before methylation with diazomethane. The latter, of course, showed additional peaks due to impurities.

Discussion

The occurrence of *N*-succinylamino acids has been noted before. The formation of *N*-succinyl-L-glutamic acid was observed by Aubert, Millet, Pineau, and Milhaud (Aubert *et al.*, 1961) in a sporulating organism at time of spore formation. *N*-Succinyl-L-diaminopimelic acid has been found by Gilvarg (1957) in an *Escherichia coli* mutant with an absolute requirement for diaminopimelic acid. Gilvarg has suggested that the succinyl compound is on the pathway leading to the formation of lysine.

D-Valine is known to inhibit the formation of the antibiotic penicillin (Demain, 1956) in *Penicillium chrysogenum*. As with actinomycin, the organism utilizes L-valine for the synthesis of antibiotic-bound D-amino acid (D-penicillamine). A similar situation occurs during the formation of polymixin D, which contains D-leucine. The formation of the antibiotic is inhibited by D-leucine (DiGirolamo *et al.*, 1964). In none of these instances is the mechanism of D-amino acid inhibition known. It would be of interest to know if succinylamino acids are formed in these related instances.

The relationship of *N*-succinyl-D-valine to the inhibition of actinomycin biosynthesis by D-valine is under investigation. The possibility exists that succinyl-D-valine is a normal intermediate. D-Valine, in high concentrations, might inhibit antibiotic formation by competition with L-valine for the succinylation reaction.

Studies are in progress to determine whether other succinylamino acids are formed during the production or inhibition of actinomycin. Isolation of *N*-succinyl-D-valine suggests the possibility that *N*-succinylamino acids may occupy an important position in the synthesis of certain peptides.

Acknowledgment

We should like to thank Dr. Sidney Udenfriend for his interest during the development of this study and Dr. John Pisano for helpful discussions.

References

Arndt, F. (1943), *Org. Syn. Collective Vol. 3*, 165.
Aubert, J. P, Millet, J., Pineau, E., and Milhaud, G. (1961), *Biochim. Biophys. Acta 51*, 529.
Brockmann, H. (1960), *Ann. N. Y. Acad. Sci. 89*, 323.
Burton, K. (1955), *Methods Enzymol. 2*, 199.
Demain, A. L. (1956), *Arch. Biochem. Biophys. 64*, 74.
DiGirolamo, M., Cifari, O., DiGirolamo, A. B., and Alkertini, A. (1964), *J. Biol. Chem. 239*, 502.
Fales, H. M., Haahti, E. O. A., Luukkainen, T., Vanden-Heuvel, W. J. A., and Horning, E. C. (1962), *Anal. Biochem. 4*, 296.
Gilvarg, C. (1957), *Biochim. Biophys. Acta 24*, 216.
Heilbron, I., and Bunbury, H. M. (1953), Dictionary of Organic Compounds, Vol. IV, New York, Oxford, p. 659.
Jackman, L. M. (1959), Nuclear Magnetic Resonance Spectroscopy, New York, Pergamon, p. 102.
Kabat, E. A. (1961), Experimental Immunochemistry, Springfield, Ill., Thomas, p. 476.
Katz, E. (1959), *Nature 184*, 1666.
Katz, E. (1960), *J. Biol. Chem. 235*, 1090.
Katz, E., and Goss, W. (1959), *Biochem. J. 73*, 458.
Katz, E., and Weissbach, H. (1963), *J. Biol. Chem. 238*, 666.
Naftalin, L. (1948), *Nature 161*, 763.
Ratner, S. (1955), *Methods Enzymol. 2*, 204.
Stein, W. H., and Moore, S. (1948), *J. Biol. Chem. 176*, 367.

Metabolic Control of Enzymes Involved in Lipogenesis and Gluconeogenesis*

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Received July 9, 1964

The activity of phosphoenolpyruvate carboxykinase and malic enzyme was measured in the 105,000 × *g* (1 hour) supernatant fractions of liver and adipose tissue from rats subjected to various dietary conditions. Phosphoenolpyruvate carboxykinase activity in liver increases rapidly during fasting but is restored to normal by refeeding diets containing carbohydrate. Refeeding carbohydrate-free diets did not depress the elevated activity of the enzyme. Insulin is essential for repression by carbohydrate. Phosphoenolpyruvate carboxykinase was not present in measurable amounts in adipose tissue. Malic enzyme activity in liver and adipose tissue is decreased by fasting. The activity increase upon refeeding is greater after a 96-hour fast than after a 48 hour fast and is also influenced by composition of the diet. A diet high in carbohydrate but containing no fat causes the greatest increase upon refeeding, while carbohydrate-free diets do not cause a significant increase in malic enzyme activity. Both liver and adipose tissue are capable of transhydrogenating DPNH to TPNH by coupling cytoplasmic malic dehydrogenase to malic enzyme. Thus malic enzyme may serve a role in lipogenesis either by direct formation of TPNH or by transhydrogenation from DPNH.

