty acid synthesis. It was demonstrated that lipogenesis decreased to near zero levels before there was any change in CCE activity in the liver of fasted rats (Srere and Foster, 1967). Foster and Srere (1968) concluded, "...that neither the amount nor the activity of citrate cleavage enzyme is rate-limiting in fatty acid synthesis." The time sequence curve for citrate cleavage enzyme versus

the time sequence curve for the rate of fatty acid synthesis reported in this thesis strongly supports the suggestion that citrate cleavage enzyme does not exert a primary regulatory effect over the lipogenic pathway. In every time sequence experiment reported in this thesis, the total activity of CCE was 25 to 65 percent lower than the control value by the end of the fourth day of meal-eating. During the same period of time, the rate of fatty acid synthesis was elevated by as much as 200%. The results reported here suggest the possibility that during the first four days of meal-eating, either: (1) the rate of synthesis of CCE is reduced while the rate of degradation remains constant, (2) the rate of synthesis remains fixed and the rate of degradation is increased, or (3) the rate of synthesis is somewhat reduced while the rate of degradation is somewhat increased. Any one of these possibilities would of course result in a net decrease in total enzyme activity. In fact, there is experimental evidence to indicate that the rate of CCE synthesis is the major determinant of the variation in net enzyme activity (Gibson et al., 1972). It is interesting to note in Gibson's report that; (1) the value of the degradation constant is greatest during maximal enzyme formation, (2) the values for the rate of synthesis and rate of degradation apparently become fixed shortly after an alteration in the nutritional state, and (3) the rate of approach to a new steady state concentration of enzyme is greatest when fasted animals are being refed.

As investigators accumulated more data concerning the physical properties of lipogenic enzymes, acetyl CoA carboxylase became an increasingly popular focal point for consideration as the regulator of the lipogenic pathway. It is known that acetyl CoA carboxylase can be activated by citrate (Vagelos et al., 1963; Numa et al., 1970), inhibited by long chain fatty acyl CoA compounds (Bortz and Lynen, 1963a, b; Numa et al, 1970; Goodridge, 1972), and assume either a protomeric (inactive) or polymeric (active) form. These observations add considerable strength to the possible regulatory role of acetyl CoA carboxylase.

The fact that acetyl CoA carboxylase activity, as reported in this thesis, decreases to less than one-third of the control value while the rate of fatty acid synthesis doubled in the same length of time does not necessarily detract from the hypothesis germane to a regulatory function for acetyl CoA carboxylase. Majerus and Kilburn (1969) have shown that changes in acetyl CoA carboxylase activity (as performed in vitro) only represent the total amount of enzyme protein present with no consideration given to the actual activity of the enzyme under the infulence of allosteric modulators in vivo. It is likely that the ratio of enzyme activity to total enzyme protein is increasing in vivo during the adaptation to meal-eating. This speculation is consistent with the observed decrease in total enzyme activity shown on the fifth day of meal-eating and the continuing upward trend of enzyme activity (finally attaining

an activity shown on the fifth day of meal-eating and the continuing upward trend of enzyme activity (finally attaining an activity that is 85% of the control value by the end of the seventh day) is not unexpected. Chakrabarty and Leveille (1968 and 1969) have shown that the total enzyme activity of acetyl CoA carboxylase in the adipose tissue of fully-adapted meal-fed rats is double that of control nibblers. Other investigators have shown that the activity of acetyl CoA carboxylase is greatly increased in the livers of fasted and refed rats (Majerus and Kilburn, 1969; Nakanishi and Numa, 1970).

Unlike acetyl CoA carboxylase, fatty acid synthetase has never been widely considered to serve a prime regulatory function in the production of fatty acids. Some investigators have suggested that the activity of fatty acid synthetase can be allosterically affected by phosphorylated sugars. It has been shown that fructose-1,6-diphosphate can increase the activity of the fatty synthetase complex in vitro. Kinetic studies have shown that fructose-1,6-phosphate decreases the K_m of fatty acid synthetase for NADPH. Presumably, the phosphorylated sugar could bind at a specific site to promote a conformational change in the enzyme, thus making it insensitive to malonyl CoA inhibition which is competitive with NADPH (Volpe and Vagelos, 1973). This hypothesis was discarded when the effect of phosphorylated sugars could not be demonstrated using purified fatty acid synthetase from rat or chicken liver. Furthermore, the

concentration of fructose-1,6-diphosphate, for example, had to be unphysiologically high to bring about a change in activity.

