RELATIVE TURNOVER RATES OF PROTEINS AND PEPTIDES OF RAT LIVER FATTY ACID SYNTHETASE

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Summary--The double label method of Arias et al. (J. Biol. Chem., 244, 3303, 1969) has been adapted to measure the relative turnover rates of proteins and peptides derived from rat liver fatty acid synthetase from starved and refed animals. Differential rates of turnover were not detected in either proteins from polyacrylamide gel electrophoresis, nor in peptides obtained by tryptic digestion.

Rat liver fatty acid synthetase (FAS) is a multienzyme complex which contains all the activities necessary for the conversion of acetyl- and malonyl-CoA to palmitic acid in the presence of NADPH (1). Other multienzyme complexes are known in which several enzymes catalyzing successive metabolic steps appear to be held together by strong noncovalent forces; and in some cases the activities can be separated and the multienzyme complex can be reconstituted in vitro (1). Attempts to separate the activities in FAS into catalytically active fragments have been unsuccessful. The total units of FAS activity found in avian and mammalian liver are a function of the nutritional state of the animal (2,3). Upon starving, FAS activity (total units) drops to a subnormal level; refeeding starved animals a fat-free diet causes the FAS activity to increase to a supernormal level. Burton et al. (3) have shown that this rise in enzyme activity in rats is due to adaptive enzyme synthesis, as evidenced by radioactive amino acid incorporation into the FAS complex during the refeed period. Although adaptive enzyme synthesis has been shown to occur, the mechanism of assembly of the FAS complex, or of any multienzyme complex, has not been well studied. In the simplest model, all the peptides of the complex are made at equivalent rates, and any protein molecule which results

from breakdown of the complex is not reutilized. Studies have been made of the protein turnover of rat liver FAS complex (4), and work is in progress in this laboratory to determine the rates of turnover of the individual proteins of the complex in steady state animals. However, the rate of FAS protein synthesis during the refeed period greatly exceeds the steady state rate (4) and the possibility exists that the FAS proteins are made at differential rates during this period. Moreover, it is possible that differential reutilization of certain of the FAS proteins from pre-starved animals occurs.

In order to examine these possibilities, the double label approach of Arias et al. (5) has been adapted to examine the FAS complex. Normal animals were injected with ^{14}C-L-leucine and after subsequent starvation and refeeding, ^{3}H L-leucine was injected. Purified enzyme obtained from such animals was examined by polyacrylamide gel electrophoresis after dissociation with phenol, acetic acid, and urea. Peptides from a tryptic digest of purified FAS were also investigated. If the simplest model discussed above is the correct one, each isolated protein, protein aggregate, or each peptide should have the same ratio of $^{3}H/^{14}C$ as every other protein or peptide. Significant differences in the ratios would indicate a more complex situation than the one discussed above.

Materials and Methods

Animals

Male Sprague Dawley rats (150 g ± 15 g) were obtained from Hilltop Lab Animals, Chatsworth, California. They were fed "Vitamin B Complex Test Diet" (Nutritional Biochemical Corp.) ad libidum for at least five days before the time of starving. Refeeding, after starvation for approximately two days, was done with "Fat-Free Diet," provided by the same source. Water was provided at all times.

Preparation of Fatty Acid Synthetase

Rats were killed at appropriate times by decapitation and exsanguination. Purified fatty acid synthetase was prepared as described by Burton, Haavik, and Porter (6), with the exception that the Sephadex G-100 gel filtration step was omitted.

Polyacrylamide Gel Electrophoresis

FAS was precipitated with acetone (90%), washed and taken up in phenol, glacial acetic acid and water, 2:1:1, and the solution brought to 2M in urea (7). An aliquot of this solution (100 to 200 λ of 5 to 15 mg per ml) was then layered under 75% acetic acid atop a 9mm x 85mm gel containing 7.5% acrylamide, prepared as described by Takayama et al. (7). Gels were run at 5 mA per tube for 4½ hr. Both upper and lower reservoirs contained 10% acetic acid. Staining was accomplished with Amido Schwartz dye and destaining by soaking in 7% acetic acid, or electrophoretically at 10 mA/tube. Bands were isolated by slicing freehand with a razor blade where separation permitted, or alternately, by piercing the gel with fine copper wires to mark the positions of the bands, freezing the gels in powdered dry ice, and then slicing the rigid, opaque gels using the protruding wires as markers. Gel slices were prepared for scintillation spectrometry essentially as described by Tishler and Epstein (8). Counting utilizing Omnifluor (New England Nuclear) was continued on a Packard Tri-Carb model 3375 spectrometer until the standard deviation of the counts was less than 3.5%. DPM for each radioisotope were calculated by a computer program from CPM of samples in each of two channels, and with the use of a curve of efficiency versus the automatic external standardization (AES) value. The ratio of $^{3}H/^{14}C$ was also calculated by this program, as was the standard deviation of the ratio, by taking into account statistical error in counts, pipetting error in preparing standards, and error incurred in interpolating with the efficiency-AES curves.

