The Effect of Fasting on Synthesis and 4'-Phosphopantetheine Exchange in Rat Liver Fatty Acid Synthetase*

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SUMMARY

The level of rat liver fatty acid synthetase is greatly decreased upon fasting. This report presents several additional observations of the effect of fasting on that enzyme. (a) The rate of synthesis is reduced during 48 hours of fasting and is 20% of the steady state rate after 12 hours. (b) The apparent half-life of the enzyme is decreased from about 70 hours to about 20 hours. (c) The exchange of the prosthetic group, 4'-phosphopantetheine, is largely eliminated, perhaps by effects on the mechanism of attachment or hydrolysis. (d) Feeding a protein-free diet mimics the effect of starvation on the enzyme levels.

In higher animals fatty acids are synthesized de novo in the liver by a multienzyme complex, fatty acid synthetase. Much of the knowledge about the mechanism and control of activity of the complex has been derived from in vitro studies (1-10). Some features which may have an influence on the control of fatty acid synthesis cannot be studied except by in vivo experimentation, since they occur as a result of complex cellular organization. In particular, regulation of the level of enzyme and controls effecting the availability of prosthetic group 4'-phosphopantetheine are of interest. A previous paper (11) has demonstrated that the fatty acid synthetase complex turns over with a half-life of about 3 to 4 days, whereas the covalently bound prosthetic group seems to be exchanged very rapidly. A similar observation of prosthetic group mobility has been made in the Escherichia coli fatty acid synthesizing system (12).

It has been known for some time that an effect of fasting on rat liver is to decrease the measured activity of enzyme and correspondingly to decrease the liver content of fatty acids (13). This correlation seems to reinforce the position that the level of fatty acid synthetase is important in the cellular control of fatty acid production. This report examines the means whereby the loss of enzyme activity is effected by various diet changes, and describes an effect of fasting on the prosthetic group exchange phenomenon.

EXPERIMENTAL PROCEDURE

Materials—Malonyl-CoA, acetyl-CoA, TPNH, and dithiothreitol were obtained from Nutritional Biochemicals Company. Polysorbate 80 and acrylamide from Sigma; DEAE, ammonium sulfate, sucrose, and 14C-carnitine acid mixture (saline profile) were obtained from Schwartz-Mann. Alternately, 14C-amino acid mixture was obtained from New England Nuclear as was the protein solubilizer, flour, and scintillation fluid (Protosol, Omnifluor, and Aquasol, all proprietary products). [3H]Pantothenate was also prepared by New England Nuclear by catalytic exchange of the hydrogen of a sample of calcium pantothenate purchased from Calbiochem. The calcium [3H]pantothenate was extensively contaminated and required purification. This was effected by chromatography of the entire sample (47 mg, 227 mCi) on Whatman No. 3MM paper in 1-butanol-acetic acid-water (25:4:10, v/v/v) followed by autoradiography to locate the resolved bands. These were cut out and eluted, the pantothenate located by comparison with standard and verified by nuclear magnetic resonance analysis (performed by Dr. Charles Klopstein, University of Oregon).

Calbiochem also supplied the Dowex 50-XS resin. Sepharose 6B was obtained from Pharmacia. Calcium phosphate gel was prepared according to the method of Keilin and Hartree (14) and aged at least a year.

Animals—All rats were Sprague-Dawley males weighing 150 ± 10 g at the time of purchase from Hilltop Lab Animals, Chatsworth, California. Upon arrival the animals were placed on the experimental diet in a controlled day-night cycle of 12 hours, daylight beginning at 7:00 a.m. The animals were kept for 5 to 7 days prior to experimentation at which time they weighed 180 to 200 g.

Diet—Several diets were used, each supplied by Nutritional Biochemicals Company: vitamin B complex test diet complete (18% casein, 68% sucrose, 10% vegetable oil, 4% salt mixture), fat free test diet (21% casein, 16.5% cellulose, 58.5% sucrose, 4% salt mixture U.S.P. XIV), normal protein test diet (27% casein, 50% sucrose, 10% vegetable oil, 4% salt mixture U.S.P. XIV), and protein free diet (70% sucrose, 15% cellulose, 10% vegetable oil, 1% cod liver oil, 4% salt mixture U.S.P. XIV). The last two diets of those listed above were especially prepared...
by Nutritional Biochemical Co. and are not listed in their catalogue. All diets except protein-free diet are supplemented with vitamins. In all experiments, animals had access to drinking water and did not avoid food when the diet was changed.

