Demonstration of the Occurrence of Inactive Fatty Acid Synthetase in Rat Liver by Immunotitration and Its in Vitro Partial Activation*

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Direct immunotitrations of rat liver fatty acid synthetase in crude tissue homogenates with monospecific rabbit anti-rat liver fatty acid synthetase antibody enabled us to make a comparison of fatty acid synthetase protein and activity (percentage of maximal activity) as a function of the nutritional state in normal, diabetic, and insulin- and glucagon-insulin treated animals. Previous results, in which large changes in fatty acid synthetase activity were related to protein synthesis and degradation rather than to enzyme activation, were confirmed. It was also shown that fatty acid synthetase activation does not occur immediately on synthesis but follows the synthesis of fatty acid synthetase protein.

In order to characterize the enzymatically inactive protein found on immunotitration and to develop an in vitro system for fatty acid synthetase activation, conditions were sought to obtain large amounts of fatty acid synthetase protein free from, or at lower in, activity. It was found that treatment of hypophysectomized rats with triiodothyronine meets these requirements, yielding milligram quantities of inactive fatty acid synthetase protein with less than 2% of maximal activity. A part of the inactive fatty acid synthetase was found to be the apoenzyme as indicated by β-ketoreductase and thioesterase activities, by its ability to incorporate label from [2H]CoA, and by its partial in vitro activation, which led to an increase in overall synthetase activity in crude and partially purified cell-free systems. The components required for activation include magnesium ion and a transferase fraction prepared from livers of 48-h fasted, 12-h refed rats.

The activity of mammalian liver fatty acid synthetase is known to be a function of the nutritional state of the animal (1, 2). For example, fasting of the animal results in a reduction of the activity of this enzyme (3, 4). Under refeding, the activity of the enzyme returns to normal, but feeding a high carbohydrate, fat-free diet causes the enzyme activity to rise to above normal levels. In recent years, hormonal factors, particularly insulin, have been shown to stimulate fatty acid synthetase activity to supranormal levels during fast-free diet feeding of diabetic rats previously fasted for 48 h (3, 5, 6). Lakshmanan et al. (6) have demonstrated that the relative rate of synthesis of fatty acid synthetase for diabetic rats treated with insulin was about 10-fold higher than that for untreated diabetic animals. On the other hand, Volpe and Vagelos (7) found that feeding a fructose diet to diabetic rats considerably increased the hepatic fatty acid synthetase level, and this stimulation was not due to enhanced plasma insulin levels. The rates of synthesis of enzyme and the levels achieved were quite different, though, from those found in the experiments with insulin. The effect of thyroid hormones on fatty acid synthetase and other lipogenic enzyme systems has also been investigated (8, 9).

Previous investigations were undertaken to understand the mechanism of enzyme regulation in vivo. Attempts were made to determine whether nutritional and hormonal variation in fatty acid synthetase activity are due to protein synthesis or modification, or both. Burton et al. (3) found a considerable increase in the rate of incorporation of [14C]leucine into fatty acid synthetase and an increase in specific activity of fatty acid synthetase between 3 and 12 h after the start of refeeding. Later, Craig et al. (10), using immunochromatographic studies, showed that it is the rate of synthesis and not that of degradation that is the major parameter in controlling the liver fatty acid synthetase content in rats subjected to nutritional stress. The role of insulin in the regulation of the rate of synthesis of fatty acid synthetase was unequivocally established (6) when a 20-fold increase was found in the actual amount of purified fatty acid synthetase from diabetics treated with insulin as compared to untreated rats. On the basis of these results, it was concluded that the diet- and insulin-induced changes in the level of fatty acid synthetase are due primarily to an adaptive increase in the rate of synthesis of this enzyme rather than an activation of previously existing enzyme.

In earlier investigations, the ratio of active to inactive enzyme species could not be determined directly in liver homogenates but had to be inferred after several purification steps from the specific activity of the enzyme. This involved the tenuous assumption that active and inactive forms of the same enzyme co-purified in the same ratio as was present in the crude extract. In the present investigation, we have estimated the amounts of active and inactive species of fatty acid synthetase by titrating a specified enzyme activity with monospecific anti-rat liver fatty acid synthetase antibody. In this procedure, equivalent units of enzyme activity will give the same endpoints on immunotitration if all enzyme molecules have the same activity, whereas in the presence of inactive species, the endpoint will be different. The immunotitration method is fast, reproducible, and sensitive. These studies have confirmed that the rate of synthesis is the major parameter in regulating the concentration of fatty acid synthetase in rats subjected to nutritional stress and insulin administration. However, administration of triiodothyronine to ad libitum-fed hypophysectomized rats resulted in the accumulation of...
an amount of inactive species of the synthetase which was much greater than observed for other dietary or hormonal regimens. The large yield of inactive species of fatty acid synthetase allowed us to partially establish the identity of this species and to develop a system which partially reactivates the inactive species in vitro.

