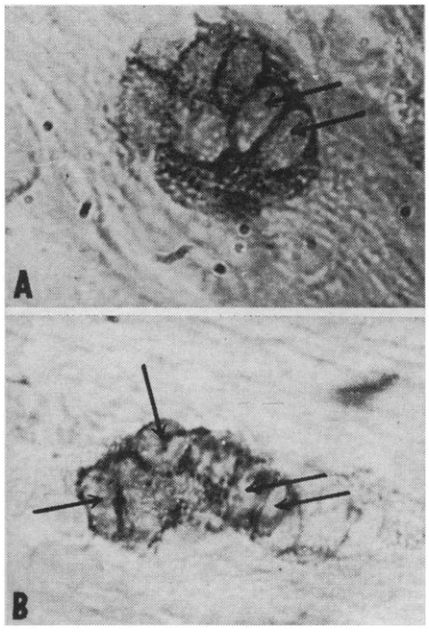


Fig. 1. (*A*) Release of calcium in the post-junctional cytoplasm of a motor endplate (diaphragm of the rat) 10 minutes after an intraperitoneal injection of Carbachol (1 mg/kg) (× 1000). (*B*) Release of calcium 30 minutes after an intraperitoneal injection of Tremorine (50 mg/kg). Arrows point to nuclei.

tion of the drug. Tremor started 4 to 10 minutes after the injection and was accompanied later by a supersecretion of the Harderian glands and salivation. Thirty to sixty minutes after the injection, the rats were killed by decapitation; the diaphragms were excised and frozen on specimen holders by means of dry ice. Longitudinal sections were cut on a cryostat, dropped immediately into acetone, floated in 2 percent sodium barbital, and stained for liberated calcium according to the method described by us earlier (*5*, *6*).

In the diaphragms of normal rats, Tremorine induced the release of calcium in well-localized areas in the post-junctional cytoplasms ("soleplates") of the myoneural junctions. The patterns obtained were virtually the same as those obtained after supramaximal electrical stimulation (*5*), or after the administration of neostigmine or Carbachol (carbaminoylcholine). Heavily stained granules outlined the fundamental cells, outlining the nuclei (Fig. 1). Smaller doses of intraperitoneally injected Tremorine (10 and 25 mg/kg) resulted in less characteristic microscopic patterns, even though the outlines of the soleplates could also be easily distinguished in these experiments.

Virtually the same pattern was observed in those animals in which the phrenic nerve had been transected 1 to 3 days prior to the administration of the drug. There was a marked decrease in the intensity of the reaction and in the number of granules showing evidence of liberated calcium on the 4th day after the nerve was transected; by the 5th day there was no evidence of liberated calcium. Thus the reaction was entirely similar to that observed earlier in animals treated with Carbachol. From the 8th day after transection onward, after injection of the same doses of Tremorine, there appeared a peculiar linear reaction on the surfaces of muscle fibers, extending up to 100 to 200 $\mu$ in length. This kind of "surface reaction" was observed previously in animals under similar experimental conditions injected with neostigmine or Carbachol. The reaction has been ascribed to the altered sensitivity of denervated muscles to acetylcholine and related compounds (*6*).

It appears, therefore, that the action of Tremorine on the myoneural junction resembles in all histochemical details that of acetylcholine-like agents. The experiments reported here support the conclusion that the main action of Tremorine is peripheral rather than central. On the basis of pharmacological studies with this drug (*1–4*, *7*), there remains little doubt that cholinergic effects on various central and ganglionic levels contribute to the general picture evoked by Tremorine. Thus, it appears that the total effect of Tremorine consists of a composite action on cholinergically mediated junctions, probably due to the liberation of acetylcholine in a variety of synapses.

Whether the actual effect observed in our experiments was due to Tremorine itself or to its activated oxo-derivate (*7*) should be decided in the future by similar experiments carried out with oxotremorine.

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## Protein Synthesis Enhanced in the Liver of Rats Force-Fed a Threonine-Devoid Diet

Abstract. *There is enhanced incorporation of leucine-$C^{14}$ into proteins in cell-free preparations from livers of young rats force-fed a threonine-devoid diet for 3 days. This increased protein synthesis in the liver is related predominantly to enhanced activity of ribosomes. The aggregates of liver ribosomes (ergosomes or polysomes) as measured in a sucrose gradient indicated a shift from lighter toward heavier ergosomes with a decrease in monomers.*