Enzyme concentrations and activities are influenced by many dietary and metabolic conditions (Potter and Ono, 1962; Niemeyer *et al.*, 1962; Vaughan and Winders, 1964; Weber, 1963). A recent report from this laboratory (Shrago *et al.*, 1963) indicated that phosphoenolpyruvate carboxykinase activity is increased by fasting and decreases to normal levels or below after refeeding with a normal diet. Conversely, malic enzyme activity was decreased by fasting and increased above normal upon refeeding. This report is the result of further investigations of the time course of changes in malic enzyme and phosphoenolpyruvate carboxykinase activity, of dietary treatments which influence or con-

* Supported in part by grants from the National Institutes of Health, the National Science Foundation, and the Eli Lilly Co.

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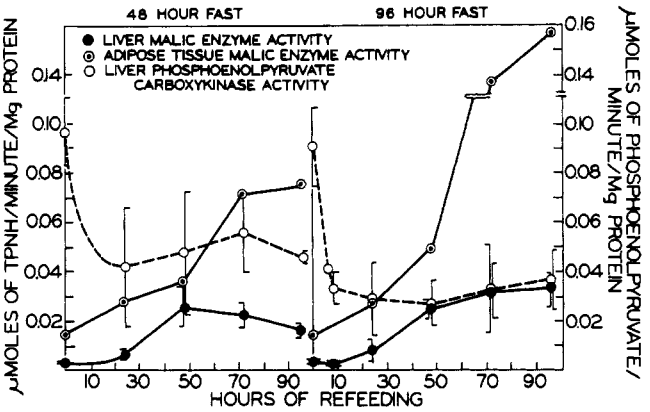


FIG. 1.—The response of phosphoenolpyruvate carboxykinase in liver and malic enzyme in liver and adipose tissue of fasted rats refed with a normal ration. Points where standard deviations are shown represent 4–6 animals. All other points represent 2 animals.

trol these enzyme activities, and of a correlation of changes in related enzymes believed to be involved in lipogenesis or gluconeogenesis.

EXPERIMENTAL PROCEDURE

Male rats, usually weighing between 200 and 250 g, were obtained from the Badger Research Corp., Madison, Wisc. Except for periods of fasting the animals were supplied *ad libitum* with either a commercial (Rockland) diet or synthetic diets containing sucrose, corn oil (Mazola), and crude casein in varying proportions. The synthetic diet was supplemented with 4% of the Phillips-Hart salt mixture IV (Phillips and Hart, 1935) and a vitamin mixture (Betheil *et al.*, 1947). Water was available at all times.

Alloxan diabetes was produced by intravenous injections of alloxan at the rate of 50 mg/kg. Animals were not used until at least 5 days after injection of alloxan.

Liver and epididymal fat pads were homogenized in nine volumes of cold 0.25 M sucrose and the 105,000 × *g* (1 hour) supernatant fractions were stored at −10° until assayed. Phosphoenolpyruvate carboxykinase was assayed as described by Nordlie and Lardy (1963), except that phosphoenolpyruvate was cleaved with mercuric ion (Lohmann and Meyerhof, 1934) and the liberated inorganic phosphate was estimated by the method of Sumner (1944).

Isocitric dehydrogenase and malic enzyme were measured by the procedures of Ochoa (1955a,b) and glucose-6-phosphate dehydrogenase was assayed by the procedure of Kornberg and Horecker (1957). Non-substrate controls, containing TPN^+, were run for all samples.

The transhydrogenation of DPNH to TPNH was assayed by allowing the TPNH generated to reduce cytochrome *c* enzymatically with purified TPNH cytochrome *c* reductase and measuring the increase in absorbancy at 550 mμ. TPNH cytochrome *c* reductase was purified according to a modification of the procedure of Haas *et al.* (1940) from brewers' yeast purchased from Anheuser Busch, St. Louis, Mo. The purified enzyme was not active with DPNH.

Protein was determined by the biuret method (Layne, 1957). After the addition of biuret reagent protein samples were ether-extracted to remove any lipid materials which might interfere with the colorimetric determination of protein. All chemicals were reagent grade and solutions were prepared in deionized glass-distilled water.

