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Regulation of synthesis of hepatic fatty acid synthetase: Polysomal translation in a cell-free system

(enzyme synthesis/enzyme control)

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ABSTRACT **Polysomes were isolated from livers of rats fed various diets and were translated in a protein-synthesizing system derived from cultured Chang liver cells. One of the labeled products was identified as complete subunit(s) of fatty acid synthetase by indirect immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the solubilized immunoprecipitate. The relative amounts of fatty acid synthetase synthesized by polysomes from livers of rats fed a normal diet, starved rats, and rats starved and refed a fat-free diet for 16 hr were 1, 0.1, and 10, respectively. Induction of synthesis of fatty acid synthetase after fat-free refeeding of starved rats began by 2 hr (3-fold increase over starved animals), was increasing rapidly by 5 hr (19-fold over starved animals), and reached a high level by 16 hr (95-fold over starved and 10-fold over normally fed).**

De novo synthesis of fatty acids in mammals is catalyzed by fatty acid synthetase (FAS). The *in vivo* level of activity of this enzyme varies markedly in response to dietary manipulation (1–3) and during development (2). Elevation of FAS activity in response to insulin has been observed in cultured animal cells (4). In rat liver, FAS activity increases after refeeding of a starved animal with a fat-free, high carbohydrate diet. Immunological methods (2, 5) and direct purification of FAS (3) have shown that these increases in activity are due to an enhanced rate of synthesis of the enzyme ("adaptive synthesis").

Insight into the biochemical processes involved in the regulation of the rate of synthesis of FAS will be gained by studying translational control more directly. Therefore, we used binding of ^{125}I-labeled antibody against FAS to isolated rat liver polysomes to demonstrate the changes in the amount of synthesis of FAS nascent chains during fat-free refeeding of starved rats (6).

In this investigation we have used an *in vitro* protein-synthesizing system and immunological isolation of labeled FAS product to study, in another way, changes in the rate of synthesis of the enzyme in the liver. Our results confirm that a marked increase in synthesis of FAS peptides occurs during "adaptive synthesis." The first demonstrable increase occurs 2–4 hr after refeeding, at the time that FAS specific activity begins to rise. The isolated labeled FAS product is similar in molecular weight to the subunit peptides of native FAS.

MATERIALS AND METHODS

Isolation of Polysomes. Rat liver polysomes were isolated as described (6, 7). After removal from the 2.5 to 1.0 M interface of discontinuous sucrose gradients, the polysomes were diluted with buffer (50 mM Tris·HCl, pH 7.4, 25 mM NaCl, 5 mM $MgCl_2$, sodium heparin, 200 μg/ml) and centrifuged at 65,000 rpm for 1.5 hr. The polysomal pellets were resuspended by gentle homogenization in buffer at a concentration of 50–200 A_{260} units/ml. Small aliquots were stored in liquid N_2 for future use in translation.

Preparation of S-30. Monolayer cultures of Chang liver cells were grown as described (4) with 10% calf serum. Cells were harvested as described (4), washed, suspended in 2 volumes of hypotonic buffer (10 mM Tris·HCl, pH 7.4, 1.5 mM MgOAc, 10 mM NaCl), and incubated at 4° for 10 min. After homogenization the lysate was adjusted to 120 mM KCl, 1 mM dithiothreitol, 20 mM Tris·HCl, pH 7.5, and 5 mM MgOAc and centrifuged at 30,000 × *g* for 15 min. The supernatant was preincubated as described by Aviv *et al.* (8) and passed over a Sephadex G-50 column equilibrated with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (pH 7.6), 1 mM dithiothreitol, 120 mM KCl, and 5 mM MgOAc. Fractions containing peak A_{260} units were pooled (usually 10–15 A_{260} units/ml), and small aliquots were frozen in liquid nitrogen for later use in translation.

