Studies on the Role of Ribosomes in the Regulation of Protein Synthesis in Hypophysectomized and Thyroidectomized Rats*

(Received for publication, June 6, 1966)

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SUMMARY

Polyribosomes were obtained from the livers of hypophysectomized and control rats. Although sucrose gradient analysis disclosed that polyribosome profiles of the livers of hypophysectomized animals were identical with those of livers derived from intact controls, the rate of amino acid incorporation into hepatic protein in vitro was lower in the polyribosomes derived from hypophysectomized animals. Furthermore, the stimulation of phenylalanine-14C incorporation that followed the addition of polyuridylic acid was less when the disaggregated polyribosomes were obtained from hypophysectomized rats than when similar preparations obtained from control animals were used. Similar findings were obtained with polyribosomes from thyroidectomized rats. These studies suggest that hypophysectomy and thyroidectomy influence protein synthesis predominantly by action at the level of the ribosome.

Studies of enzyme induction in microorganisms have suggested that the rate of protein synthesis is controlled by the concentration of messenger ribonucleic acid. It has been shown that the ribosomes exert no regulatory influence on this process (1, 2). In higher organisms numerous studies (e.g. References 3-6) have suggested that hormones induce protein synthesis by a similar mechanism, i.e. by the activation of mRNA synthesis. However, evidence has subsequently been presented that in mammals mRNA is relatively stable (7, 8), while ribosomes turn over at a more rapid rate than initially suspected (9). In several instances it was also shown that the synthesis of predominantly ribosomal RNA was stimulated by the hormones (thyroxine, hydrocortisone, growth hormone) that concomitantly induced an increase in the rate of synthesis of hepatic protein (3, 10-12). Moreover, the evidence that administration of hormones stimulated ribosomal RNA synthesis was relatively direct, while that for mRNA synthesis was more dependent upon inference.

This suggested the present studies, in which the effects on hepatic protein synthesis of changing the hormonal environment of the rat by hypophysectomy or thyroidectomy were studied. It is concluded that the hormonal state of the animal influences the ribosomal regulation of protein synthesis.

EXPERIMENTAL PROCEDURE

Materials

L-Phenylalanine-14C, uniformly labeled (specific activity, 300 mC per mmole), and ornithine-6-14C (specific activity, 8 mC per mmole) were obtained from New England Nuclear; polyuridylic acid from Miles Laboratories, Elkhart, Indiana; phosphoenolpyruvate kinase, sodium phosphoenolpyruvate, ATP, GTP, and nonradioactive amino acids from Sigma; sodium deoxycholate and sodium dodecylsulfate from Fisher Chemical Company, New York, New York.

Animals

All rats were maintained on Purina laboratory chow and fasted for 15 hours before they were killed (in all experiments). Hypophysectomized and thyroidectomized rats were used only if a significant decrease in the animals' growth rate was seen (thyroidectomy was accomplished either surgically or with 131I). Female rats of the Charles River strain (Charles River Company, Wilmington, Massachusetts) weighing approximately 60 g were hypophysectomized and used from 3 to 5 weeks later. Intact littermates served as controls. At the time of the experiments, the hypophysectomized rats weighed approximately 90 g, and the controls, 172 g. Male Sprague-Dawley rats (Badger Company, Madison, Wisconsin) weighing approximately 80 g were surgically thyroidectomized and used from 3 to 5 weeks later. Alternatively, similar male rats placed on an iodine-free diet for 1 week were given intraperitoneal injections of 0.5 mC of 131I and used approximately 5 to 6 weeks later. Controls were their untreated littermates. The thyroidectomized animals and the untreated controls weighed approximately 182 and 247 g, respectively, when used in the experiments.

* This paper was presented in part at the 48th meeting of the Endocrine Society, Chicago, June 1966.