MATERIALS AND METHODS

RESULTS

Effect of T3 and Hydrocortisone on the Ratio of Enzymatically Active to Inactive Fatty Acid Synthetase in Hypophysectomized (Ad libitum-fed) Rats—The administration of T3 to hypophysectomized animals did not result in any significant change in fatty acid synthetase activity over a period of 48 h. Immunotitration confirmed these results by showing very low levels of active enzyme which remained almost constant up to 48 h after T3 injection. However, there was a dramatic increase in the levels of inactive fatty acid synthetase species at 16, 24, and 32 h after T3 injection (100 μg/100 g of body weight) (Table VI). These data are in agreement with earlier reports (5, 22), inasmuch as there is an increase in protein synthesis in response to T3 administration. It may be pointed out here that Tata (23) has shown that the increase in protein synthesis on T3 administration is dependent on the dose and that there is a lag period of 12 to 48 h. In the present studies, a T3 dosage level of 100 μg/100 g of body weight greatly stimulated the synthesis of the fatty acid synthetase complex, whereas 10 μg/100 g of body weight produced a much smaller stimulation. However, if one had followed the increase in enzyme activity as an indicator of protein synthesis, one would have concluded the absence of enzyme synthesis and missed the inactive enzyme species present. Interestingly, hydrocortisone administration had no effect on the induction process. There was no difference in the levels of active and inactive enzyme over a time period of 0 to 48 h from the time of injection.

Incorporation of [14C]Pantetheine into Active and Inactive Fatty Acid Synthetase—Aliquots of the dialyzed DEAE-cellulose-purified fatty acid synthetase proteins were mixed with equal volumes of 4 N KOH in small test tubes. The contents of the tubes were heated to boiling over a small flame, cooled to room temperature, and neutralized with a slight excess of 2 N acetic acid. The mixtures were assayed in dioxane for radioactivity. The results shown in Table VII indicate that the incorporation of [14C]pantetheine into the fatty acid synthetase of triiodothyronine-treated hypophysectomized rats is less by an order of 10-fold that in normal rats.

Retention of the [14C]Pantotenate Pool—The cytosol was counted for radioactivity in order to determine whether nonincorporation of [14C]pantetheine into inactive fatty acid synthetase could be accounted for by a failure of the hypophysectomized rats to retain [14C]pantetheine. The 100,000 × g supernatant solution of liver homogenate from normal rats 24 h after refeeding and 21 h after the first injection of [14C]pantetheine contained 68,000 dpm/ml or a total of 675,000 dpm per rat liver (average of 4 rats). The 100,000 × g supernatant solution of liver homogenate from hypophysectomized T3-injected rats contained 93,000 dpm/ml or 450,000 dpm per rat liver (average of 2 rats). Thus, the retention of the [14C]pantotenate pool in the two sets of animals is quite similar, which means that the nonincorporation of [14C]pantetheine into fatty acid synthetase could not be attributed to a premature excretion of substrate. The results indicate, therefore, that the inactivity of the fatty acid synthetase in T3-treated hypophysectomized rats is due to a lack of 4-phosphopantetheine, and that the inactive enzyme complex may, at least in part, consist of the apo form of the enzyme.

Evidence of Enzymic Transfer of 4'-Phosphopantetheine from [G-3H]CoA into Inactive Fatty Acid Synthetase—The incorporation of radioactivity from [G-3H]CoA into inactive rat liver fatty acid synthetase is shown in Table VIII. The data of this table show a requirement for the supernatant solution of liver homogenate from 48-h fasted, 12-h refed rats and for

| Source | Total fatty acid synthetase | Fatty acid synthetase synthesized/total enzyme | Radioactivity in fatty acid synthetase | Source | Total | Synthesized |
|--------|-----------------------------|---------------------------------------------|-------------------------------------|--------|-------|------------|
| 3 h    | 7.5                         | 0.892                                       | 2276                                | Normal, 24-h refed | 20.28 | 0.923      |
| 24 h   | 2.0                         | 0.28                                        | 190                                 | Hypophysectomized, 24-h fed + T3 | 20.28 | 0.923      |

TABLE VIII

Incorporation of [3H]CoA into inactive fatty acid synthetase Values represent immunoprecipitate from one-fourth aliquot of reaction mixture: 62 μg of fatty acid synthetase.

| Source | Total | Synthesized |
|--------|-------|------------|
| Complete | 10,580 |           |
| Transferase | 2,560 |           |
| Acceptor (apoenzyme) | 500 |           |
| Mg++ | 600 |           |

The assay system contained 4 mM EDTA.