***In Vitro* Protein-Synthesizing System.** Assays were carried out according to the methods of Aviv *et al.* (8) with minor modifications. Each 50-μl reaction mixture contained 20 mM Hepes (pH 7.6), 2 mM dithiothreitol, 80 mM KCl, 2.5–4.0 mM MgOAc, 8.0 μg of creatine phosphokinase, 40 μM each of 19 nonradioactive amino acids, 0.6 mM CTP, 10 mM creatine phosphate, 100 μM GTP, 1 mM ATP, 9 μCi of radioactive amino acid ([^{35}S]methionine, 250 Ci/mmol, Amersham-Searle, or [^{3}H]leucine, 50 Ci/mmol, New England Nuclear), 0.08–0.15 A_{260} units of S-30 (3.2 mg/ml of protein), and 0.4–1.5 A_{260} units of polysomes. The reaction mixtures were incubated at 25° for 60 min, followed by immersion in an ice bath to stop the reaction. Five microliter aliquots were removed and placed in 50 μl of unlabeled amino acid (2 mM), to which 0.5 ml of trichloroacetic acid was added. The mixture was heated 10 min at 90° and the precipitate was collected over 0.45 μm Millipore filters and washed five times with 5% trichloroacetic acid. Radioactivity of the filters was determined in 10 ml of 3a70 liquid scintillation fluid (Research Products International) using a Packard 3380 scintillation counter.

Antibody Preparation. Rat liver FAS was purified by the methods of Alberts *et al.* (6) and subjected to sodium dodecyl sulfate ($NaDodSO_4$)-polyacrylamide gel electrophoresis (9). The single band migrating with a molecular weight (M_r) of 240,000 was cut out of the gel, homogenized in phosphate-buffered saline (20 mM Na_2HPO_4, pH 7.0, 150 mM NaCl), and injected into the foot pads of rabbits (250 μg each week for 3 weeks and 250-μg boosters every 6 weeks). The rabbits were bled 2–3 weeks after antigen boosters.

Abbreviations: FAS, fatty acid synthetase; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; S-30, supernatant after centrifugation at 30,000 × *g* of Chang liver cell lysate; M_r, molecular weight.

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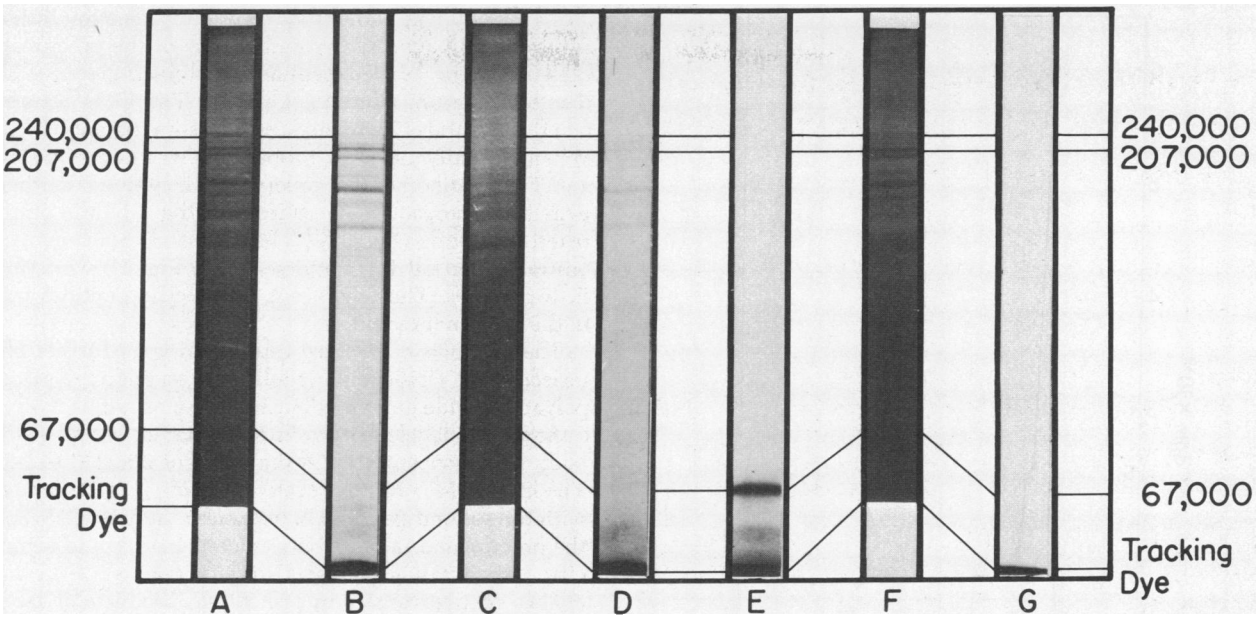