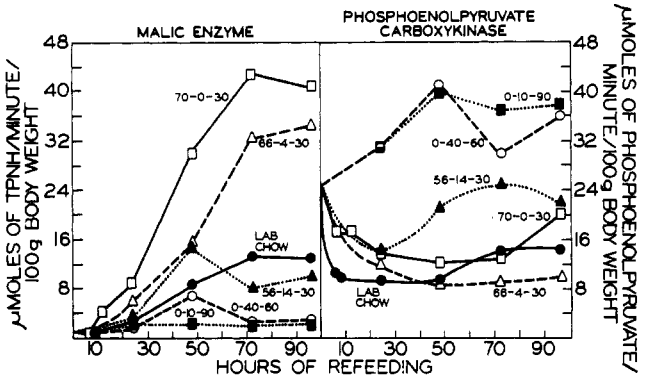


FIG. 2.—The effect of fasting 96 hours and refeeding with various diets upon the activity of malic enzyme and phosphoenolpyruvate carboxykinase in rat liver. The synthetic diets are designated by the percentages of sucrose, corn oil, and casein, respectively. The number of animals per point for the respective diets are as follows: 70–0–30, 3; 66–4–30, 2; lab chow, 4–6 except at 6 and 8 hours, where there are 2 and 3 animals, respectively; 56–14–30, 1; 0–40–60, 1; and 0–10–90, 1.

TABLE I
THE EFFECT OF FASTING UPON THE ACTIVITY OF MALIC ENZYME AND PHOSPHOENOLPYRUVATE CARBOXYKINASE IN LIVER AND ADIPOSE TISSUE OF RATS

Treatment	Malic Enzyme Activity (mμmoles TPNH formed/min per mg protein)		Phosphoenolpyruvate Carboxykinase Activity (mμmoles PEP formed/min per mg protein)
	Liver	Adipose Tissue	Liver
Control (6)[a]	6.0 ± 1.5[b]	19.2 ± 4.5	53 ± 6.9
24-hour fast (3)	6.9 ± 0.3	19.2 ± 7.0	115 ± 4.8
48-hour fast (3)	2.4 ± 0.5	14.1 ± 6.3	97 ± 14.9
72-hour fast (3)	3.7 ± 3.3	11.0 ± 1.0	90 ± 10.8
96-hour fast (6)	3.5 ± 1.0	8.3 ± 2.8	91 ± 16.0

[a] Number of animals. [b] Standard deviation.

RESULTS

The relation between length of fast and malic enzyme and phosphoenolpyruvate carboxykinase in liver and adipose tissue is given in Table I. The activity of liver malic enzyme does not decrease greatly until between 24 and 48 hours of fasting, at which time it falls to about one-half the normal activity and thereafter remains relatively constant. In adipose tissue malic enzyme also began to decrease between 24 and 48 hours but continued to decrease slowly up to 96 hours of fasting. The change in liver phosphoenolpyruvate carboxykinase activity occurs rapidly, with a 2-fold increase appearing during the first 24 hours of fasting. We have not detected phosphoenolpyruvate carboxykinase activity in adipose tissue under any condition.

Figure 1 shows the response of phosphoenolpyruvate carboxykinase in liver, and malic enzyme in liver and adipose tissue, upon refeeding a normal diet after either a 48- or a 96-hour fast. Plotting the data from the liver enzymes on the basis of total activity per 100 g body wt led to the same conclusions presented herein.

As in the case of fasting, phosphoenolpyruvate carboxykinase responded very rapidly to refeeding. After a 96-hour fast, 6 hours of refeeding reduced the activity to slightly below normal. Refeeding animals that had been fasted 48 hours did not result in as great

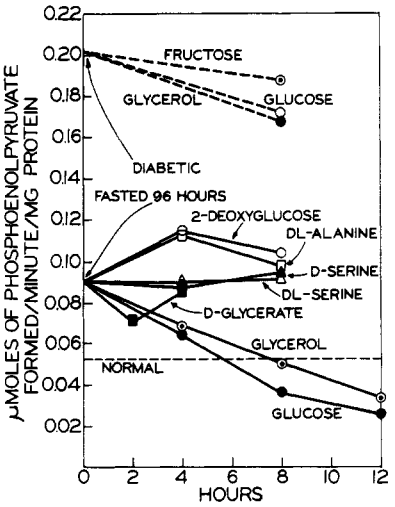


FIG. 3.—The ability of various compounds to suppress the elevated phosphoenolpyruvate carboxykinase levels in liver caused by fasting or by alloxan diabetes. All animals were given 0.5 g of the respective compound in 3 ml of solution every 2 hours via stomach tube until the time of sacrifice. The D-glycerate, which was a gift of Dr. H. Sallach, Dept. of Physiological Chemistry, University of Wisconsin, was neutralized approximately 50% with NaOH-KOH. The points for the diabetic experiment represent from 4 to 9 animals. The points for fasted animals given glucose or glycerol represent 2 animals and all other points represent either 1 or 2 animals.