Trypsin Digestion of FAS

To a solution of purified FAS in 0.05M phosphate buffer, pH 7.0, and 1 mM in EDTA and 2-mercaptoethanol, a quantity of 1M HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid) (Calbiochem.), pH 8.4 at 0.10M, was added, until its concentration was 0.10M. Twice crystallized trypsin (Nutritional Biochemicals Corp.) was added directly in a quantity equal to 15% by weight of the FAS. The solution was then incubated at 38° for 18 hrs, at the end of

which time, sufficient 1M HCl was added to bring the pH to 2. The solution was then spun briefly at 12,000 x g and the supernatant applied to a cation exchange column.

Cation Exchange Column Chromatography

Peptides resulting from tryptic digests of FAS were chromatographed on an Aminex A-5 (Bio-Rad) cation exchange column 9 mm x 20 cm, at 50° and 75-100 psi, by eluting with 50 ml of pyridine-acetate buffer, pH 3.3, 0.1M (80 ml glacial acetic acid and 8.1 ml pyridine per liter), and then with a linear gradient to pyridine-acetate, pH 4.8, 2.0M (144 ml glacial acetic acid and 161 ml pyridine per liter). Eight to 10 ml fractions were collected. Each fraction was transferred to a scintillation vial, and taken to dryness under a stream of hot air. The dry samples were wetted successively with 75 λ of water, 0.5 ml NCS solubilizer (Nuclear Chicago Corp.), and scintillation fluid.

Results and Discussion

The double label method of Arias et al. (5) has been modified for use in these experiments. Two differently labeled forms of the same amino acid were injected at separate times. At the first time point, 6 hours before removal of food, ^{14}C-leucine was injected, thus labeling the FAS complex in steady state animals. The rats were then starved, allowing the complex to fall to an amount less than normal. Upon refeeding the rats a fat-free diet, the level of FAS was induced to rise to a supernormal quantity. Two hours after refeeding, the animals were again injected, this time with ^{3}H-leucine, which was then incorporated during the period of the adaptive enzyme synthesis. Since the level of the enzyme never reached zero during the period of starvation, the FAS purified at the end of the experiment would be expected to contain both isotopes, although not necessarily in the same individual molecules.

Thus any differences in the rates of turnover of component enzymes of the complex would be reflected in differences in the $^{3}H/^{14}C$ ratio found in individual component polypeptides. For example, if one component of the complex were preferentially spared degradation during starvation, then the

$^{3}H/^{14}C$ ratio would be expected to be lower than that of the other components.

Controls consisted of rats subjected to these same conditions except that both radioactive isotopes of L-leucine were injected at the same time, 2 hours after refeeding. Variations in the $^{3}H/^{14}C$ ratio in proteins from these animals were attributed to experimental error. Rats were killed and FAS isolated 46 hours after refeeding in order to avoid co-purification of a contaminating protein which appears in FAS preparations from starved rats. This contaminant is no longer detected 36 hours after refeeding (9).