FIG. 1. Immunologic isolation of proteins synthesized *in vitro*. A translation reaction in 350 μl of sample was performed with liver polysomes from rats starved 48 hr and refed a fat-free diet for 16 hr. Incorporation of [^{35}S]methionine into protein was 6.5 × 10^6 cpm/350 μl. Aliquots (50 μl) were treated as described below and then electrophoresed on a 6% $NaDodSO_4$-polyacrylamide gel with M_r markers (bovine serum albumin, 67,000; myosin, 207,000; rat liver FAS, 240,000) run in parallel slots. The gel was stained, dried, and autoradiographed for 6 days (see *Materials and Methods*). (A) 50 μl of the original translation reaction sample. (B) 50 μl of translation reaction sample was subjected to indirect immunoprecipitation with purified anti-rat liver FAS antibody (see *Materials and Methods*). The immunoprecipitate was then electrophoresed. (C) The supernatant of immunoprecipitation in (B). (D) Unlabeled rat liver FAS (8 μg) was added to a 50-μl aliquot which was immunoprecipitated as in (B). The precipitate was then electrophoresed. (E) A 50-μl aliquot was subjected to indirect immunoprecipitation with partially purified anti-rat serum albumin antibody and the precipitate was electrophoresed. (F) Supernatant of the immunoprecipitation in (E). (G) Partially purified rabbit IgG and goat anti-rabbit IgG were added to a 50-μl aliquot in amounts sufficient to give an equal-*sized* precipitate to that in (B). The precipitate was electrophoresed. (A), (C), and (F) were reproduced from the original autoradiograph at one-third the density of (B), (D), (E), and (G) to assure printability.

Anti-FAS antibody was purified by ammonium sulfate precipitation, DEAE-Sephadex chromatography, and affinity chromatography as described (6).

Indirect Immunoprecipitation. Translation reaction mixtures were adjusted to 0.5% sodium deoxycholate, 0.5% Triton X-100, and 0.02% sodium azide and centrifuged at 10,000 rpm for 10 min. Monospecific rabbit antibody (anti-rat liver FAS or anti-rat serum albumin) or partially purified rabbit IgG was added to the supernatant (final concentration of 0.2–0.5 mg/ml), and the mixture was allowed to stand at 25° for 30 min and overnight at 4°. After centrifugation at 7000 rpm at 0° for 10 min, purified goat anti-rabbit IgG (Gateway Scientific) was added to the supernatant in the amount necessary to quantitatively precipitate all rabbit IgG. After incubation for 1 hr at 25°, the suspension of the precipitate was layered over 400 μl of 1 M sucrose in phosphate-buffered saline, and the samples were centrifuged at 1500 rpm for 20 min. The supernatant above the sucrose was removed, the interface washed twice with 200 μl of phosphate-buffered saline, and all liquid above the pellet decanted. The pellet was washed with phosphate-buffered saline and then prepared for gel electrophoresis.