TABLE VI

Amount of enzymatically active and inactive rat liver fatty acid synthetase in ad libitum-fed hypophysectomized rats after T3 administration

| Time after injection | Average weight of livers | Inactive | Active | Fatty acid synthetase |
|----------------------|--------------------------|----------|--------|-----------------------|
| 0 h                  | 4.0                      | 0.45 ± 0.1 | <0.050 |                      |
| 24 h                 | 3.9                      | 0.87 ± 0.15 | <0.050 |                      |
| 24 h                 | 4.1                      | 3.2 ± 0.2  |        |                      |
| 8 h                  | 5.5                      | 3.6       | 0.30   | 0.087                 |
| 16 h                 | 5.2                      | 6.2       | <0.1   | <0.016                |
| 24 h                 | 4.8                      | 8.5       | <0.1   | <0.012                |
| 32 h                 | 5.0                      | 5.9       | <0.1   | <0.015                |
| 48 h                 | 4.8                      | 4.0       | 0.14   | 0.032                 |
| 48 h                 | 5.0                      | 2.05      |        |                      |

* 0.9% saline only.
+ 10 μg of T3/100 g of body weight.
+ 100 μg of T3/100 g of body weight.
magnesium for this reaction. Assuming that 50% of the label of [14C]CoA is on the pantetheine moiety, a calculation based on data of Table II yields the result of ~0.11 nmol of pantetheine incorporated per 0.125 nmol of fatty acid synthetase protein. Ammonium sulfate fractionation of the crude preparation from 48-h fasted, 12-h refed rats indicates that this “transferase” activity precipitates between 20 and 35% of saturation (data not shown).

**Fig. 5. Increase in units of fatty acid synthetase activity as a function of time of incubation and apoenzyme, CoA and transferase concentrations.** A. increase in units of fatty acid synthetase activity versus amount of transferase fraction added. Reaction mixtures contained 70 μl (160 μg of inactive enzyme protein) of a 20 to 33% ammonium sulfate precipitated protein fraction of the 100,000 X g supernatant solution of liver homogenate from hypophysectomized, T4-treated rats. Also present were 20 to 35% heat-treated ammonium sulfate fraction, 10 to 60 μl (15 to 90 μg protein), from livers of 12-h refed rats, 7.5 μl of 0.01 M CoA, and transferase reaction buffer in a final volume of 0.180 ml. The reaction was started by the addition of the CoA solution and carried out at 30 °C for 30 min. Reactions were terminated by the addition of 15 μl of 0.1 M EDTA. A 100-μl aliquot of each incubation mixture was pipetted into 0.35 ml of reassociation buffer. Incubation of this mixture was carried out for 60 min at 30 °C. Fatty acid synthetase activity was determined after preincubating 6 min with 25 μl of 2 mM NADPH. After the blank NADPH oxidation was determined, acetyl- and malonyl-CoA, 25 μl of 0.67 mM and 2 mM, or 0.33 and 1.0 μmol, respectively, were added and the rate of NADPH oxidation with substrate was determined. The rates of fatty acid synthetase activity were corrected to zero CoA concentration. B, increase in units of fatty acid synthetase activity versus time of incubation of inactive fatty acid synthetase with the transferase at 30 °C. The incubation mixture had 300 μl (0.6 mg inactive enzyme protein) of a 20 to 33% ammonium sulfate-precipitated fraction, 180 μl (100 μg of protein) of heat-treated transferase fraction, and the reaction buffer, in a final volume of 0.57 ml. The final Mg2+ concentration was 0.01 M. The reaction was started by the addition of 30 μl of 0.01 M CoA (final concentration, 5 X 10−3 M). Aliquots (100 μl) were withdrawn at 0, 5, 10, 25, and 40 min, pipetted into 0.35 ml of reassociation buffer containing 0.5 mM potassium phosphate, pH 7.0, 3.3 mM EDTA, and 1 mM diithothreitol, and incubated at 30 °C for 45 min. Fatty acid synthetase activity was assayed as reported in the legend for A. C, increase in units of fatty acid synthetase activity versus CoA concentration. Reaction mixtures contained 30 μl (70 μg of protein) of a 20 to 33% ammonium sulfate-precipitated fraction of the 100,000 X g supernatant solution of liver homogenate from T4-treated hypophysectomized rats, 30 μl (20 μg of protein) of a 20 to 35% ammonium sulfate fraction, diluted one-tenth and heat-treated (see text), from the 100,000 X g supernatant solution of homogenate of livers from 12-h refed rats, and designated concentrations of CoA that were made up to 0.10 ml with reaction buffer. Reactions were started by the addition of the 20 to 33% ammonium sulfate-precipitated protein fraction. Incubations were carried out at 30 °C for 15 min. The reactions were stopped by the addition of 10 μl of 100 mM EDTA. CoA was separated from the reaction mixtures by passing through a Sephadex G-50 column (0.5 X 5 cm) in 0.2 mM potassium phosphate, pH 7.0, containing 1 mM EDTA. The fractions containing protein were combined (total, 0.8 ml), and the reaction buffer, in a final volume of 0.57 ml. The samples were brought to 10 mM with respect to diithothreitol and incubated at 30 °C for 45 min. A 0.450-ml aliquot of each incubation mixture was assayed for fatty acid synthetase activity as described for A. D, increase in units of fatty acid synthetase activity versus quantity of inactive fatty acid synthetase. For each determination, a control without transferase (heat-treated 20 to 35% ammonium sulfate-precipitated fraction of 48-h fasted, 12-h refed rats) was run. Assays with the complete system included 30 μl (20 μg of protein) of the transferase fraction, 10 mM magnesium chloride, 1.5 X 10−3 M CoA, and designated amounts of the 20 to 30% ammonium sulfate-precipitated fraction of inactive fatty acid synthetase (0.6 mg of protein/ml), and the reaction buffer, in a volume of 0.1 ml. Reactions were initiated by adding CoA, carried out at 30 °C for 60 min, and then terminated by pipetting the mixtures into 0.350 ml of the reassociation buffer. The remaining steps of the assays were carried out as described for A.
Stability of Apo-Fatty Acid Synthetase—Assays of mixtures of the apoenzyme before and after activation of fatty acid synthesis by the transferase reaction indicated that the specific activity of the partial reaction, β-ketoacyl reductase, did not change. Therefore, the total β-ketoacyl reductase activity represents the sum of the ketoreductase activities of apo- and holoenzymes and could be used to determine the quantity of apoenzyme (which could be converted to active enzyme) in a sample in conjunction with assays for fatty acid synthetase activity. However, it was found that apoenzyme loses its β-ketoacyl reductase activity after one or two freezings and thawings unless it is stored in 15% glycerol. The first ammonium sulfate step also causes a decrease in β-ketoacyl reductase activity of the apoenzyme, even though the recovery of fatty acid synthetase-related protein is quantitative.