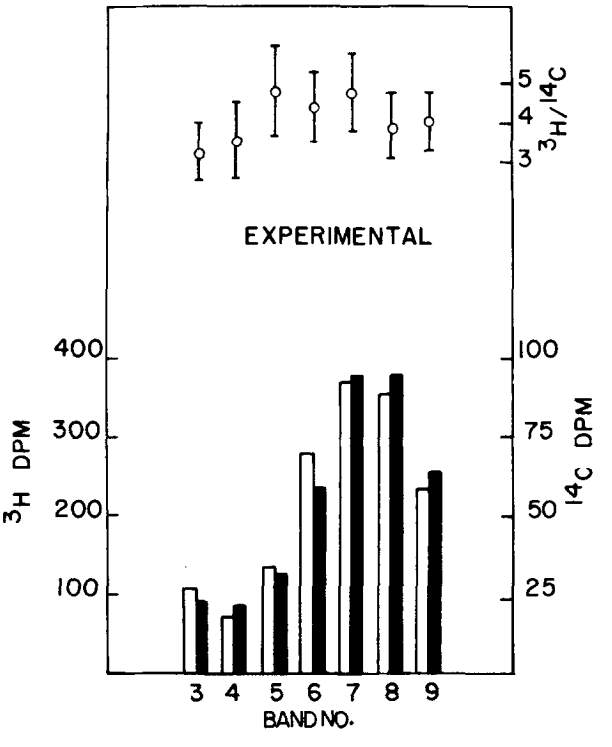

Figure 1

Polyacrylamide gel electrophoresis of purified FAS isolated from livers of two rats that had been starved 42 hours, refed a fat-free diet for 49 hours, and killed. 25 uci of ^{14}C uniformly labeled L-leucine (256 mci/mmole) was injected intraperitoneally six hours before removal of food. A second injection of 100 uci of 4, 5 ^{3}H-L-leucine (1 ci/mmole, Amersham Searle) was given two hours after refeeding on a fat-free diet. Bands 1 through 3 and 10 through 13 contained insufficient counts for analysis. Bands from five gels were isolated, pooled and counted as described in materials and methods. Black bars represent ^{14}C DPM; light bars, ^{3}H DPM. The ratio of $^{3}H/^{14}C$ DPM is shown in the upper portion of the figure, with 1 standard deviation limit indicated for each ratio.

Figure 1 shows the result of polyacrylamide gel electrophoretic separation of subunits of the FAS complex which was labeled with both isotopes, at separate times, as described above. These subunits were obtained by treatment of the purified complex with phenol, acetic acid, and urea. Within the limits of the experiment, all the bands in the gel show the same $^{3}H/^{14}C$ ratio. The variation in the value of this ratio is essentially comparable to that obtained in a control experiment in which both labels were injected at the same time (Figure 2). The patterns of bands in these gels consistently show 10 to 13 components. At present it is not known if any band is an enzyme aggregate, an intact separated enzyme, or subunits of the various enzymes of the complex. Since it is possible that the heavier bands in the gel are aggregates of two or more enzymes

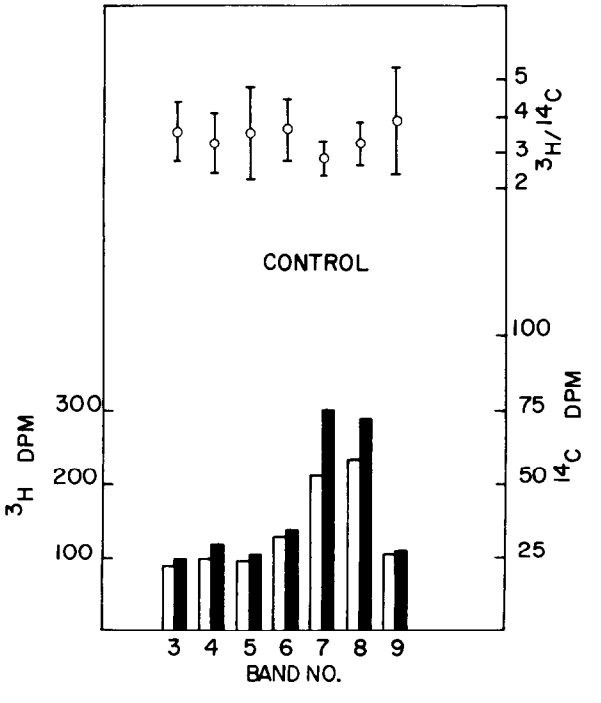

Figure 2

Control polyacrylamide gel electrophoresis of FAS from two rats treated as described in the legend to Figure 1, with the exception that both radioactive isotopes of L-leucine were injected at the same time, two hours after refeeding a fat-free diet. Bands from three gels were combined.

of the complex,* which do not separate or release subunits under these conditions, it is possible that differential turnover of a protein which constitutes a relatively small percentage of one of the more densely staining bands would not be detected in this experiment.