$NaDodSO_4$-Polyacrylamide Gel Electrophoresis. Preparation of samples for electrophoresis (both whole translation product analysis and indirect immunoprecipitates) was performed as described by Schutz *et al.* (10). Translation reaction mixtures were adjusted to 1% $NaDodSO_4$ and 0.1 M dithiothreitol, and immunoprecipitates were dissolved in Tris-glycine buffer, pH 8.5 (2 mM Tris, 20 mM glycine), and adjusted to 1% $NaDodSO_4$ and 0.1 M dithiothreitol. Samples were heated 10 min at 90°, and 0.05% bromophenol blue in 50% glycerol was added as tracking dye. Six percent polyacrylamide gels with a stacking gel of three percent acrylamide containing 0.1% $NaDodSO_4$ were used (9). After electrophoresis the gels were stained, destained, and dried. For determination of ^{3}H, the dried gels were cut into 2-mm slices and dissolved in 0.5 ml of H_2O_2 by incubation for 4 hr at 80°; radioactivity was determined in 10 ml of 3a70. For ^{35}S autoradiography, the dried gels were exposed to Kodak RP Royal X-Omat film for 1.5–7 days.

RESULTS

The *in vitro* protein-synthesizing system utilizing the 30,000 × *g* supernatant from cultured Chang liver cells gave efficient translation of rat liver polyribosomes. Various S-30s and various polysome preparations stimulated incorporation into protein of [^{35}S]methionine 20- to 50-fold over S-30 alone. [^{3}H]Leucine incorporation was 20–120 times background (data not shown). The most efficient translations were obtained with 0.15 A_{260} unit/50 μl (0.064 mg of protein) of S-30 and 0.4 A_{260} unit/50 μl of rat liver polysomes (data not shown).

FAS is a small percentage of the protein synthesized by rat liver (1, 2). Therefore, we isolated labeled FAS from the *in vitro* translation reaction by indirect immunoprecipitation using purified rabbit anti-rat liver FAS and purified goat anti-rabbit IgG. A large excess of anti-FAS antibody was used in these precipitations (at least 5-fold greater than might crossreact with Chang liver FAS present in the S-30). Specificity and purity of the anti-FAS antibody have been described (6).

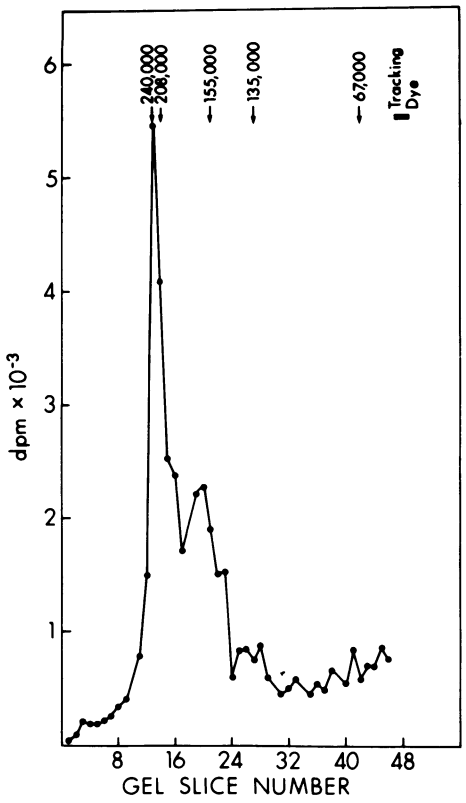

FIG. 2. $NaDodSO_4$-polyacrylamide gel of immunoprecipitate of *in vitro* translation after [^{3}H]leucine incorporation. Isolated polysomes from rats that had been starved 48 hr and refed a fat-free diet for 16 hr were translated (150-μl reaction mixture with total protein dpm = 1.8×10^6). After indirect immunoprecipitation with 30 μg of purified anti-liver FAS antibody and 250 μg of purified goat anti-rabbit IgG, the solubilized precipitate was electrophoresed as in Fig. 1. The dried gel was sliced and radioactivity was determined (see *Materials and Methods*). M_r markers run in a parallel slab of the same gel are rat liver FAS (240,000), myosin (208,000), *E. coli* RNA polymerase (165,000 and 155,000), β-galactosidase (135,000), and bovine serum albumin (67,000).