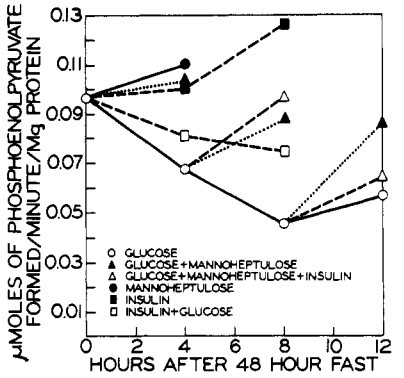


FIG. 4.—The effect of mannoheptulose and insulin upon the suppression of liver phosphoenolpyruvate carboxykinase caused by feeding glucose after a 48-hour fast. Glucose was administered via stomach tube at the rate of 0.5 g in 3 ml of aqueous solution every 2 hours. Mannoheptulose was given in 2 ml of aqueous solution, and 2 units of insulin (Iletin) was given in saline. Both mannoheptulose and insulin were injected subcutaneously at the time indicated when their curves deviate from the curve of glucose alone. Mannoheptulose was isolated and crystallized by V. Paetkau. Each point represents 2 animals.

a depression of activity as did refeeding animals fasted for 96 hours. Again, malic enzyme activity responded less rapidly than did phosphoenolpyruvate carboxykinase, with only a slight increase in activity in either liver or adipose tissue during the first 24 hours of refeeding. From the values obtained at 6 and 8 hours of refeeding after a 96-hour fast, it would appear that a majority of the decrease in activity of phosphoenolpyruvate carboxykinase is completed before any stimulation of malic enzyme occurs in liver.

It is obvious from Figure 1 that the longer period of fasting led to a greater refeeding response of malic enzyme in both liver and adipose tissue. During the first 48 hours of refeeding, malic enzyme activity increased similarly in both groups. Thereafter, malic enzyme activity began to decrease toward normal in animals fasted 48 hours, while in animals fasted 96 hours activity continued to increase up to 96 hours of refeeding. In adipose tissue, malic enzyme activities were equal after 24 hours of refeeding following a 48- or a 96-hour fast but thereafter increased much more rapidly in animals previously fasted 96 hours, and after 4 days reached values twice as high as in animals previously fasted for 48 hours.

In an effort to determine which dietary components might be influencing these enzyme activities, diets of varying compositions were given to animals previously fasted 96 hours. This study is presented in Figure 2. The synthetic diets will be referred to by weight percentages of sucrose, corn oil, and casein, respectively, before supplementation with minerals and vitamins.

The results on the left half of Figure 2 show that refeeding diets high in carbohydrate increase activity of malic enzyme, while diets lacking in carbohydrate do not. Comparison of the 70–0–30, the 66–4–30, and the 56–14–30 diets indicate that even though malic enzyme activity is induced by carbohydrate, such induction also is influenced by the amount of lipid in the diet. Lipid suppression of malic enzyme induction was illustrated conclusively by feeding the 66–4–30 diet to rats that had been refed either 24 or 48 hours with 70–0–30. In all cases, changing to the 66–4–30 diet resulted in a decrease in malic enzyme activity and the only difference in the diets was 4% corn oil. Thus, it appears that malic enzyme in liver is induced by carbohydrate and suppressed by fat in the diet.

In contradistinction to malic enzyme, the amount of phosphoenolpyruvate carboxykinase increases in total activity when fasted animals are refed with carbohydrate-free diets and decreases upon refeeding with carbohydrate-containing diets (see Shrago *et al.*, 1963). The increased total activity upon refeeding carbohydrate-free diets was related to the increase in liver weight, as activity per unit of protein remained relatively constant. In all other instances changes in total activity and specific activity were essentially the same. The more rapid decrease of phosphoenolpyruvate carboxykinase in animals refed with lab chow as compared to synthetic carbohydrate-containing diets is probably related to food intake as the rats were not accustomed to synthetic rations. These data suggest that available carbohydrate, by exerting a suppressing effect, is a major factor controlling phosphoenolpyruvate carboxykinase levels in the liver. This was emphasized further by changing rats from a lab chow diet to a 0–10–90 synthetic diet which in 24 hours caused the normal liver phosphoenolpyruvate carboxykinase activity to increase 2-fold. Dietary lipids do not appear to influence levels of phosphoenolpyruvate carboxykinase.

The effects of various carbohydrates and carbohydrate precursors upon phosphoenolpyruvate carboxykinase activity elevated by a 96-hour fast or by alloxan diabetes was investigated (Fig. 3). The administration of glucose or glycerol decreased phosphoenolpyruvate carboxykinase to levels below normal, while 2-deoxyglucose, DL-alanine, D-serine, DL-serine, and D-glycerate did not. Although fructose, glucose, and glycerol given to alloxan diabetics resulted in a slight decrease of activity after 8 hours, the decrease was not statistically significant when tested by the *t* test (Snedecor, 1956). In view of the fact that alloxan-diabetic rats produce some insulin, the data of Figure 3 suggest that insulin may be essential for glucose, glycerol, and carbohydrate diets to exert a depressing effect upon phosphoenolpyruvate carboxykinase. This possibility was in-