Dependence of Reactivation of Fatty Acid Synthetase on Quantity of Transferrase Fraction—A linear dependence in the increase in fatty acid synthetase activity on the volume of the transferrase fraction added is shown in Fig. 5A.

Dependence of Reactivation of Fatty Acid Synthetase on Time of Reaction—A linear increase in fatty acid synthetase activity with time of transferrase action is shown in Fig. 5B.

Dependence of Reactivation of Fatty Acid Synthetase on Concentration of CoA—In this experiment, CoA was removed from each reaction mixture by molecular filtration before assay of fatty acid synthetase activity. The experiment was designed so that the time of reaction at the highest level of CoA concentration permitted the reaction of no more than one-third of the apoenzyme. Under these conditions, the rate of activation of the apoenzyme showed a linear dependence on the concentration of CoA substrate at low levels. At higher concentrations, the reaction rate was nonlinear with CoA (Fig. 5C). An apparent $K_a$ (pseudo-first order reaction) of approximately $1.5 \times 10^{-4}$ M for CoA was obtained.

Dependence of Increase of Fatty Acid Synthetase Activity on Quantity of Apeoenzyme—The reaction for each level of apoenzyme concentration was allowed to go to completion. Fig. 5D indicates that the limit of increase of fatty acid synthetase activity is directly proportional to the quantity of apoenzyme in the reaction mixture.

Comparison of NADPH Disappearance with Long Chain Fatty Acid Formation due to Increase in Fatty Acid Synthetase Activity—The increase in NADPH oxidized in a specified time interval (determined spectrophotometrically) and the increase in fatty acid synthesis in the same time interval (determined radiochemically) are plotted against time of transferrase reaction in Fig. 6A. Fig. 6B is a plot of the increase in fatty acids synthesized against the increase in NADPH oxidized. NADPH disappearance and fatty acids formed are related by the stoichiometry of the following equation:

\[
\text{Acetyl-CoA} + 7[^{14}C]\text{malonyl-CoA} + 14\text{NADPH} \rightarrow
\]

\[
[^{14}C]\text{pantetheine} + 7\text{CO}_2 + 8\text{COASH} + 14\text{NADP}^+ + 7\text{H}_2\text{O}
\]

These plots indicate a linear relationship between NADPH disappearance and fatty acid formation with time. The plots also indicate the stoichiometric equivalence between substrate consumed (NADPH) and product formed (long chain fatty acids considered as palmitate) due to newly activated fatty acid synthetase.