^{35}S-Labeled peptides were identified by autoradiography of $NaDodSO_4$-polyacrylamide gels after electrophoresis of either total translation products or antibody precipitates of the translation reaction (Fig. 1). The autoradiograph in Fig. 1A shows the total labeled products of translation of liver polysomes from rats starved 48 hr and refed a fat-free diet for 16 hr. Most of the ^{35}S label is seen in peptides of M_r less than 70,000. However, several larger peptides are seen, one of which comigrated with the equal-sized (240,000 M_r) subunits of rat liver FAS. When an equal aliquot of this translation reaction was subjected to immunoprecipitation with anti-FAS antibody (Fig. 1B), at least 11 labeled peptides were present in the precipitate. These ranged in M_r from 155,000 to 240,000, and the highest M_r band comigrated with authentic FAS. Autoradiography of the supernatant of this anti-FAS precipitation showed selective loss of all 11 immunoreactive peptides and demonstrated the massive amount of label that did not precipitate (Fig. 1C). In a control experiment excess, unlabeled, dissociated rat liver FAS was added to the translation reaction mixture before indirect immunoprecipitation. The 11 previously precipitated peptides were nearly completely absent from the precipitate (Fig. 1D, compare to 1B) and were present in the supernatant (not shown). As a further control, rabbit anti-rat albumin antibody was added to a translation reaction instead of anti-FAS antibody. An autoradiograph of the precipitate revealed a single labeled peptide which comigrated with authentic rat serum albumin (Fig. 1E). The 11 FAS immunoreactive peptides remained in the supernatant (Fig. 1F). When partially purified rabbit IgG was added instead of anti-FAS antibody, the precipitate contained neither the FAS immunoreactive peptides nor the albumin immunoreactive peptide (Fig. 1G). These control experiments demonstrated that all 11 peptides seen in Fig. 1B were, indeed, FAS peptides. They were not nonspecific peptides trapped in the immunoprecipitate.

The products of *in vitro* translation were further characterized after [^{3}H]leucine labeling by slicing of $NaDodSO_4$-polyacrylamide gels and determination of radioactivity. Fig. 2 shows that labeled peptides precipitated by anti-FAS antibody were concentrated in the range of 200,000 to 240,000. The highest M_r immunoreactive peptide (Fig. 2) comigrated with the subunit peptides of authentic rat liver FAS. Some of the precipitated radioactivity (slices 24–46) could be nonspecific precipitation, labeled immunoreactive FAS nascent chains, or posttranslation proteolytic products of larger labeled FAS peptides.

We attempted to distinguish among these possibilities by pelleting the nascent-chain polysome complexes with high speed ultracentrifugation. The "released" peptides in the supernatant (10) were then subjected to indirect immunoprecipitation (Fig. 3). Anti-FAS antibody selectively bound high M_r peptides (>155,000) when compared to the radioactivity (in this region) precipitated with control rabbit IgG. The radioactivity precipitated below M_r 130,000 (slices 25–44) was nearly equal in the control rabbit IgG immunoprecipitate and the anti-FAS antibody precipitate. These results suggest that the radioactivity precipitated in the smaller M_r region was due to nonspecific trapping in the immunoprecipitate. However, the peak of radioactivity in the M_r range of 155,000 to 240,000 was almost entirely specific FAS peptides.

Use of the procedures outlined above allowed us to determine the amounts of FAS that were being synthesized in rat liver in various nutritional states (Table 1). Liver polysomes were isolated from rats fed a normal diet, from rats starved for 48 hr, and from rats starved 48 hr and refed with a fat-free diet for the times indicated. After translation, the amount of labeled FAS was identified by indirect immunoprecipitation. Polysomes from starved animals made very low, but detectable, amounts of FAS immunoreactive peptides. Polysomes from normally fed rats incorporated nine times as much label into FAS peptides. Fat-free refeeding induces high levels of FAS activity, which has been shown to be due to increased synthesis of the enzyme (1–3, 5). The data in Table 1 demonstrate that this induction of FAS synthesis, as shown in the *in vitro* translation system, began by 2.5 hr after refeeding and increased dramatically by 5 hr after refeeding. By 16 hr after refeeding an extremely high rate of synthesis (4–10 times normal in several experiments) of FAS was achieved.