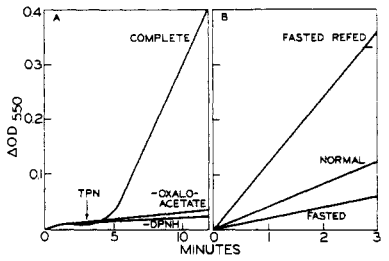


FIG. 5.—Transhydrogenation from DPNH to TPNH by the coupling of malic dehydrogenase and the malic enzyme from the high-speed supernatant fraction of rat liver homogenate. The reaction mixture contained 75 μmoles glycylglycine buffer, *p*H 7.4, 0.76 μmole oxalacetate neutralized to *p*H 7.4 with 2 N KOH, 0.2 μmole DPNH, 0.1 μmole TPN^+, 0.15 μmole cytochrome *c*, 0.03 μmole FMN, 3 μmole $MnCl_2$, 5 units purified yeast, TPNH–cytochrome *c* reductase (1 unit = 1 μmole cytochrome *c* reduced/min), and 0.1 ml enzyme (high-speed supernatant fraction of rat liver homogenate). The reaction was carried out in a volume of 3.0 ml at 25°. The reduction of cytochrome *c* was followed at 550 mμ; in (B), only the linear part of the reaction curves are plotted. The supernatant fractions were obtained from rats that were fasted 96 hours and refed with commercial chow diet for 96 hours, normal, and fasted 96 hours.

vestigated further by refeeding glucose to animals fasted 48 hours and administering various combinations of insulin and mannoheptulose, which prevents insulin release from the pancreas (Coore *et al.*, 1963).

The results (Fig. 4) show that mannoheptulose given when glucose feeding is started prevents the decrease of phosphoenolpyruvate carboxykinase and that mannoheptulose given later, after glucose had initiated a decrease in phosphoenolpyruvate carboxykinase activity, immediately reversed the decrease in activity. Insulin given alone did not cause a decreased activity in the fasted animal (Fig. 4) as it does in the fed diabetic rat (Shrago *et al.*, 1963). Insulin appeared to lessen the effect of glucose in suppressing the level of phosphoenolpyruvate carboxykinase. Insulin given with mannoheptulose at 8 hours' refeeding prevented, as expected, much of the increase of phosphoenolpyruvate carboxykinase caused by mannoheptulose during the next 4 hours. However, this was not true for animals treated at 4 hours. This discrepancy, along with the failure of glucose plus insulin to cause as great a decrease as glucose alone, is difficult to explain, but may result from the fact that excessive insulin increases glucose entrance and utilization in peripheral tissues to such an extent that concentrations of glucose in liver were too low to exert a maximum repressive effect. Despite this discrepancy these results indicate that both insulin and glucose or a utilizable carbohydrate source are required to suppress the elevated levels of phosphoenolpyruvate carboxykinase resulting from fasting. In a separate experiment it was found that mannoheptulose caused an increase in phosphoenolpyruvate carboxykinase levels after 24 hours refeeding of lab chow following a 96-hour fast.

Sources of TPNH for Lipogenesis.—In order to evaluate more fully the potential role of malic enzyme in lipogenesis, changes in specific activity were compared with that of glucose-6-phosphate dehydrogenase and isocitric dehydrogenase in the soluble fractions of liver and epididymal fat pads (Table II). Both malic enzyme and glucose-6-phosphate dehydrogenase respond in a similar manner to starvation and refeeding. In contrast, isocitric dehydrogenase does not seem to be adaptive and furthermore is relatively lower in the fat pad than in the liver.

Figure 5A is a representative experiment illustrating transhydrogenation from DPNH to TPNH which is mediated through the coupled reactions of soluble malic dehydrogenase and malic enzyme. The reaction mixture is given in the legend and TPNH formation is measured at 550 mμ by its reduction of cytochrome *c* with the purified yeast TPNH–cytochrome *c* reductase which does not react with DPNH. Oxalacetic acid is necessary for activity, indicating that a soluble transhydrogenase is not active in this system. Figure 5B is a comparison of the rate of transhydrogenation of supernatant fractions obtained from the livers of normal, fasted (96 hours), and fasted-refed (96 hours) rats. The increased activity noted in the refed animal is in keeping with the elevation of malic enzyme. Low activities were also found in diabetic animals with an increase above normal after insulin treatment for 3 days. This transhydrogenation reaction was observed in adipose tissue also.

DISCUSSION

Phosphoenolpyruvate carboxykinase rapidly doubles in activity when rats are fasted (Table I), increases even more when fasted rats are refed with a carbohydrate-free diet (Fig. 2), but decreases rapidly when animals are refed with a diet containing high levels of carbohydrate (Figs. 1, 2). Hence changes in activity of this enzyme in liver are concomitant with the need for gluconeogenesis. The quantitative response of phosphoenolpyruvate carboxykinase has been shown (Shrago *et al.*, 1963) to be similar to changes in glucose-6-phosphatase, fructose 1,6-diphosphatase, and glutamic-pyruvic transaminase under similar conditions; however the response is more rapid (Lardy *et al.*, 1964a) than for the other enzymes, indicating a closer relationship to the mechanism which controls gluconeogenesis.