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The abbreviation used is: mRNA, messenger ribonucleic acid.
Methods

Crude Ribosomes—Ribosomes were isolated by deoxycholate treatment as previously described (13). All procedures were performed at temperatures between 0° and 5°. Rats were decapitated and the livers were rapidly removed and homogenized with a motor-driven homogenizer in 2 volumes of a medium containing 0.25 M sucrose, 0.05 M Tris-chloride at pH 7.6, 0.005 M MgCl₂, and 0.025 M KCl (Littlefield's medium (14)). After centrifugation at 600 X g for 10 min, the supernatant fluid was removed and centrifuged at 20,000 X g for 10 min. The pellet was discarded, and 2.7 ml of 10% deoxycholate were added per 18 ml of supernatant fraction. The preparation, kept for 20 min at 0-5°, was centrifuged in the Spinco preparative ultracentrifuge at 100,000 X g for 2 hours. The precipitated ribosomal pellet was resuspended in medium containing 0.35 M sucrose, 0.07 M KCl, 0.05 M Tris-chloride at pH 7.8, 0.004 M MgCl₂, and 0.006 M mercaptoethanol.

Purified Ribosomes—The postmitochondrial deoxycholate-treated supernatant obtained as described above was layered over a discontinuous gradient containing 0.5 M sucrose over 2 M sucrose in Littlefield's medium as described by Wettstein, Staehelin, and Noll (15). After centrifugation in the Spinco ultracentrifuge for 36 hours at 100,000 X g, the supernatant was decanted, the tube wall wiped clean, and the translucent, purified ribosomal pellet resuspended as described above.

Polysome Sucrose Gradient Analysis—Sucrose-free Littlefield's medium (0.25 ml) containing 5 optical density units (at 260 nm) of ribosomes was layered over a 10 to 30% sucrose gradient as modified for polysome analysis by Wettstein, Staehelin, and Noll (15). This was centrifuged in the SW 25.1 rotor of the Spinco L2-65 ultracentrifuge at 25,000 rpm for 2 hours. After centrifugation, the bottom of the tube was punctured and the effluent was allowed to pass through a Gilford model 2000 recording spectrophotometer equipped with a flow through cell of 1.0-cm light path.

Amino acid incorporation was measured according to the method of Lowry et al. of the Folin-Ciocalteau method (17) as modified for polysome analysis by Keller and Zamecnik (18), with the use of ribosomes isolated by deoxycholate treatment as described above.

Ribosomal RNA sucrose gradient analysis was performed as described by Staehelin et al. (19). Sodium dodecylsulfate, 0.5% in 0.05 M Tris-chloride at pH 7.6 (0.5 ml), was added to 10 optical density units (at 260 nm) of ribosomes. After warming at 37° for 10 min the RNA was considered to be released from the ribosomes, and the mixture was layered over 28 ml of a 5 to 20% sucrose gradient in Littlefield's medium. The tubes were placed in the SW 25.1 rotor and centrifuged for 18 hours at 25,000 rpm. After centrifugation the tubes were punctured and the effluent was analyzed as described below. Protein was determined by the method of Lowry et al. of the Folin-Ciocalteau method (19). DNA and RNA were determined as described by Schmeider (21). Ferritin contamination of the ribosomal fraction was corrected by the method of Wilson and Hoagland (22).

RESULTS

Confirming previous reports (e.g. Reference 5), the rate of amino acid incorporation into liver protein in vitro was diminished when the ribosomes used were obtained from hypophysectomized rats (Fig. 1). Similar results were obtained with either crude ribosomes or ribosomes purified by centrifugation through 2 M sucrose as described.

Since an identical pH 5 fraction (23) obtained from control animals was used in all studies, the diminished rate of amino acid incorporation of the hypophysectomized group was attributed to differences in the ribosomal fraction. It has been shown that ribosomes that are active in protein synthesis occur as aggregates (polysomes), and it has been proposed that these ribosomes are held together by a strand of messenger RNA (15, 24). Therefore, the diminished rate of amino acid incorporation into liver protein of the experimental group could be explained by postulating either a decrease in the concentration of mRNA or a diminished efficiency in the utilization of available mRNA by the ribosomes of this polysome fraction.