DISCUSSION

The protein-synthesizing system used in these experiments allows completion of nascent peptide chains bound to the polyribosomes isolated intact from rat liver. These chains were initiated *in vivo*. Therefore, isolation from the *in vitro* system of the completed, labeled FAS peptides reflects the amount of enzyme that was being translated *in vivo* at the

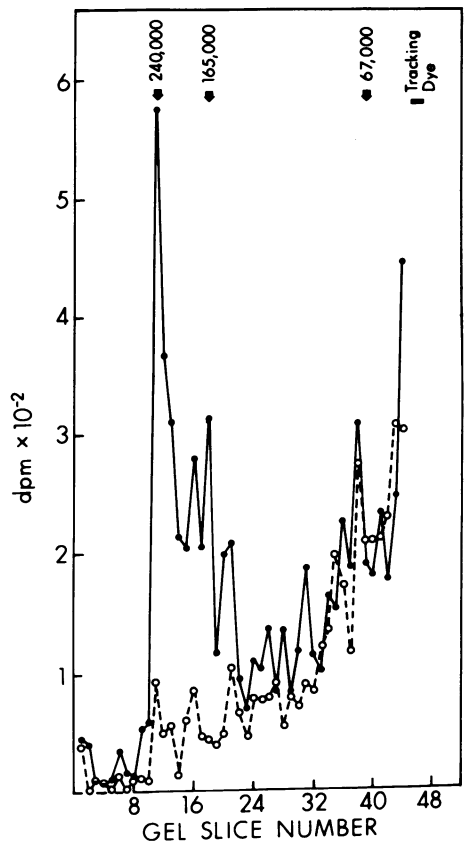

FIG. 3. Immunoprecipitation of "released" peptides from *in vitro* translation. [^{3}H]Leucine was incorporated into protein using rat liver polysomes as in Fig. 2 in a 1-ml translation reaction mixture (24 × 10^6 dpm). This was centrifuged for 90 min at 100,000 × *g* in a 65 rotor. Fifty microliters of the supernatant (363,000 protein dpm) was immunoprecipitated with anti-rat liver FAS antibody and analyzed as in Fig. 2 (●-●). To a second 50 μl of the supernatant were added amounts of partially purified rabbit IgG and goat anti-rabbit IgG to give a precipitate of equal size to the above FAS precipitate. This was analyzed as in Fig. 2 (○- -○). Arrows indicate position of M_r markers in adjacent slabs of the same gel.

time of polyribosome isolation. It is not possible to distinguish whether differences in amount of synthesis of FAS are due to differences in rate of initiation, quantity of translatable FAS mRNA, or both.

We initially used polyribosomes from rats starved and then refed a fat-free diet for 16 hr because previous investigations have shown that synthesis of FAS is nearly maximal at this time (5, 11). Translation and indirect immunoprecipitation of the products of translation using these polysomes allowed us to identify FAS peptides with a high degree of certainty (Figs. 1–3). Purified anti-FAS antibody selectively precipitated peptides not precipitated with either anti-albumin antibody or rabbit IgG. Furthermore, unlabeled, native rat FAS could effectively compete for anti-FAS antibody, thereby leaving labeled FAS peptides in the supernatant. Schutz *et al.* (10) and Taylor and Schimke (7) have used similar controls in order to demonstrate the identity of the immunoprecipitated, labeled peptides.