The question of what controls levels of phosphoenolpyruvate carboxykinase is not settled. Figures 1 and 2 seem to indicate that the presence or absence of "normal" carbohydrate levels in the diet is a major controlling factor. However, it must be remembered that a lack of carbohydrate necessarily means an increase in protein utilization for carbohydrate synthesis; thus other factors may influence control of the enzyme. Both glucose and glycerol are effective in depressing phosphoenolpyruvate carboxykinase levels after fasting (Fig. 3), whereas alanine, serine, and glycerate, which can give rise to glycolytic intermediates, and 2-deoxyglucose, which is phosphorylated but not metabolized further (McComb and Yushok, 1964) are not effective. It is interesting also that both glucose and glycerol administration, as well as refeeding certain high-carbohydrate diets, not only depress the elevated levels of the enzyme but decrease the activity to less than normal. One theory compatible with these results would be that increased intracellular levels of some metabolic intermediate regulate the formation of a repressor for phosphoenolpyruvate carboxykinase. Catabolite repression by glucose of β-galactosidase synthesis (Monod, 1947) has been recognized as a general regulatory mechanism which contributes to the control of the formation of numerous inducible enzyme systems in bacteria (Magasanik, 1962). Though both insulin and carbohydrate are required to depress elevated levels of phosphoenolpyruvate carboxykinase, it is not clear whether or not one exerts a primary effect and the other a secondary effect.

Phosphoenolpyruvate carboxykinase responds to various treatments in a manner opposite to the response of malic enzyme, lipogenesis, and enzymes related to

TABLE II
SPECIFIC ACTIVITY OF MALIC ENZYME, GLUCOSE-6-PHOSPHATE DEHYDROGENASE, AND TPN–ISOCITRIC DEHYDROGENASE IN LIVER AND ADIPOSE TISSUE

Experimental Condition	Malic Enzyme	Glucose-6-P Dehydrogenase	Isocitric Dehydrogenase
	(mμmoles of TPNH formed/min per mg protein)		
	Liver		
Normal (6)[a]	6.0 ± 1.5[b]	11.2 ± 1.1	146.0 ± 6.0
Fasted 96 hours (4)	2.9 ± 2.0	5.5 ± 2.5	129.0 ± 11.0
Fasted 96 hours, refed[c] 96 hours (5)	37.6 ± 15.4	104.0 ± 5.0	119.0 ± 13.0
	Adipose Tissue		
Normal (6)	19.5 ± 4.5	63.0 ± 10.1	31.8 ± 6.7
Fasted 96 hours (4)	8.8 ± 3.6	37.8 ± 15.5	22.9 ± 5.1
Fasted 96 hours, refed[c] 96 hours (5)	113.2 ± 21.7	189.0 ± 79.0	44.4 ± 18.4

[a] Number of animals. [b] Standard deviation. [c] Refed with Rockland Lab Chow.

lipogenesis. Furthermore, malic enzyme seems to respond only after most of the phosphoenolpyruvate carboxykinase response is completed.

It has already been established (Shrago *et al.*, 1963) that malic enzyme is not directly involved in gluconeogenesis and it has been suggested that this enzyme is an important source of TPNH for fat synthesis (Lardy *et al.*, 1964a). The present work further implicates malic enzyme in fat synthesis.

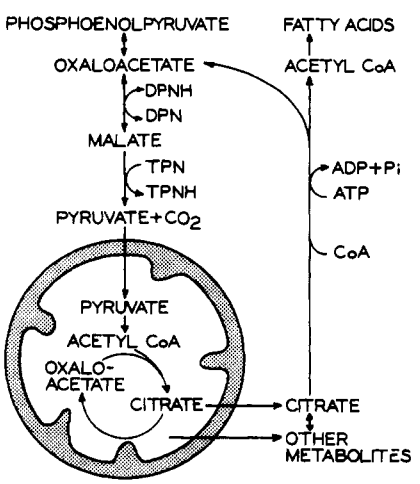
Lipogenesis is decreased during fasting (Van Bruggen *et al.*, 1952; Hutchens *et al.*, 1954; Lyon *et al.*, 1952; Chareonchai and Johnson, 1963) and increases rapidly to levels above normal upon refeeding (Medes *et al.*, 1952; Chareonchai and Johnson, 1963). The changes reported in this paper, when correlated with the results of the above-cited workers, illustrate that malic enzyme adapts in a manner identical to over-all lipogenesis.