Purification of Fatty Acid Synthetase—Rats were killed by decapitation. Liver supernatant solutions and fatty acid synthetase preparations were prepared according to the procedure of Burton et al. (15) with the exception that the Sephadex G-100 gel filtration step was omitted. A further purification step was necessary in order to rid the preparations of contaminating 7 S species (16). This purification was achieved either by sucrose density gradient centrifugation (with a SW41 or SW56 rotor) in 5% to 20% sucrose prepared in 0.5 M potassium phosphate, pH 7.0, and 2 mM dithiothreitol, or by gel filtration on a Sepharose 6B column 50 cm long equilibrated with the above buffer. In each case the enzyme from the DEAE purification (15) was activated for 30 min at 35° prior to application (17). Tubes corresponding to the leading edge of the peak and containing enzyme activity were pooled.

Use of the Sepharose 6B column revealed a macromolecular species traveling with the void volume (exclusion limit 4 x 104) which exhibited variable activity (90 to 250 nmoles per min per mg). This fraction, when precipitated with ammonium sulfate and rechromatographed, reform was a small amount of 12 S material, which appeared in the eluate. These results suggest the existence of an aggregated species of fatty acid synthetase.

The conclusion of Collins et al. (16) that the contaminating 7 S protein species is not related to fatty acid synthetase as a breakdown product is here confirmed. This species does not contain pantothenate, which tends to eliminate a large portion of the fatty acid synthetase molecule. More convincingly, however, a pulse of labeled amino acids given to rats in various stages of fasting yields, after sucrose gradient sedimentation, a 7 S species which is never lower in specific radioactivity than the enzyme and which reaches a specific radioactivity three times that of the enzyme. In addition, the pattern of 7 S formation as a function of time bears no resemblance to that of enzyme. These observations are not consistent with a precursor product relationship between fatty acid synthetase and 7 S protein.

All enzyme assays were performed by observation of the malonyl-CoA dependent oxidation of TPNH (18). Maximal specific activities of about 1300 nmoles per min per mg were occasionally observed, but the usual range observed was 1000 to 1200 nmoles per min per mg.

Protein determinations were made by measuring the spectrophotometric 260:280 ratio (19). Characteristic ratios of 0.55 to 0.60 for fatty acid synthetase and 0.9 for the "aggregate" species mentioned above were noted.

CoA Determination—When the specific radioactivity of CoA was determined, the supernatant fraction from the second ammonium sulfate precipitation (15) was boiled to remove protein and made 50% in methanol to precipitate salt, followed by evaporation under a stream of nitrogen. The concentrated CoA was then isolated by use of a DEAE column (20) and assayed by the phosphotransacetylase reaction (21).

Amino Acid Specific Activity—One milliliter of liver supernatant solution (15) was precipitated with 10% trichloroacetic acid and the resulting pellet washed with 10% trichloroacetic acid. The pooled supernates were diluted with 40 ml of water and applied to a 5-ml column of Dowex 50-XS which had previously been washed with 1 M NaOH, 1 M HCl, and finally water. After loading, the column was eluted with 0.02 M HCl followed by 15 ml of 4 M HCl. The ammonia fraction was evaporated to dryness over concentrated H2SO4 in a vacuum, washed with 1 M KOH, and re-evaporated. The resulting salt was taken up in 1 ml of 0.2 M citrate, pH 5, titrated with citric acid to pH 5, and used in the quantitative ninhydrin procedure of Cocking and Yemm (22). An aliquot of the sample was also counted and from these data the specific activity of the amino acid pool was determined. The above procedure yielded the same values for specific activity as the unchromatographed trichloroacetic acid-treated supernatant solution for periods after fasting up to 24 hours. After this time, however, the amount of labeled non-amino acid material became significant. The above procedure failed to retain glutamic and aspartic acid on the Dowex resin.

When the total liver protein was determined, a 1-ml aliquot of the liver supernatant solution was precipitated by making it 10% in perchloric acid, the pellet washed and counted. The protein content was determined in this case by the biuret method (23).

Counting—All radioactivity counting was performed in a Nuclear Chicago 720 series liquid scintillation spectrometer employing either Aquasol or a toluene-ethanol-N-methyl-2-pyrrolidinone mixture as a scintillation fluid. All protein was counted after complete solution in Protosol. All counting was performed to better than ±2% (confidence level of two standard deviations), and all samples were quench corrected by internal standardization.

Results and Discussion

The control of enzyme levels in higher animals has been reviewed by Schimke and Doyle (24). The question of how fasting effects a change in fatty acid synthetase levels can be conveniently considered in two parts: (a) Is the half-life of the enzyme altered? (b) Is the rate of synthesis altered? Since fatty acid synthetase levels drop by 80% to 90% after 2 days of fasting (13), this is the time period of interest.