Other investigators demonstrated a decline in fatty acid synthetase activity in the presence of palmityl CoA. This observation immediately pointed to the possibility of a feedback inhibition for the control of fatty acid synthetase. This hypothesis was subsequently dispelled as it became increasingly clear that the detergent properties of palmityl CoA were responsible for the inhibition of the enzyme complex (Volpe and Vagelos, 1973).

Currently, the emphasis in the regulation of fatty acid synthetase is directed toward the actual changes in the amount of the enzyme protein as an effect of enzyme synthesis and degradation. The most influential factors controlling the rates of synthesis and degradation are; (1) fasting and refeeding (Volpe and Vagelos, 1973), (2) meal-eating (Chakrabarty and Leveille, 1969), (3) fat-feeding (Volpe and Vagelos, 1973), and (4) hormonal and growth changes (Volpe and Vagelos, 1973). It appears that changes in fatty acid synthetase activity (in vivo) are entirely related to changes in enzyme content. Guynn et al. (1972) performed a series of experiments in which the concentration of a number of different metabolites and cofactors were measured in freeze-clamped livers of rats that had been meal-fed for 3 hours daily. The results of their experiment led them to state that "...short term

control of fatty acid synthesis did not appear to be exerted by free mitochondrial [NAD^+]:[NADH], free cytoplasmic [NAD^+]:[NADH] or [$NADP^+$]:[NADPH], 'energy charge' or phosphorylation state." Furthermore, Guynn et al. (1972) present convincing evidence showing that the short term control of fatty acid synthesis lies before the fatty acid synthetase step--probably through an inhibition of acetyl CoA carboxylase by long chain CoA derivatives. There is no evidence to indicate that fatty acid synthetase exercises control over fatty acid synthesis allosterically.

In every experiment reported in this thesis the activity of fatty acid synthetase was seen to decrease as much as 65 percent by the end of the fourth day of meal-eating. The activity of the enzyme then started to increase and by the end of the seventh day of meal-eating the activity of fatty acid synthetase was definitely approaching the control levels of activity.

Unlike the fatty acid synthetase complex, the role of the decarboxylating malic enzyme is somewhat indirect relative to fatty acid synthesis. For the purpose of the experiments presented here, malic enzyme is viewed as a way of generating NADPH to support the increased rates of fatty acid synthesis that materializes as a function of meal-eating. Previously, in this discussion, the same kind of supportive role was ascribed to adaptive changes in the hexosemonophosphate shunt. Assigning the production of NADPH to two different sources is not contradictory. Under

conditions of hyperlipogenesis, Flatt and Ball (1964) have demonstrated that the pentose pathway is capable of providing only about sixty percent of the reducing equivalents required to support the hyperlipogenesis in rat adipose tissue. Indeed, Kather et al. (1972) found that the pentose pathway was only able to support lipogenesis entirely when measured in the adipose tissue of starved rats. In fact, the conversion of oxaloacetate (formed in the citrate cleavage reaction) to malate by way of malate dehydrogenase and the conversion of malate to pyruvate by malate enzyme constitute a transhydrogenation pathway which provides NADPH at the expense of NADH.

The adaptive nature of malic enzyme and its positive correlation to lipogenesis has been reported numerous times (Tepperman, J. and Tepperman, H., 1958; Leveille and Hanson, 1966; Leveille, 1970). The adaptive response of malic enzyme as reported in this thesis is in complete agreement with the changes that have been previously reported to occur in rat adipose tissue when the animals are started on a time restricted pattern of food intake (Leveille, 1966). Generally, the malic enzyme activity decreased thirty to fifty percent during the first four or five days of the experiment. By the end of the seventh day of meal-eating the malic enzyme activity was increased to control activity or more. There is no reason to doubt that increased rates of fatty acid synthesis create a high demand for NADPH which is met by increasing the rate of de novo synthesis in malic enzyme

(Gibson, 1972) and ultimately providing the necessary reducing equivalents.