It is interesting that malic enzyme is present in high concentrations in adipose tissue (Fig. 1) and increases upon refeeding in proportion to the length of the previous period of fasting. After a 96-hour fast more lipogenesis would be required to restore body-fat stores to normal than after a 48-hour fast. Malic enzyme and glucose-6-phosphate dehydrogenase, an enzyme known to contribute to lipogenesis (Cohn and Joseph, 1959), change in a similar manner in fasting and refeeding (Potter and Ono, 1962), with malic enzyme showing a greater increase than glucose-6-phosphate dehydrogenase under conditions conducive to intensive lipogenesis (Table II). These data add credence to the hypothesis that malic enzyme is related to lipogenesis.

Of some importance may be the relatively low amount of TPN^+-linked soluble isocitric dehydrogenase in adipose tissue which does not adapt to increased lipogenesis. This may be an indication that the enzyme is not directly related to fatty acid synthesis as a TPNH-generating system. Similar conclusions have been reached, but for different reasons, by Kallen and Lowenstein (1962).

The close correlation of glucose oxidation by the hexose monophosphate pathway and concomitant increased glucose-6-phosphate dehydrogenase activity with lipogenesis has been shown (Tepperman and Tepperman, 1958; Cohn and Joseph, 1959). This pathway would provide a partial source of the TPNH necessary for fatty acid synthesis. More recently Bhaduri and Srere (1963), Kornacker and Lowenstein (1964), and Lardy *et al.* (1964b) have implicated the citrate-cleavage enzyme as an important regulator of lipogenesis in liver, particularly since the enzyme could form acetyl CoA extramitochondrially. The other product of the reaction, oxalacetate, could be reduced by DPNH and the cytoplasmic malic dehydrogenase to malate which in turn could react with the malic enzyme to form pyruvate, CO_2, and TPNH. One effect would be a transhydrogenation from DPNH to TPNH, as was shown, an advantage to the organism if lipogenesis is occurring at a rapid rate. Flatt and Ball (1964) have noted that TPNH generation through the hexose monophosphate pathway is not sufficiently rapid to account for the TPNH required for lipogenesis in adipose tissue. The malic enzyme and the coupled transhydrogenase mechanism could provide additional TPNH.

The following diagram illustrates how certain soluble enzymes could exert a concerted effect in lipogenesis.



The malic dehydrogenase–malic enzyme system and phosphoenolpyruvate carboxykinase would be expected to compete for the oxalacetate formed. Elevated carboxykinase levels would divert oxalacetate to carbohydrate formation, while elevated malic enzyme would divert this 4-carbon acid to pyruvate. It should be noted that at low *p*H and in the presence of TPN^+ the malic enzyme could also react with oxalacetate and decarboxylate it directly to pyruvate (Ochoa *et al.*, 1948; Rutter and Lardy, 1958). We have assayed the citrate-cleavage enzyme, cytoplasmic malic dehydrogenase, and the malic enzyme in rat livers under the extreme metabolic conditions of alloxan diabetes and after treatment with insulin. Both the citrate-cleavage enzyme and the malic enzyme would be rate-limiting steps while the soluble malic dehydrogenase is present in excessive amounts under all conditions studied. These results would be compatible with the proposed scheme.