In earlier studies (*1–3*) we found that young rats force-fed for 3 to 8 days purified diets devoid of single essential amino acids developed pathologic changes that closely resembled many of those found in infants with kwashiorkor (*4*). The morphologic changes consisted of a periportal fatty liver, excess hepatic glycogen, and atrophy of the pancreas, submaxillary gland, stomach, spleen, and thymus. Among the biochemical changes in the livers of these experimental animals were increases in lipid, glycogen, total RNA, and in the incorporation in vivo of amino acids into protein (*2*, *3*). The last-mentioned finding was particularly surprising in an animal in which obvious pathologic changes were readily produced by a regimen deficient in an amino acid. However, subsequent reports by others (*5*) substantiated the occurrence of enhanced protein synthesis in the livers of animals with amino acid or protein deficiencies under a variety of experimental conditions. The studies in vitro which we are now reporting show that the increased protein synthesis in the livers of rats force-fed a threonine-devoid diet is related predominantly to enhanced activity of ribosomes.

Male and female Sprague-Dawley rats (70 g) were force-fed for 3 days a purified complete diet or one devoid of threonine (*3*). Each animal received

three feedings (totaling 7 g) of diet daily. On the morning of the 4th day the rats were anesthetized with ether and exsanguinated approximately 16 hours after the last feeding. The livers were removed, and ribosomes were prepared according to Korner's method (*6*). In several experiments sucrose-gradient patterns of ribosomes were obtained according to the method of Wettstein *et al.* (*7*).

In four experiments (Table 1) there was an average increase of 65 percent in the extent of leucine incorporation into proteins in cell-free preparations from the livers of rats force-fed a threonine-devoid diet in comparison with similar preparations from animals force-fed the complete diet. In one experiment animals were force-fed the diets 7 days instead of 3 days and the average stimulation was 220 percent in the experimental group.

The stimulation of amino acid incorporation is principally accounted for by the activity of the ribosomes. Ribosomes from the experimental animals showed a 74 to 76 percent increase in activity (Table 2) regardless of the source of the postmicrosomal supernatant fraction of liver homogenates. However, the supernatant from liver homogenates of the experimental animals was slightly more active (21 percent) than that from control animals when tested with control ribosomes. This increase in incorporation of amino acid due to the supernatant is consistent with the results of Mariani *et al.* (*8*), which indicate an increased activity of amnio acid-activating enzymes in the livers of protein-depleted rats. In two experiments supernatant (which had been frozen and then thawed) of liver homogenate of a rat fed a stock diet were incubated with the liver ribosomes of control and experimental animals. The liver ribosomes from the experimental animals were 30 percent more active than those from control animals.

The present results of enhanced incorporation of leucine in vitro into liver protein are in agreement with results obtained in experiments in vivo. Studies in vivo (*2*) of 7 days' duration revealed a 37 to 169 percent enhancement of incorporation of amino acid (L-leucine, L-valine, or L-isoleucine) into liver protein and a 45 to 123 percent enhancement of incorporation into plasma protein of animals force-fed the threonine-devoid diet in comparison with those fed the complete diet. These animals also showed a 37 percent increase in total liver RNA. Recently we have repeated these experiments twice, using rats force-fed the complete and threonine-devoid diets for 3 days and injecting 1.7 or 5.0 $\mu$c (0.04 or 0.11 $\mu$mole) of uniformly labeled L-leucine-$C^{14}$ intraperitoneally 3 hours before the animals were killed on the morning of the 4th day. The results, expressed as the radioactivity in total liver protein corrected for the radioactivity in the acid soluble fraction, revealed a 30 to 116 percent increase in the livers of experimental animals in comparison with livers of control animals. Total liver RNA was increased 16 to 37 percent in animals fed the deficient diets for 3 days.

Ribosome aggregates (ergosomes or polysomes) obtained from the livers of control animals force-fed the complete diet and of experimental animals force-fed the threonine-devoid diet for 3 days were separated by size in a sucrose gradient. The sedimentation patterns were constant for the ribosomes of control animals (Fig. 1*A*), while the pattern for the experimental animals always showed a shift of aggregate size from lighter toward heavier ergosomes, with a concomitant decrease in monomers. However, the degree of this shift varied in different experiments. The pattern obtained from a highly responsive experimental animal is illustrated in Fig. 1*B*. For quantitative analysis, aggregates larger than dimers are considered active polysomes or ergosomes held together by messenger RNA (mRNA). In the control animal, 51 percent of the ribosomes are ergosomes and 49 percent are free monomers and dimers (Fig. 1*A*), while in the animal fed the threonine-devoid diet 77 percent are ergosomes and 23 percent are free monomers and dimers (Fig. 1*B*). Under control conditions, the number of ribosomes in a fraction of given size of ergosomes is proportional to the length or molecular weight of its mRNA (*9*), and therefore the size distribution of ergosomes should reflect the size distribution of mRNA. The obvious shift in the ergosome pattern toward heavier aggregates under the experimental pathological conditions could be due to a different size distribution of mRNA's, a closer spacing of ribosomes on the same size distribution of messengers or a more stable population of ergosomes which are less susceptible to breakdown during their preparation.