**DISCUSSION**

The preparation of monospecific antirat liver fatty acid synthetase antibody by affinity chromatography on a stationary phase containing homogeneous antigen makes it possible to estimate the antigen directly in crude homogenates. It had been established earlier (14) and in this work that immunotitration is a suitable method for determining the amount of active and inactive enzyme protein in 100,000 x g supernatant solutions of animals subjected to different hormonal and dietary regimens. In this report, we have shown that under conditions of partial inhibition of fatty acid synthetase activity by the monospecific immunoglobulin, the quantity of immunoprecipitate formed by the antibody is a function of quantity of the antibody and not antigen. The quantity of antigen removed from an excess by a given amount of antibody is constant. These results further confirm the validity of Equations 1 and 2. Determination of the endpoint in the titration (Fig. 2) depends on a determination of the intercept on the y axis. The use of linear regression analysis for determining the intercepts gave standard deviations of less than ±0.3 mmol/ min. This corresponds to a value of ±0.5 mg of antigen protein/ml for 48-h refed liver supernatant solution and ±0.023 mg of antigen protein/ml for 0-h refed animals.

There is a rapid increase in the ratio of active enzyme to total enzyme in the initial 6- to 12-h period after the commencement of refeeding fasted rats a high carbohydrate, fat-free diet. Similarly, the refeeding of diabetic rats treated with insulin resulted in the production of rapidly increasing amounts of enzymatically active hepatic fatty acid synthetase. This shows that feeding of a fat-free diet and administration of insulin increases the rate of synthesis of the fatty acid synthetase. This confirms the earlier work (6, 10) and for the first time provides a means to quantitate directly the actual amount of active enzyme present in a particular hormonal or nutritional state. However, the amount of inactive species in the animals in these nutritional and hormonal states remains almost at the same level during the initial 8-h period. It seems probable that the accumulation of low levels of inactive species in the initial period is due to the low rate of conversion of inactive species into active species, which implies the absence or low level of an enzyme involved in such a conversion in the initial period. The disappearance of the inactive species after an 8-h period would then, suggest that the transferase activity involved in the conversion of the enzymatically inactive to active species is being generated at a later time than the fatty acid synthetase. It is tempting to suggest that the inactive species is apo-fatty acid synthetase. Earlier investigators (10, 24, 25) have shown the presence of apoenzyme in rat liver. Yu and Burton (24) found that in fasted, refed animals, incorporation of $[^{14}C]$pantetheine into fatty acid synthetase does not occur until approximately 4 h from the start of refeeding, whereas the incorporation of $^3$H-labeled amino acids into immunoprecipitable protein commences with the time of refeeding. Consequently, it was suggested that the enzyme converting the apoenzyme to holoenzyme, viz. 4'-phosphopantetheine transferase may be present in the 100,000 x g supernatant solution of liver homogenates from 12-h refed rats.

The role of glucagon in inhibiting the induction of fatty acid synthetase in insulin-treated animals was demonstrated earlier (6). Our data obtained by immunotitration demonstrate the absence of active enzyme in glucagon-treated animals, whereas the amount of inactive species stays constant (Table V). It is clear from our results that immunotitration gives a reliable estimate of the amount of active and inactive enzyme in the 100,000 x g supernatant solution obtained from animals in different hormonal and nutritional states. This method is fast, reproducible, and sensitive.

The only hormonal state in which large amounts of inactive fatty acid synthetase species were found was in hypophysectomized rats 24 h after a T3 injection. Even though growth hormone was absent in these animals (as shown by an average of zero weight gain in one week), T3 injection at the level of 10
and 100 μg/100 g of body weight stimulated an approximately 2- to 10- to 20-fold increase, respectively, in fatty acid synthetase protein. It is of significance to note that even though there is not a large increase in fatty acid synthetase protein in liver during refeeding fasted normal rats a regular diet, the percentage of maximal activity increases sharply. Only when normal rats are refed a fat-free diet after fasting are high levels reached for both fatty acid synthetase protein and percentage of maximal activity for that protein. This results in a 10- to 20-fold increase in capacity to produce fatty acids.