References

Betheil, J. J., Wiebelhaus, V. D., and Lardy, H. A. (1947), *J. Nutr. 34*, 431.
Bhaduri, A., and Srere, P. A. (1963), *Biochim. Biophys. Acta 70*, 221.
Chareonchai, A., and Johnson, R. M. (1963), *J. Nutr. 81*, 387.
Cohn, C., and Joseph, D. (1959), *Am. J. Physiol. 197*, 1347.
Coore, H. G., Randle, P. J., Simon, E., Kraicer, P. F., and Shelesnyak, M. C. (1963), *Nature 197*, 1264.
Flatt, J. P., and Ball, E. G. (1964), *J. Biol. Chem. 239*, 675.
Haas, E., Horecker, B. L., and Hogness, T. R. (1940), *J. Biol. Chem. 136*, 747.
Hutchens, T. T., Van Bruggen, J. T., Cockburn, R. M., and West, E. S. (1954), *J. Biol. Chem. 208*, 115.
Kallen, R. G., and Lowenstein, J. M. (1962), *Arch. Biochem. Biophys. 96*, 188.
Kornacker, M. S., and Lowenstein, J. M. (1964), *Science 144*, 1027.
Kornberg, A., and Horecker, B. L. (1957), *Methods Enzymol. 3*, 323.
Lardy, H. A., Foster, D. O., Shrago, E., and Ray, P. D. (1964a), *in* Advances in Enzyme Regulation Vol. II, Oxford, Pergamon Press, p. 39.
Lardy, H. A., Shrago, E., Young, J. W., and Paetkau, V. (1964b), *Science 144*, 564.
Layne, E. (1957), *Methods Enzymol. 3*, 450.
Lohmann, K., and Meyerhof, O. (1934), *Biochem. Z. 273*, 60.
Lyon, I., Masri, M. S., and Chaikoff, I. L. (1952), *J. Biol. Chem. 196*, 25.
McComb, R. B., and Yushok, W. D. (1964), *Cancer Res. 24*, 193.
Magasanik, B. (1962), *Cold Spring Harbor Symp. Quant. Biol. 26*, 249.
Medes, G., Thomas, A., and Weinhouse, S. (1952), *J. Biol. Chem. 197*, 181.
Monod, J. (1947), *Growth 11*, 223.
Niemeyer, H., Clark-Turri, L., Garces, E., and Vergara, F. E. (1962), *Arch. Biochem. Biophys. 98*, 77.
Nordlie, R. C., and Lardy, H. A. (1963), *J. Biol. Chem. 238*, 2259.
Ochoa, S. (1955a), *Methods Enzymol. 1*, 699.
Ochoa, S. (1955b), *Methods Enzymol. 1*, 739.
Ochoa, S., Mehler, A. H., and Kornberg, A. (1948), *J. Biol. Chem. 174*, 979.
Phillips, P. H., and Hart, E. B. (1935), *J. Biol. Chem. 109*, 657.
Potter, V. R., and Ono, T. (1962), *Cold Spring Harbor Symp. Quant. Biol. 26*, 355.
Rutter, W. J., and Lardy, H. A. (1958), *J. Biol. Chem. 233*, 374.
Shrago, E., Lardy, H. A., Nordlie, R. C., and Foster, D. O. (1963), *J. Biol. Chem. 238*, 3188.
Snedecor, G. W. (1956), Statistical Methods, 5th ed., Ames, Iowa, Iowa State College Press, p. 90.
Sumner, J. B. (1944), *Science 100*, 413.
Tepperman, H. M., and Tepperman, J. (1958), *Diabetes 7*, 478.
Van Bruggen, J. T., Hutchens, T. T., Claycomb, C. K., Cathey, W. J., and West, E. S. (1952), *J. Biol. Chem. 196*, 389.
Vaughan, D. A., and Winders, R. L. (1964), *Am. J. Physiol. 206*, 1081.
Weber, G. (1963), *in* Advances in Enzyme Regulation Vol. I, Oxford, Pergamon Press, p. 1.

Enzymatic Synthesis of Benzoylornithines*

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Received May 25, 1964; revised August 13, 1964

An enzyme system has been prepared from chicken kidney which is capable of catalyzing the synthesis of monobenzoylornithine and ornithuric acid in the presence of benzoic acid, ornithine, adenosine triphosphate, and coenzyme A. Evidence has been obtained for the participation of benzoyladenylic acid and benzoyl-coenzyme A as intermediates in the reaction. The principal monobenzoylornithine synthesized is 5-*N*-benzoylornithine, though both the 2-*N*- and 5-*N*-benzoyl derivatives serve as substrates for ornithuric acid synthesis. Preliminary fractionation studies suggest the involvement of at least three enzymes in the overall reaction.

It has long been known that chickens fed benzoic acid will conjugate it to ornithine and excrete the dibenzoyl derivative, ornithuric acid (Jaffe, 1877). More recently McGilvery and Cohen (1950) described the acylation of 2-*N*- and 5-*N*-benzoylornithines by *p*-aminobenzoic acid catalyzed by the 2000-*g*-sedimentable fraction of chicken kidney homogenates. There has been a very brief report of the formation of acetyl-, *n*-valeryl-, and benzoyl-L-ornithines catalyzed by extracts of acetone powders of chicken and duck kidneys (Schachter *et al.*, 1955). The present paper describes the preparation and partial characterization of an enzyme system from chicken kidney which catalyzes the synthesis of mono- and dibenzoylornithines.

* Supported by a U. S. Public Health Service research grant (GM-05475).

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Experimental

Materials.—L-Ornithuric acid was synthesized by the benzoylation of L-ornithine with benzoyl chloride according to a general acylation method (Schulze and Winterstein, 1898). The product had a melting point of 187–188°, which is in agreement with the value reported for ornithuric acid (Sörensen, 1905). 5-*N*-Benzoyl-L-ornithine was synthesized by the benzoylation of the copper chelate of L-ornithine (McGilvery and Cohen, 1950). The product melted at 243–246°, corresponding closely to the 243–245° previously reported (Baldwin *et al.*, 1960). 2-*N*-Benzoyl-L-ornithine was prepared by the benzoylation of 5-*N*-carbobenzoxy-L-ornithine, followed by removal of the carbobenzoxy group by catalytic hydrogenation. The method used was similar to that of Baldwin *et al.* (1960). The product melted at 238–241°, in good agreement with the 239° previously reported (Baldwin *et al.*, 1960). The 2-*N*-benzoyl-L-ornithine could