Meal-feeding has been shown to alter glycogen metabolism as well as enzyme activities. The concentration of liver glycogen in 48 hour fasted rats decreases to about 20 percent of the normal concentration. Within 12 hours of refeeding, the glycogen content of the liver is twice the amount in unfasted, ad libitum fed rats (Tepperman, H. and Tepperman, J., 1958). Leveille (1966) has shown that even more dramatic changes take place in the adipose tissue of meal-fed rats. The glycogen levels measured in adapted meal-fed rats were found to be about 42 ug per g of tissue prior to the meal. After feeding the meal-eaters, the glycogen levels were increased by a factor of 10. Based on this observation, Leveille (1966) suggested that the glycogen stored in the adipose tissue might serve as a primordial source of α-glycerophosphate in the period of fast between meals. Since the activity of glycerol kinase in the adipose tissue is low, one could reasonably postulate the need for storing α-glycerophosphate, in the form of glycogen, for the purpose of fatty acid reesterification. Leveille (1967) proposed that fatty acid synthesis in the adipose tissue might be inhibited by the high levels of free fatty acids in the adipose tissue and that this inhibition could be removed by substrates such as glucose and pyruvate which are convertible to α-glycerophosphate.

The glycogen values obtained as part of this thesis

may be interpreted as testimony to the importance of glycogen in the adipose tissue of meal-eaters. Though there is a 32 percent decrease (based on ug glycogen per mg of fat-free adipose tissue) in the amount of glycogen present by the end of the fourth day of meal-eating, the glycogen content by the end of the sixth day is equal to the levels found in the control rats. The remaining two days of the study show the glycogen levels to be far in excess of the amount of glycogen found in the adipose tissue of the ad libitum fed rats. This rapid accumulation of glycogen in the adipose tissue of meal-fed rats is congruent with the results reported by Leveille (1967) in rats adapted to a meal-eating regimen for three weeks. In addition, Wiley and Leveille (1970) observed marked increases in the activities of glycogen synthetase and other enzymes involved in glycogen synthesis from glucose-6-phosphate in the adipose tissue of adapted meal-fed rats.

SUMMARY

When the results of all of the experiments are integrated, a paradoxical interplay between enzyme activities and lipogenesis becomes evident. The activities of the lipogenic enzymes in every experiment in this thesis initially decreased when the ad libitum feeding mode was supplanted by a meal-eating regimen. The paradox lies in the fact that the rates of fatty acid synthesis increase despite the decreasing activities of the lipogenic enzymes. To resolve the apparent contradiction, one need only to realize that the discovery of an enzyme unit of activity in vitro cannot be taken a priori as proof that it is actively catalyzing reactions in vivo. In fact, the experiments reported in this thesis clearly show that the lipogenic enzyme concentrations in the adipose tissue of rats adapting to a meal-eating regimen are in sufficient excess to support increased rates of fatty acid synthesis even though the total enzyme concentrations are initially decreasing.

The overall conclusion to be drawn from this thesis necessitates a movement away from a simplistic approach to the regulation of fat synthesis. Rather than a single control point (for example, acetyl CoA carboxylase) there appears to be an integrated system of substrate

concentration, enzyme activity and concentration and probably a genetic regulation governing the rates of enzyme synthesis and degradation. If one imagines that the concentration and availability of the substrate serves as the prime regulator for all of the ensuing metabolic changes, then one has grasped the essence of what is meant by metabolic flux. The concept of metabolic flux--the concentration and rate of flow of various substrates through metabolic pathways--suffices, in a general way, to explain how metabolic adaptation is forced to occur; however, the discrete steps of metabolic adaption must still be elucidated and related to metabolic flux before any irrefutable conclusions can be drawn to the in vivo situation.

It is within the framework of metabolic flux that meal-eating excels as a research technique. Many of the experiments performed to examine enzyme induction and activation have utilized a procedure of fasting and ad libitum refeeding in order to measure the degree of metabolic change. While this technique certainly has its uses, the experimental design creates an all or nothing response that leaves little opportunity to observe the most pristine and subtle metabolic changes. Meal-feeding experiments, on the other hand, are unique inasmuch as they expand the time scale for metabolic adaptation. This is primarily due to the gradual and time-limited realimentation of the animal after a 24 hour fast.

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