These investigators previously have found a single large peak of labeled protein using immunoprecipitation. Our immunoprecipitates, however, contained several large peptides. When [^{35}S]methionine was used to label translation products (Fig. 1B), the most heavily labeled immunoreactive FAS peptides were of M_r 165,000 and 180,000, and multiple

Table 1. Effect of nutritional state on rate of FAS synthesis

Nutritional status*	dpm/FAS peak†	FAS specific activity‡ (units/mg of protein)
Normally fed	1,500§	5.2
Starved	166	1.2
1 Hr refed	175	1.2
2.5 Hr refed	545	1.2
4.5 Hr refed	1,190	2.4
5 Hr refed	3,160	—
16 Hr refed	5,560 (1.5 A_{260}/assay)	12.5
	15,600 (4.5 A_{260}/assay)	

* "Normally fed" rats received a regular diet for at least 5 days prior to polysome isolation. "Starved" rats were starved for 48 hr. "Refed" rats were starved for 48 hr and then refed a fat-free diet for the indicated times. Polysomes were prepared and translated and the entire translation mixture was subjected to indirect immunoprecipitation with anti-rat liver FAS antibody (see *Materials and Methods*). The precipitates were analyzed as in Fig. 2.

† Dpm in gel slices corresponding to numbers 12–16 of Fig. 2 were totaled and dpm of the same slices of a rabbit IgG control precipitate subtracted (approximately 250 dpm).

‡ FAS assay was performed as described (4).

§ Each translation assay contained 1.5 A_{260} units of polysomes per 150 μl except for the second assay of polysomes from a 16 hour refed animal, which contained 4.5 A_{260} units/150 μl.

distinct bands were observed. When [^{3}H]leucine was used as a label, the 240,000 M_r FAS peptide contained most of the radioactivity (Figs. 2–3), and only one or two major peaks were observed. Possible explanations for these differences include (*a*) more uniform labeling with [^{3}H]leucine, (*b*) instability (presence of sulfoxides) of [^{35}S]methionine, (*c*) partial degradation of polyribosomal mRNA (to explain smaller than expected size peptides), (*d*) premature termination of translation, (*e*) release of incomplete nascent chains, and (*f*) proteolysis of large labeled FAS peptides. However, there is no doubt (especially with [^{3}H]leucine labeling) that most of the immunoprecipitated FAS label is contained in peptides of molecular weight similar to that of the two FAS subunits (Figs. 2–3). This result suggests that rat FAS is synthesized as either (*a*) two large peptides with M_r 240,000, or (*b*) smaller peptides that are rapidly, covalently linked into two large peptides. The first explanation would appear more likely in view of our previous data (6) showing binding of iodinated anti-FAS antibody to the nascent chains of large polyribosomes.

One of our purposes in developing this translation system, with immunological isolation of labeled FAS product, was to quantitate the amount of FAS synthesized under various nutritional states. Investigators (reviewed in ref. 12) previously have shown that in starved animals FAS is synthesized at 1/5 the rate of normally fed animals. After the refeeding of starved animals with a fat-free diet, peak FAS synthesis was 14 times faster than in normally fed animals. Our data (Table 1) show similar differences in rate of synthesis with different nutritional states.

Synthesis of FAS [or apo-enzyme lacking 4′-phosphopantetheine (5, 11)] is induced by fat-free refeeding of starved rats after a lag period variously reported as from minutes to 6 hr (3, 5, 11). The data shown in Table 1 demonstrate a

slight increase in rate of *in vitro* synthesis of FAS peptides by polysomes from rats refed for 2.5 hr. Moreover, the rate of *in vitro* synthesis of FAS by polysomes from 16 hr refed rats was 10–30 times faster than at 2.5 hr. No difference was detected in the rate of *in vitro* FAS synthesis by polysomes from starved rats and 1 hr refed animals. Our results do not distinguish whether the labeled FAS peptides synthesized were holo- or apo-enzyme. However, the increase in rate of FAS synthesis by polysomes *in vitro* closely paralleled the induction of FAS specific activity found in animals refed for various periods of time (Table 1). This suggests that any apo-FAS synthesized was rapidly converted to active enzyme.

Our results show that a marked increase in the rate of *in vitro* translation of FAS peptides from polysomes is critical in the "adaptive synthesis" of FAS after fat-free refeeding of starved rats. We are unable to distinguish whether this increase is due to an increase in initiation rate of FAS mRNA, an increase in number of FAS mRNA molecules, or both. *In vitro* translation of purified liver mRNA with isolation and quantitation of amount of FAS produced should resolve this question.

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