Because it was postulated that the proportion of aggregated ribosomes (polysomes) was determined by the concentration of mRNA, polysome patterns from the livers of normal and hypophysectomized rats were compared. Sucrose gradient analyses disclosed no consistent differences between the polysome profiles of control and hypophysectomized groups. Indeed, the differences observed in the polysome profiles from experiment to experiment were as profound within each group as between
polysomes (A and B) and for polysomes purified through 2 su-
crose (C and D). Figure 2, where the patterns are shown for crude
region of the gradient, the monosome peak almost entirely
disappears, indicating that in this fraction of the liver 90 to 95%
of the ribosomes are aggregated and sediment with a constant
rected for contamination by ferritin, which sediments in the 70 S
of 110 S or greater. This phenomenon was seen in both control
and hypophysectomized groups (Fig. 2). Because Korner (5) reported a decrease in the proportion of
aggregated ribosomes after hypophysectomy, the following
study was undertaken in order to provide a control over possible
differences in the conditions of homogenization and analysis
shown in Fig. 3, 18 hours after radioactive orotic acid is injected
intraperitoneally into rats the radioactivity is largely located
in the ribosomal RNA. Therefore, in the following experiment,
50 μC of 14C-otic acid were injected intraperitoneally into the
hypophysectomized rats, and 600 μC of 3H-otic acid into the
control animals. The rats were killed 18 hours later, the livers
were removed and homogenized, and the experimental groups
were arranged as follows. (a) The livers from the control and hypophy-
ysectomized groups were homogenized in separate vessels,
and the sucrose gradient patterns of the polysome fractions were
obtained independently, then plotted together (Fig. 4A). (b) The
polysome fractions from the control and experimental groups
were obtained separately, but mixed immediately prior
to sucrose gradient analysis (Fig. 4B). (c) The livers from the
control and hypophysectomized groups were homogenized
together, and the polysomes obtained were analyzed in the same
sucrose gradient (Fig. 4C). These studies showed that the
polysome patterns of the samples from control and hypophyse-
tomized rats were identical when they were handled together
throughout the entire procedure. However, the differences
in the patterns observed when the groups were analyzed sepa-
ately, although greater than the differences observed when the
groups were handled together, did not appear to be significant,
a finding similar to those with unlabeled polysomes described
above (Fig. 2). Indeed, the plot of the separately prepared polysomes in the experiment depicted in Fig. 4A suggests a slightly,
but insignificantly, greater concentration of aggregated
ribosomes in the hypophysectomized group. Of course, this
technique measures only the ribosomes which incorporated

the control and hypophysectomized groups. Examples of this
are shown in Fig. 2, where the patterns are shown for crude
polysomes (A and B) and for polysomes purified through 2 m
sucrose (C and D).
As previously described (22), when these patterns were cor-
corrected for contamination by ferritin, which sediments in the 70 S
region of the gradient, the monosome peak almost entirely
disappears, indicating that in this fraction of the liver 90 to 95% of
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of the polysomes from control and experimental groups. As
shown in Fig. 3, 18 hours after radioactive orotic acid is injected
intraperitoneally into rats the radioactivity is largely located
in the ribosomal RNA. Therefore, in the following experiment,
50 μC of 14C-otic acid were injected intraperitoneally into the
hypophysectomized rats, and 600 μC of 3H-otic acid into control rats; B and D, polyribosomes from hypophyse-
tomized rats.

FIG. 2. A and B, sucrose gradient analyses of crude polyribo-
somes. Five optical density units (at 260 μm) of polyribosomes
were carefully layered over 16 ml of a 10 to 30% sucrose gradient
(see “Methods”). This was centrifuged in the SW 25.3 rotor of
the Spinco L-2 ultracentrifuge at 25,000 rpm for 2 hours. The
bottom of the tube was punctured and the effluent was allowed to
pass through a Gilford model 2000 recording spectrophotometer
which sediments in the 70 S region of the gradient indicates the ferritin contamination. C and D, sucrose gradients
of 2 μ sucrose-purified polyribosomes. The sucrose gradient
analyses were performed exactly as described for crude poly-
ribosomes, with the exception that 2 μ sucrose purified polyribo-
somes were used (see “Methods”). A and C, polyribosomes from control rats; B and D, polyribosomes from hypophysec-
tomized rats.