During this period of fasting animals are not in the steady state and therefore the rate of synthesis of the enzyme must be determined separately from the rate of degradation. The simplest approach would be to measure loss of enzyme after synthesis has been stopped. This can be approximated if the determination is made after 12 hours. Fig. 1 shows a curve fitted by the method of least squares to a series of determinations of enzyme activity per liver as a function of time after removal of all food. The data represent determinations from four separate experiments and are expressed as percentage of zero time activity in the high speed supernatant solution. The scatter is partly due to the difficulty of obtaining uniform supernatant preparations. The slope of this line corresponds to a half-life of 20 hours. The half-life in the normal steady state is about 70 hours (11), so it appears that fasting affects the degradation of the enzyme. This result is not unexpected since a 70-hour half-life could not account for almost total loss of activity in 48 hours. The approximate fit of the data to a single curve suggests a rapid change to a new half-life as opposed to a slower incremental change. However, it is not clear whether this loss of enzyme activity represents total degradation of the enzyme complex or rather a change to some inactive form. For example, the prosthetic group could be removed upon fasting and later replaced on refeeding, a possibility to be discussed later. During the 2-day fast period the animal weight decreased only slightly. The liver, however, after a brief lag period, rapidly lost weight to...
Fig. 1. Total enzyme units per liver were determined in a series of high speed supernatant fractions (two animals for each point) obtained from animals fasted for varying lengths of time. The ordinate is the natural logarithm of the percentage of the steady state level of a particular experiment. Rats were fed the vitamin B complex test diet complete and fasting was begun at 9:00 a.m.

Fig. 2. Each point represents data derived from the liver of a single animal, converted to percentage of the maximal value observed for data of a particular type. Each rat was injected via the intraperitoneal route with 12.5 μCi of U-14C-amino acid mixture (Schwarz/Mann) in 0.25 ml of neutralized 0.01 M HCl 1 hour prior to each time point. For information about the means of obtaining the various data see "Experimental Procedure." Rats were fed the vitamin B complex test diet complete. X-X, liver wet weight, maximum 12 g; O--O, enzyme level, maximum 8900 units per liver; Δ-Δ, specific radioactivity of total soluble protein, maximum 1600 dpm per mg; •-•, total soluble protein per liver, maximum 900 mg.

Fig. 3. Each point represents data derived from pooled livers of two animals. One hour prior to each time point each animal received 17 μCi of U-14C-amino acid mixture (New England Nuclear) in 0.3 ml of neutralized 0.01 M HCl. Rats were fed the vitamin B complex test diet complete prior to fasting (9:00 a.m.). ●-●, data corrected as described in the text; ○-○, the uncorrected data.

Some of this weight loss can be attributed either to degradation or to transport of soluble protein. The weight losses, as well as the probable production of amino acids from degraded protein, make it difficult to be certain that the concentration of amino acids in the liver amino acid pool is constant throughout the experiment. Since the specific activity of injected label is calculated to be equal to approximately 1% of the pool of fatty acid synthetase molecules being synthesized in any hour and since the rate of synthesis seems to drop much more rapidly than the pool size upon fasting, it seems reasonable to neglect this objection.

The corrected curve in Fig. 3 was obtained from the points of the uncorrected curve by multiplying the value of each point by a factor:

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\frac{\text{protein specific activity (cpm/mg)}(t = 0)}{\text{protein specific activity (cpm/mg)}(t = x)} \times \frac{\text{enzyme units/liver} (t = x)}{\text{enzyme units/liver} (t = 0)}
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From the corrected curve it can be seen that within 6 hours after food was removed the rate of synthesis dropped and continued to fall until the end of the experiment. The ability of the liver to respond to the absence of food after such a short period is striking. This may be due in part to the timing of the starvation which was begun in all cases between 8:00 and 9:00 a.m., an hour after daylight in the programmed light cycle. In a preliminary effort to determine what diet component is the cause of this change in
rate, the experiment represented in Fig. 4 was performed. This experiment was different from those previously described in that the equilibrating diet was not the vitamin B complex test diet complete, but rather the normal protein test diet. The change was necessary to achieve a better matching of the control and experimental diets. The values for the rate of synthesis in Fig. 4 were corrected as indicated above. The sudden drop in the rate of synthesis indicated by all of the curves in that figure possibly reflects a difficulty in “changing over” metabolically to the requirements of a new diet. While the rate of synthesis resulting from change to a fat free diet began to rise after 12 hours, that resulting from the protein-free diet continued to decrease. This could be an indication that the protein component in the diet was responsible for the effect of fasting on enzyme level observed after 12 hours. Further similarity between those conditions is indicated by a second experiment described in Table I. There it can be seen that over a longer period of time the effect of the protein-free diet on enzyme levels mimics that observed for fasting. In addition, feeding a fasted animal with protein-free food did not change the level of enzyme, but feeding both fasted and protein-deprived animals with fat-free food had a similar enhancing effect. Since fatty acid synthetase represents a sizeable portion of the total soluble protein (0.8/o, as determined from a comparison of the specific activity of crude supernatant solution with purified enzyme) of the steady state liver, it is expected that the enzyme may play an important part in the adjustment to a negative nitrogen balance. The results of experiments which utilized a fat-free diet are also expected in view of the effect of that diet on fasted animal and the similar effect recorded for mouse liver (25). The results of Table I indicate that fasting prior to fat-free feeding produced a change in the level of enzyme additional to that expected from the simple diet change. Thus there are additional phenomena to be considered in analyzing the effect of refeeding a fat-free diet. Part of the effect could be due to reactivation of an inactive enzyme species (mentioned below) as well as the de novo synthesis already verified (26). The levels of enzyme activity found in the supernatant fraction from animals fed the vitamin B complex test diet complete as well as that from animals fed these diets may be comparable.