Consequently, another aliquot of each of the purified FAS samples described above was subjected to trypsin digestion and subsequently subjected to chromatography on Aminex A-5 as described by Jones (10). Figure 3 shows that

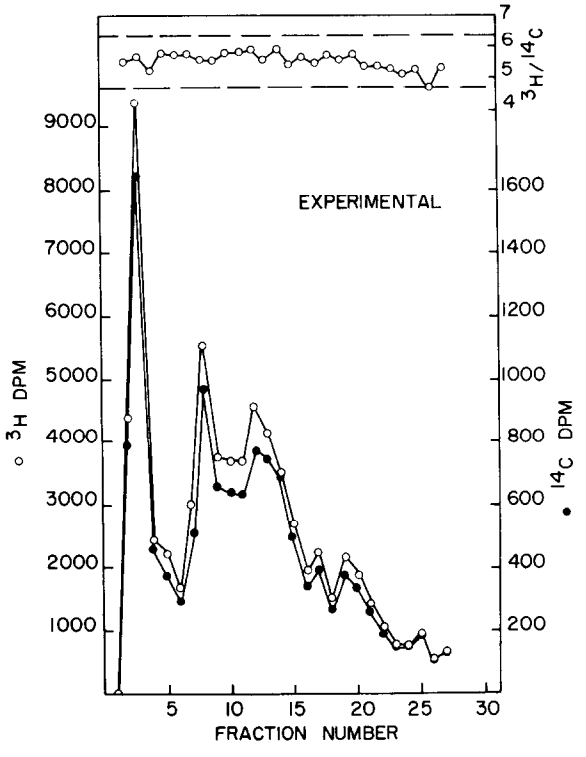

Figure 3

Aminex A-5 column chromatograph of a tryptic digest of FAS obtained from livers of the two rats described in Figure 1. Standard deviation shown for the $^3H/^{14}C$ ratio shown in the upper portion of the figure (±.8), is taken from that obtained for the control experiment (Figure 4). The amount of protein loaded onto the column was 9.1 mg.

*Polyacrylamide gel electrophoresis of FAS labeled in the 4'-phosphopantetheine prosthetic group has yielded bands with label distributed among several bands, indicating that the complex is most likely incompletely dissociated (unpublished observation).

the $^{3}H/^{14}C$ ratio of the various fractions of the partially purified trypsin digest did not vary significantly more than fractions derived from a similar experiment in which both isotopes were injected at the same time (Figure 4).

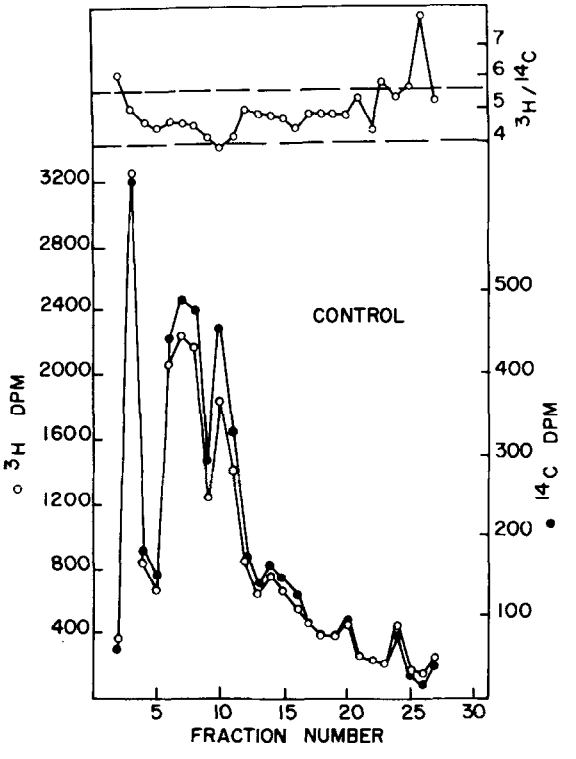

Figure 4

Control Aminex A-5 column chromatograph of FAS purified from livers of the rats described in Figure 2. Standard deviation of the values for ratios of $^{3}H/^{14}C$ is shown by the horizontal lines bounding the mean ratio. The standard deviation was calculated from the values reported in this figure and not as described in materials and methods for the polyacrylamide gel experiments. The amount of protein loaded was 4.2 mg.

Although we recognize that small differences in the rate of turnover of the enzymes of FAS during the period of adaptive enzyme synthesis described here would not be detected, and that the possibility still remains that real differences may not have been detected by the present experiments, we feel that the results reported here strongly support the conclusion that the various enzymes of the complex are synthesized at similar rates with no significant re-

utilization of parts derived from breakdown of the FAS complex during the period of starvation preceeding refeeding a fat-free diet. Moreover, the coincidence of the turnover rates of the component proteins of this multienzyme complex would support its proposed occurrence *in vivo*. Rat liver proteins show widely varying protein turnover rates (11) and if multienzyme complexes are artifacts of isolation (see 11, 12 for a discussion of this point) then differential turnover rates of the individual proteins might be expected to occur.

Acknowledgements

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