FIG. 3. Orotic acid incorporation into ribosomal RNA. Eight-
teen hours after the intraperitoneal injection of 50 μC of 14C-otic
acid into hypophysectomized rats and the injection of 600 μC of
H-otic acid into control rats, the animals were killed, the livers
removed, and ribosomes obtained as described in “Methods.”
Sodium dodecyl sulfate, 0.5% in 0.05 M Tris-chloride buffer at pH
7.6 (0.5 ml), was added to 10 optical density units (at 200 μm)
of ribosomes. The RNA was differentially assayed in each fraction with a liquid
scintillation counter.
FIG. 4. Sucrose gradient analyses of radioactively labeled polyribosomes. Ribosomes were radioactively labeled as described in the legend to Fig. 3. Crude ribosomes were obtained from the hypophysectomized and control rats, and sucrose gradient analyses were performed as described in the legend to Fig. 2, with the exception that 30-drop fractions were collected into 10 ml of Bray's solution (25) and the radioactivity was assayed in the liquid scintillation counter. In each figure, the bottom of the gradient is at the left, the top at the right. A, sucrose gradient analysis was performed separately on 5 optical density units (at 260 mp) of crude, radioactively labeled polyribosomes from control and hypophysectomized rats. The radioactivity patterns obtained from each group are plotted together. B, crude, radioactively labeled polyribosomes (5 optical density units at 260 mp) from control and hypophysectomized rats were mixed just prior to being placed on the sucrose gradient. In these presentations, there is no evidence that the diminished rate of amino acid incorporation into liver protein in vitro in the hypophysectomized group might be explained by a change in polysome fraction (5) and consequently a decreased amount of mRNA in this fraction. Moreover, the entire ribosomal fraction obtained from liver, whether or not purified through 2 M sucrose, consists almost entirely of polysomes. This occurs not only in controls, as previously described by Wilson and Hoagland (22), but also in the hypophysectomized group (Fig. 2).

Since there was no evidence for a decrease in the concentration of mRNA, the possibility was considered that the diminished rate of amino acid incorporation of the hypophysectomized group might be explained by a change in the efficiency of the ribosomes' reading of the available mRNA. The following experiments were designed to test this possibility.

As previously shown, when polysomes were incubated for 30 min at 37° in the complete amino acid-incorporating system, the aggregates disassembled, predominantly into single ribosomes (26). In Fig. 5, the profiles of these treated polysomes obtained from control and hypophysectomized rats are compared, and appear to be similarly disaggregated. It was also previously shown, and confirmed in the present experiments, that this treatment results in the inactivation of the polysomes for incorporation of radioactive amino acids (at 260 ma) into protein. The 14C and 3H radioactivities in each fraction were differentially determined as in B. In these experiments the ribosomes were obtained from four control and six hypophysectomized rats.
Fig. 6. Effect of hypophysectomy on polyuridylic acid stimulated phenylalanine-14C incorporation. Polyribosomes were obtained from four control rats and six hypophysectomized rats and purified by passage through 2 M sucrose as described in "Methods." Ribosomal protein (0.4 mg) and 1.2 mg of protein of the pH 5 fraction were added to incubation mixtures containing 0.35 m sucrose, 0.07 m KCl, 0.05 m Tris-chloride buffer at pH 7.8, 1 \times 10^{-3} \text{M} \text{ATP}, 6 \times 10^{-4} \text{M} \text{mercaptoethanol}, 1 \times 10^{-4} \text{M GTP}, 5 \times 10^{-3} \text{M} \text{sodium phosphoenolpyruvate, 20 \mu g of crystalline phosphoenolpyruvate kinase, 19 mC-1-amino acids minus the } 14C \text{-amino acid, } 1 \times 10^{-4} \text{M each, and } 5 \times 10^{-4} \text{M MgCl}_2. \text{ The final volume was 0.37 ml, and the initial incubation was at 37° for 30 min. Following this, ATP, GTP, sodium phosphoenolpyruvate, and phosphoenolpyruvate kinase were added in the same concentrations as above, and, in addition, 1 mg of MgCl}_2, 0.02 \mu \text{ mole of phenylalanine, containing 0.1 } \mu \text{C of } 14C \text{ radioactivity, and } 80 \mu \text{g of polyuridylic acid were added. The final volume was brought to 0.5 ml, and the samples were incubated at 30°. The rate of phenylalanine-14C incorporation after the addition of polyuridylic acid was determined after subtracting the radioactivity of a sample which acid was not added, the rate of phenylalanine-14C incorporation was between 20 and 50 cpm over the background, which was approximately 28 cpm. The radioactive incorporation is expressed as counts per min per mg of ribosomal protein.