The rationale for feeding the hypophysectomized rats on a regular, instead of a fat-free, diet is based on the fact that the rats are unable to synthesize endogenous fatty acids. This will ultimately result in fatal consequences if fatty acids are not externally replenished. Hypophysectomized animals eat very little, and many lose 5 to 10% in weight during the week after the operation. As a result, they are virtually in a state of fasting. Hypophysectomy causes a condition of thyroid and adrenal deficiency, which in turn affects lipid metabolism. It is known that thyroid hormones enhance lipid synthesis (21-23), though the mechanism of stimulation is not known. That factors in addition to thyroid hormones appear necessary is shown by the fact that the amount of active fatty acid synthetase is reduced in hypophysectomized animals to about 1% of that in 48-h fasted, 48-h refed normal animals. Similar decreases in the specific and total activity of fatty acid synthetase after hypophysectomy were reported earlier (9). On the other hand, in the T₃-treated hypophysectomized rats, the amount of inactive enzyme increases, whereas the amount of active fatty acid synthetase decreases during the period of protein synthesis (Table VI). Kumar et al. (9) reported that T₃ at 100 μg/100 g of body weight elevates the specific activity of fatty acid synthetase in the hypophysectomized animals 8- to 9-fold over control animals 3 days after the injection. However, the data reported by these authors indicate that, even after this increase, the level of active enzyme reached only 1 mg/g of liver tissue or 0.4 mg/ml of homogenate.

The inactive enzyme found in our work appears to be apo-fatty acid synthetase, as characterized by the following experiments: (a) approximately quantitative transfer of the label from [G-³H]CoA to inactive fatty acid synthetase in the presence of Mg²⁺ and a fraction from 48-h fasted, 12-h refed rats, and (b) increase in overall fatty acid synthetase activity in the presence of CoA, Mg²⁺, and the transferase fraction. Sucrose density and sodium dodecyl sulfate-gel electrophoresis indicate that the inactive protein has a molecular weight close to that of the active protein and is homogeneous, as shown by a single peak corresponding to that of the active complex and a single band, respectively, corresponding to that of the half-molecular weight subunit.

While it appears that all of the inactive protein immunologically cross-reacting with fatty acid synthetase is apo-fatty acid synthetase within the uncertainty of the distribution and exchange with H₂O of the label in [³H]CoA, the recovery of overall activity of the enzyme upon incorporation of pantetheine is incomplete. The generation of overall activity is, however, dependent upon the parameters which characterize a transformation from apo- to holoenzyme. It remains to be shown whether another step in modification of the enzyme protein is needed to bring about complete reactivation or whether, in fact, some of the material, while of high molecular weight, is in the initial stage of degradation. Indeed, the reductase and thioesterase partial reactions of the apoenzyme are much less stable than those in the holoenzyme, indicating that the presence of 4'-phosphopantetheine is needed for stability of some portions of the complex as well as for the overall reaction mechanism. Our results are similar to those of Werkmeister et al. (26), who recently reported that only a 20% increase in the specific activity of the in vitro-prepared holoenzyme was obtained, as compared to in vivo-synthesized enzyme, when partially purified yeast cell extract was utilized as a source of apoenzyme 4'-phosphopantetheine transferase activity.

Further work is being carried out on the characterization of the apoenzyme and on the quantitative determination of the prosthetic group in both the apo- and holoenzyme, as well as in the product of the transferase reaction.

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Immunotitration of Rat Liver Fatty Acid Synthetase
were and Hypophysectomized Rats after 12-mlmistration of 14C-Butyrate. A group of control rats were injected with saline, and a group of rats were injected with 14C-Butyrate. The mixture was injected at 2.8 ml/kg body weight. The animals were sacrificed 2 h after the injection.

Preparation of Liver Homogenates - The liver homogenates were prepared as previously described (13). The liver tissue was minced with a scalp knife and homogenized in hypotonic saline (100,000 g supernatant solution of liver homogenate) using a Teflon/glass homogenizer. The volume of the homogenate was adjusted to 8 ml/liver using saline. The homogenate was then centrifuged at 100,000 g for 30 min. The supernatant was collected and used for the assay.

Determination of Protein - Protein was determined by the method of Lowry et al. (17) as modified by Bio-Rad (18).

Immunofluorochromatography of Rabbit Antiserum Against Fatty Acid Synthetase - A rabbit antiserum against rat fatty acid synthetase (prepared in this laboratory) was used. The antiserum was diluted 1:10 in PBS containing 0.1% bovine serum albumin.

Preparation of Fatty Acid Synthetase - The fatty acid synthetase was isolated from rat liver homogenate as described previously (13). The homogenate was centrifuged at 100,000 g for 30 min. The supernatant was collected and used for the assay.

Flushing and Perfusion - The liver was flushed and perfused with saline as described previously (13).

Immunoeffiency - The antibody was added to the perfusion buffer at a concentration of 1:10 in PBS. The mixture was then incubated at 37°C for 30 min. The reaction was stopped by the addition of 2 M HCl. The mixture was then centrifuged at 100,000 g for 30 min. The supernatant was collected and used for the assay.

Immunoprecipitation - The mixture was then incubated at 37°C for 30 min. The reaction was stopped by the addition of 2 M HCl. The mixture was then centrifuged at 100,000 g for 30 min. The supernatant was collected and used for the assay.