We now turn to a discussion of the effects of fasting on the pantothenate incorporation into fatty acid synthetase. In a previous publication (11) it was concluded that pantothenate compounds in the cytosol are rapidly exchanged with 4'-phosphopantetheine bound to the enzyme. From Fig. 5 it can be seen that for animals in the steady state, a rapid rise in specific radioactivity of enzyme was found as previously observed (11), and therefore it can be concluded that, under these dietary conditions also, the exchange phenomenon is occurring. In the case of

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**Fig. 4.** For each point, two animals received 21 μCi of U-14C-amino acid mixture (Schwarz/Mann) in 0.25 ml of neutralized 0.01 M HCl 1 hour prior to sacrifice for isolation of fatty acid synthetase. Animals were fed normal protein test diet for 7 days prior to the indicated diet change at time zero. ●—●, fasted; ▲—▲, protein-free diet; ■—■, fat free diet.
animals fasted for 12 hours prior to administration of isotope, the exchange of prosthetic group was not as readily discerned. Even after 48 hours, the specific radioactivity of the protein failed to reach the levels found in the steady state. This is not due to a failure of the label to enter the pantothenate pool since in all cases the measured specific radioactivity of CoA from the fasted series was higher than that observed for the 20-hour steady state point. If CoA is a precursor of pantothenate in fatty acid synthetase it can be concluded that fasting interferes with the exchange process somewhere between CoA and the protein, possibly directly inhibiting the enzyme responsible for attaching or removing the prosthetic group. This conclusion is supported by the results shown in Fig. 5. By making the assumption that the turnover of enzyme reported earlier (11) is similar to that observed here in the steady state, and by making use of the pool size of enzyme units and CoA specific activity per liver as well as the changes in rate of synthesis and degradation described above, a rough calculation of the amount of pantothenate expected to be incorporated solely as a result of de novo synthesis of fatty acid synthetase can be made. This calculated result, when compared with the actual measured incorporation of labeled pantothenate, allows an estimate of incorporation due to exchange. The disparity between the steady state experimental and calculated curves indicates the high degree of exchange mentioned above. However, after 12 hours fasting the experimental curve lies relatively close to the calculated curve indicating that most of the incorporation of label is due to de novo synthesis. This result is consistent with the conclusion that in fasted animals a precursor of the protein bound 4'-phosphopantetheine is labeled, but that this precursor is not being exchanged with the facility achieved in the steady state.

The pantetheine exchange phenomenon might involve the over-all cellular control of CoA metabolism, but at present this relation is obscure. The exchange might have an effect on fatty acid metabolism by controlling the presence of prosthetic group on the enzyme complex. Thus, if the enzyme which synthesizes pantetheine is inhibited during fasting then one could account for the observed loss of enzyme activity in terms of removal of prosthetic group by a hydrolase. Such loss would not be replaced from the precursor pool until food is restored. A study of the loss of enzyme during fasting as measured by an anti-fatty acid synthetase antobody should help clarify this point.

Studies by Majerus (27, 28) on rat acetyl-CoA carboxylase bear a resemblance to those reported here. Acetyl-CoA carboxylase is the enzyme responsible for the synthesis of malonyl-CoA from acetyl-CoA and $\text{CO}_2$. Fasted rats show an increased rate of degradation and a decrease in the rate of synthesis of of this enzyme. Changes in the level of enzyme were shown to be changes in the level of enzyme content in the liver rather than activation or inhibition of previously formed protein. Animals fed diets deficient in biotin led to an accumulation of apo-acetyl-CoA carboxylase in the rat epididymal fat pad. Subsequent injection of labeled biotin led to a rapid appearance of labeled enzyme. The analogous experiment cannot be accomplished utilizing pantothenate-deficient animals. Such animals show a normal level of fatty acid synthetase in the epididymal fat pad up until the time of death due to vitamin deficiency (20).

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