corporating amino acids into protein.\textsuperscript{2} With this treatment, therefore, ribosomes freed of mRNA were obtained from both control and hypophysectomized rats. Because it had been shown (17) that the addition of polyuridylic acid to this system reactivated it and specifically stimulated the incorporation of phenylalanine-14C, it appeared that ribosomal activity from control and hypophysectomized rats could be compared in a system in which mRNA was not limiting. Thus, in the following experiment the phenylalanine-14C-incorporating activities of ribosomes derived from control and hypophysectomized rats were determined by this method. In these studies, the concentration of ribosomes used was shown to be limiting (i.e. increasing ribosomal concentration in either the control or hypophysec-

\textsuperscript{2} E. S. Maxwell, personal communication.

Table I

<table>
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from the hypophysectomized group. For it can be reasoned that, if the amount of mRNA was diminished and the concentration of single ribosomes concomitantly increased in the hypophysectomized group, then more sites would be available on these ribosomes for polyuridylic acid attachment. Thus, contrary to Korner's actual findings, an increased rate of phenylalanine incorporation would have been expected in these polysomes in response to the addition of polyuridylic acid (e.g. see Dietz, Reid, and Simpson (27)). Recently, however, Korner reviewed this discrepancy in his original studies and offered several alternative mechanisms to explain these results (28, 29).

Similar studies were performed with ribosomal fractions obtained from thyroidectomized rats, which exhibited a decreased growth rate. Confirming previous findings (12, 30), polyribosomes from livers of thyroidectomized rats showed a diminished rate of amino acid incorporation into protein. However, when these polyribosomes were first incubated in the complete amino acid-incorporating system to form inactive ribosomes, and then phenylalanine incorporation was stimulated with polyuridylic acid, the stimulated rate of amino acid incorporation in the thyroidectomized group was still less than that of the controls, despite the addition of excess polyuridylic acid and of the same pH 5 fraction as the controls. Table II shows that, similar to the findings with the hypophysectomized rats, considerable variation occurred from experiment to experiment, but that results within each experiment were consistent with this interpretation.

**DISCUSSION**

These investigations indicate that the ribosomes from hypophysectomized rats incorporate amino acids into protein less efficiently than controls. This conclusion is based on the following findings. (a) While the proportion of aggregated ribosomes (polysomes) remained unchanged, the polysomes from the hypophysectomized animals showed a diminished rate of amino acid incorporation in vitro. (b) The addition of polyuridylic acid to ribosomes freed of mRNA stimulated the incorporation of phenylalanine less effectively in ribosomes obtained from hypophysectomized animals than in those obtained from control animals. Similar findings were obtained with ribosomes from thyroidectomized animals.

Findings in line with these studies have been reported by Rampersad and Wool (31) with the use of ribosomes obtained from muscle of diabetic animals. Dietz, Reid, and Simpson (27) suggested that the decreased amino acid-incorporating activity found in yeast gathered during certain growth phases also represented control of protein synthesis at the ribosomal level. Although several different observations have been reported to explain the diminished rate of protein synthesis which occurs in reticulocytes with aging, the finding that the proportion of aggregated ribosomes remained unchanged during reticulocyte maturation (32) suggests the possibility that diminished ribosomal function contributes to this phenomenon.