Immunofluorochromatography of Rabbit Antiserum Against Fatty Acid Synthetase - A rabbit antiserum against rat fatty acid synthetase (prepared in this laboratory) was used. The antiserum was diluted 1:10 in PBS containing 0.1% bovine serum albumin.

Preparation of Fatty Acid Synthetase - The fatty acid synthetase was isolated from rat liver homogenate as described previously (13). The homogenate was centrifuged at 100,000 g for 30 min. The supernatant was collected and used for the assay.

Flushing and Perfusion - The liver was flushed and perfused with saline as described previously (13).

Immunoeffiency - The antibody was added to the perfusion buffer at a concentration of 1:10 in PBS. The mixture was then incubated at 37°C for 30 min. The reaction was stopped by the addition of 2 M HCl. The mixture was then centrifuged at 100,000 g for 30 min. The supernatant was collected and used for the assay.
Immunotitration of Rat Liver Fatty Acid Synthetase

**FIG. 2.** Immunoregulation of monoclonal antibody with rat liver fatty acid synthetase. Antibody (2.4 µg) was titrated with 1.1 to 15 µg of the rat liver enzyme. Each assay mixture contained 1.0 µg of antibody, 5 µg of fatty acid synthetase in 50 µl of 0.05 M potassium phosphate buffer, pH 7.0. 100 µg bovine serum albumin (BSA) and 0.1 M sodium metabsulfite, pH 7.0, and 0.05 µM MnCl₂. The rate of the reaction was followed spectrophotometrically at 410 nm.

This method assumes that the same amount of antibody will always bind to antigen when fatty acid synthetase is in solution. Then the intercepts will be proportional to the specific activity of the fatty acid synthetase. From this consideration, the concentration of inactive enzyme protein in a sample is calculated from the relation:

\[ Y = \frac{A + B}{C} \]

where \( Y \) is the intercept when all the fatty acid synthetase is in solution and \( C \) is the concentration of (fully active) enzyme protein added to the titration mixture. The quantities of antibody and fatty acid synthetase are specific activity, \( A \), and the concentration, \( B \), of fatty acid synthetase. Antibody (2.6 µg) was dialyzed sodium metabsulfite at pH 7.0, and 100 µg bovine serum albumin in 1.45 ml of 0.5 M potassium phosphate buffer (pH 7.0), 1 mM EDTA, and 1 mM dithiothreitol, and incubated for 30 min. NADPH, 25 µl of 20 mM (0.05 µM), acetyl-CoA, 20 ml of 0.67 M, and malonyl-CoA, 2 µM of 0.03 M, respectively, were added and the reaction was monitored spectrophotometrically at 340 nm. The supernates were then dialyzed and assayed for protein as described previously.

**Determination of Immunoregulatory Activity of Fatty Acid Synthetase in Fractions in Different Nutritional States**

The concentration of immunoregulatory activity of fatty acid synthetase was determined by the immunotitration method. The concentration of immunoregulatory activity of fatty acid synthetase in the initial 6-h feeding period was very low, whereas the amount of inactive species showed very little change, thus maintaining the ratio of active to inactive species almost constant. In the period between 12 h and 24 h of feeding, the most drastic increase in the ratio of immunoregulatory activity of fatty acid synthetase was found. Table II presents the results in rats fasted for 6 h and then refed for 24 h. There is a progressive decrease in the amount of enzyme with fasting time, although the decrease is slower in the total 6% of the ratio of immunoregulatory activity of fatty acid synthetase shows a decrease in the 6-12 h period.

**TABLE I**

| Duration of fasting | Average weight of rats (g) | Active fatty acid synthetase (mg protein/ml) | Inactive fatty acid synthetase (mg protein/ml) | Total fatty acid synthetase (mg protein/ml) |
|---------------------|---------------------------|---------------------------------------------|-----------------------------------------------|--------------------------------------------|
| 0 h                 | 350                        | 4.0                                        | 0.5                                           | 4.5                                         |
| 6 h                 | 340                        | 3.5                                        | 0.3                                           | 3.8                                         |
| 12 h                | 330                        | 3.0                                        | 0.2                                           | 3.2                                         |
| 24 h                | 320                        | 2.5                                        | 0.1                                           | 2.6                                         |

*All animals were fasted for 48 h and then refed a fast-free diet.