According to the hypothesis of the tape mechanism of protein synthesis (*10*) mRNA moves like a tape through the active site of each ribosome, thus delivering its encoded message for the translation into the colinear amino acid sequence of the growing peptide chain. This process begins with the attachment of ribosomes at one end of the

Table 1. Incorporation of leucine-$C^{14}$ into proteins of cell-free preparations from the livers of young rats force-fed a threonine-devoid diet (TD) or a complete diet (C). In a total volume of 1 ml the incubation mixture contained: 0.2 ml ribosomes (0.2 to 0.8 mg RNA) in 0.045*M* tris-buffer, *p*H 7.6, containing 7.5 m*M* $MgCl_2$, 0.12*M* KCl, and 0.075*M* NaCl; 0.3 ml of supernatant in 0.44*M* sucrose, and 0.5 ml solution containing 0.02*M* tris-buffer (*p*H 7.6), 5m*M* $MgCl_2$, 0.08*M* KCl, and 0.05*M* NaCl, in which were dissolved components in concentration to give 1m*M* adenosine triphosphate, 0.6m*M* guanosine triphosphate, 0.825m*M* mercaptoethanol, 10m*M* phosphoenolpyruvate, 30 $\mu$g pyruvate kinase and 0.5 $\mu$c (0.01 $\mu$mole) L-leucine-$C^{14}$ in the 1 ml final volume. Samples in triplicate were incubated at 37°C for 1 hour. The reaction was stopped with 1 ml of 10 percent trichloroacetic acid (TCA). Samples were washed three times with cold 10 percent TCA (first wash containing 5 mg of nonradioactive leucine per milliliter), extracted once with an ethanol-ether mixture (3:1) for 5 minutes at 60°C and twice with 10 percent TCA for 15 minutes at 90°C, and washed once each with ethanol, ethanol-ether, and ether. Samples were plated and counted in a thin window gas-flow counter with an efficiency of 25 percent for $C^{14}$. Protein was determined gravimetrically.

| Group | No. of rats | Specific activity* (count/min) | Effect (%) |
|---|---|---|---|
| C | 3 | 1249 ± 180 | |
| TD | 6 | 1631 ± 136 | +31 |
| C | 4 | 518 ± 65 | |
| TD | 6 | 899 ± 84 | +74 |
| C | 4 | 967 ± 173 | |
| TD | 5 | 1808 ± 314 | +87 |
| C | 4 | 655 | |
| TD | 4 | 1094 | +67 |

* Counts per minute per milligram of ribosome RNA. Each value for the first three experiments is the means ± standard error of individual determinations in the group. The values of the fourth experiment are on pooled specimens.

Table 2. The incorporation of radioactive leucine-$C^{14}$ into proteins in different liver fractions from rats force-fed a threonine-devoid (TD) or complete (C) diet.

| Source of homogenate fraction | | Percentage of control* |
|---|---|---|
| Ribosomes | Supernatant | |
| C | C | 100 |
| C | TD | 121 |
| TD | C | 176 |
| TD | TD | 174 |
| C | N† | 100 |
| TD | N | 130 |

* The activity of the control flask containing components of the livers of rats force-fed the complete diet was arbitrarily set at 100. The values represent averages of three experiments. Experimental conditions are the same as described in Table 1. † Supernatant from sample (frozen and then thawed) from the liver of a rat fed ad libitum a stock diet.

Table 3. The relation of liver ribosomes, classified as total ribosomes and ergosomes, to incorporation of amino acids into liver protein in vivo.

| Characteristic | Control | Experimental |
|---|---|---|
| *a*: Total ribosomes per liver | 100 | 137* |
| *b*: % of ribosomes in ergosomes (> dimers) | 51 | 77 |
| *c*: Total mass of ergosomes per liver = (a × b)/100 | 51 | 106 |
| *d*: Total amino acid incorporation per liver (in vivo) | 100 | 216† |
| Amino acid incorporation per unit total ribosomes = *d*/*a* | 1.00 | 1.58 |
| Amino acid incorporation per unit ergosomes = *d*/*c* | 1.96 | 2.04 |

* Value based on increase in total liver RNA in rats fed the threonine-devoid diet. † Value based on increase in corrected total liver amino acid incorporation in rats fed the threonine-devoid diet.