Numerous additional studies suggesting that regulation of protein synthesis occurs by mechanisms involving translation of mRNA rather than mRNA synthesis have been reported from several laboratories (e.g. References 33–38). Indeed, even in microorganisms, in which the turnover of mRNA is comparatively extremely rapid, it has been postulated that in certain instances protein synthesis can be regulated by mechanisms affecting translation as well as synthesis of mRNA (39, 40). The recent studies of Davies, Gilbert, and Gorini (41), and of Anderson, Gorini, and Breckenridge (42), involving bacterial ribosomes, even indicate the possibility that under certain circumstances ribosomes can influence the specificity of protein synthesis. Previous studies (3–6) on the regulation of hepatic protein synthesis by hydrocortisone, thyroxine, or growth hormone were interpreted as indicating an increased amount of messenger and ribosomal RNAs after hormonal administration. The evidence that hormones stimulate the synthesis of mRNA is based on the finding of an increased concentration of heterogeneous, rapidly labeled nuclear RNA, and the finding that nuclear RNA stimulates the amino acid-incorporating system in vitro which is apparently responsive to mRNA (e.g. References 3, 4).

Although these observations may be in line with a finding of stimulation of mRNA synthesis by hormones, the evidence is far from conclusive. Despite the interpretation in early studies that rapidly labeled RNA represented mRNA, it was subsequently shown that even in bacteria the bulk of the rapidly labeled RNA was ribosomal precursor rather than mRNA (43, 44). It was also shown that following special treatment ribosomal RNA can also stimulate an amino acid-incorporating system in vitro which hitherto was believed to respond specifically to mRNA (45, 46).

It has been shown that the administration of growth hormone stimulates predominantly nuclear RNA, the nucleoli apparently being the site of ribosomal RNA synthesis (11). Because the pituitary gland secretes growth hormone and controls the secretion of corticosterone and thyroxine, it is not surprising that after hypophysectomy, as shown previously and confirmed in the present study, the concentration of ribosomes per cell, i.e. with respect to DNA, decreased to approximately 50% of that in the control animals. The aforementioned findings by others and the evidence from the present study suggest the speculation that certain hormones may regulate hepatic protein synthesis by stimulating the synthesis of ribosomal RNA rather than mRNA, and that the ribosomes in turn regulate the rate.

**TABLE II**

<table>
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<th>Experiment</th>
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of protein synthesis by acting at the level of translation of stable mRNA.

Of course these data do not rule out the possibility, or even probability, that these hormones also act by stimulating mRNA synthesis, but rather indicate the paucity of available evidence along this line, and suggest another possible mechanism to be considered as an explanation for the regulation of protein synthesis by certain hormones.

In the present study, the mechanism of the change in ribosomal function which followed hormonal deprivation has not been elucidated. Moreover, the possibility that the reported findings are the results of nutritional or metabolic changes that follow the removal of pituitary or thyroid function, rather than direct responses to the change in hormonal environment, has not been ruled out. Nevertheless, the data show that, even in the presence of optimal concentrations of mRNA, when ribosomes are obtained from thyroidectomized or hypophysectomized rats, a decreased rate of incorporation of amino acids into protein occurs. This lends further support to the hypothesis that the rate of protein synthesis can be regulated at the level of ribosomal function.3

Acknowledgment—We are indebted to Dr. Elisabeth Maxwell for her aid in setting up the amino acid-incorporating system.

REFERENCES


3 Note Added in Proof—After the acceptance of this manuscript for publication, communication with Dr. Asher Kornner revealed that the main difference in our findings concerns the number of polysomes present in the liver after hypophysectomy. Both laboratories isolate polysomes by different techniques which may account, at least in part, for the dissimilar observations. The differences in interpretation are further minimized by Dr. Kornner's conclusion that the changes in the concentration of polysomes which he observes in the livers of hypophysectomized rats are not due to a change in the concentration of mRNA. In addition, he agrees with the conclusion presented here that hypophysectomy causes a decrease in ribosomal translation of mRNA and has published results to this effect. Dr. M. Staehelin called our attention to his study (Biochem. Z., 342, 459 (1965)) from which he concluded that the decreased incorporating activity of ribosomes obtained from hypophysectomized rats is due to both a lack of mRNA and a reduced capacity of the ribosomal acceptance of mRNA.
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