**TABLE II**

| Duration of fasting | Average weight of rats (g) | Active fatty acid synthetase (mg protein/ml) | Inactive fatty acid synthetase (mg protein/ml) | Total fatty acid synthetase (mg protein/ml) |
|---------------------|---------------------------|---------------------------------------------|-----------------------------------------------|--------------------------------------------|
| 0 h                 | 350                        | 4.0                                        | 0.5                                           | 4.5                                         |
| 6 h                 | 340                        | 3.5                                        | 0.3                                           | 3.8                                         |
| 12 h                | 330                        | 3.0                                        | 0.2                                           | 3.2                                         |
| 24 h                | 320                        | 2.5                                        | 0.1                                           | 2.6                                         |

*All animals were fasted for 48 h, followed by a fast-free diet for 48 h and then refed.

**Effect of Insulin and Glucagon on the Immunoregulatory Activity and Inactive Activity of Rat Liver Fatty Acid Synthetase.**

The results of immunoregulatory activity of fatty acid synthetase were obtained during the course of feeding in rats on a fast-free diet, after insulin treatment, after glucagon administration, and after insulin and glucagon administration. The results presented in Table III are the average of duplicate or triplicate experiments. The results from Table III that diabetic rats showed the same ratio of enzyme activity to total enzyme activity as the amount of active enzyme activity in the fed rats. The sum of active enzyme activity and inactive enzyme activity is greater than the initial lag period of insulin but is still during the 6-h period. The animals are fasted for 6 h. The animals were placed in insulin with insulin after the 6-h period. The results from Table III that diabetic rats showed the same ratio of enzyme activity to total enzyme activity as the amount of active enzyme activity in the fed rats. The sum of active enzyme activity and inactive enzyme activity is greater than the initial lag period of h in normal rats (Table II). The amount of active enzyme activity at 48 h of refed was not observed for normal rats that were subjected to an identical dietary regimen. These results are in excellent agreement with earlier work in which the highest enzyme activities at 24 and 48 h of refed were determined for diabetic animals and insulin and non-insulin animals.
Table III

| Duration of refeeding | Average weight of livers | Inactive | Active | Total fatty acid synthetase |
|-----------------------|--------------------------|---------|--------|-----------------------------|
|                       | g                        | mg protein/ml | ratio  |                             |
| 0                     | 7.3                      | 0.120   | 0      | 5                           |
| 2                     | 9.7                      | 0.065   | 0.12   | 0.65                        |
| 4                     | 12.0                     | 0.248   | 0.22   | 0.47                        |
| 8                     | 12.8                     | 0.45    | 0.04   | 0.56                        |
| 12                    | 12.0                     | 0.420   | 1.15   | 0.73                        |
| 24                    | 11.0                     | 0.810   | 2.76   | 0.77                        |

Rats were made diabetic by injecting streptozotocin (65 mg/kg body weight) into the tail vein of 48-h fasted rats.

Table IV

| Duration of refeeding | Average weight of livers | Inactive | Active | Total fatty acid synthetase |
|-----------------------|--------------------------|---------|--------|-----------------------------|
|                       | g                        | mg protein/ml | ratio  |                             |
| 0                     | 6.7                      | 0.39    | 0      | 0                           |
| 2                     | 9.8                      | 0.50    | 0.15   | 0.23                        |
| 4                     | 11.6                     | 0.15    | 0.75   | 0.89                        |
| 8                     | 12.8                     | 0       | 5.30   | 1.0                         |
| 12                    | 15.2                     | 0       | 5.50   | 1.0                         |

Insulin was administered subcutaneously at a dose of 3 units/day per 100 g of body weight. For short refeeding times, insulin was given as a dose of 1 unit/100 g of body weight was given 1, 4, and 8 h before sacrifice.

The data presented so far show that insulin is involved in the dietary induction of hepatic fatty acid synthetase. Since glucagon is a known physiological antagonist of insulin (6,7), we also studied the effect of glucagon on the amount of active and inactive enzyme. Table V shows the effects of insulin and glucagon treatments on the amount of enzymatically active and inactive rat liver fatty acid synthetase in diabetic rats at different times of refeeding. It is evident from these data that glucagon inhibits the formation of active enzyme during the first 8 h of refeeding.

Table V

| Duration of refeeding | Average weight of livers | Inactive | Active | Total fatty acid synthetase |
|-----------------------|--------------------------|---------|--------|-----------------------------|
|                       | g                        | mg protein/ml | ratio  |                             |
| 0                     | 7.2                      | 0.24    | 0      | 0                           |
| 2                     | 9.3                      | 0.30    | 0      | 0                           |
| 4                     | 9.3                      | 0.42    | 0      | 0                           |
| 8                     | 11.7                     | 0.48    | 0      | 0                           |
| 24                    | 21                       | 0.48    | 2.0    | 0.81                        |
| 48                    | 18                       | 0.38    | 1.0    | 0.63                        |

Glucose was administered at a dose of 100 mg/100 g of body weight 3 h before the insulin administration. Insulin was administered as in Table IV (see also Section on Methods).