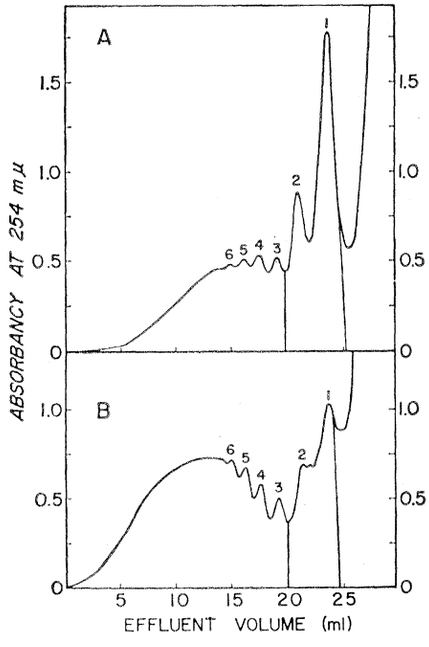


Fig. 1. Sucrose-gradient patterns of ribosomes from: *A*, control animal force-fed the complete diet for 3 days; *B*, experimental animal force-fed the threonine-devoid diet for 3 days. The supernatant of liver homogenate after removal of unbroken cells, debris, nuclei, and mitochondria was treated with deoxycholic acid (sodium salt) and an amount corresponding to approximately 0.15 g liver was layered in the form of an inverted gradient on top of 27.5 ml of a [convex exponential] sucrose gradient (11 to 34 percent) containing 0.05*M* tris-buffer at *p*H 7.6, 0.005*M* $MgCl_2$, and 0.025*M* KCl. The gradient tubes were centrifuged in a Spinco SW 25 rotor at 24,000 rev/min for 3 hours at 4°C. The bottoms of the tubes were punctured with a hypodermic needle, and the fluid was passed through a continuous flow ultraviolet absorption cell of 4 mm diameter attached to a stripchart recorder. A constant flow rate was maintained by a precision pump. The absorbency scale is corrected for a 1-cm light pathway.

mRNA strand and terminates with their release at the other end, each with its finished peptide chain. The spacing of ribosomes on mRNA is therefore determined by the rate at which ribosomes are attached to the "head" of mRNA (*V'*) and by the speed at which the ribosomes move along the mRNA (*V''*). Any alteration in the ratio *V'*:*V''* changes the spacing of ribosomes on mRNA and concomitantly produces a change in the ratio of free ribosomes to polysomes as well as a change in the size distribution of polysomes. An increased ratio of *V'*:*V''* could account for a closer spacing of ribosomes in the experimental animals. This could be achieved under two extreme conditions: (i) by increasing the rate of ribosomal attachment *V'* or (ii) by decreasing the speed of ribosomal movement *V''*. In the first case the rate of protein synthesis per mRNA molecule, and therefore per cell or organ, would increase proportionally to the number of ribosomes drawn from the free monomer pool and added to the ergosome population. In the second case, the rate of protein synthesis per mRNA molecule and per cell or organ would remain the same, even though more ribosomes are engaged in protein synthesis, because the ribosomes are working more slowly.

From the data derived from Fig. 1 and data previously presented from a selected highly responsive 3-day experiment in vivo, we have made approximate calculations for the incorporation of amino acid for the total mass of ribosomes and for the total mass of ergosomes (Table 3). These calculations are based on the assumptions that (i) the size of the ribosomes is the same in control and experimental animals and (ii) the mass of ribosomes per liver is proportional to the total hepatic RNA content in both groups of animals. The second assumption is based on the observation that by far the largest (60 to 70 percent) fraction of RNA in the normal liver is cytoplasmic ribosomal RNA. The results reveal a moderate increase in specific activity of total ribosomes of the experimental animals and almost identical specific activities of polysomal ribosomes in the control and experimental animals. The latter results indicate essentially no change in the speed at which the ribosomes move along the mRNA. The data are therefore compatible with either a marked shift in the size distribution of mRNA toward larger molecules or a significant increase in the number of ribosomes for each mRNA strand. If the speed at which the ribosomes move along the mRNA (*V''*) has not decreased, a closer spacing could only result by increasing the rate of ribosomal attachment to mRNA (*V'*). If this hypothesis is correct the mechanism of attachment would be a key point in control of cytoplasmic protein synthesis.

The results of our previous studies and of the present study clearly demonstrate that protein synthesis in the liver is accelerated in rats force-fed a threonine-devoid diet in comparison with those fed the complete diet, when protein synthesis is measured as incorporation of a radioactive amino acid into protein either in vivo or in vitro. The ribosomes in the experimental animals are mainly responsible for the increased incorporation of amino acid. Since enhanced incorporation of amino acid into liver protein has been found in studies with other agents producing hepatic injury (*5*, *11*) it is conceivable that a common reaction pattern may exist in response to a variety of injurious agents.

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