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ASPECTS OF CONTROL OF PROTEIN SYNTHESIS IN NORMAL AND REGENERATING RAT LIVER, II. A MICROSOMAL INHIBITOR OF AMINO ACID INCORPORATION WHOSE ACTION IS ANTAGONIZED BY GUANOSINE TRIPHOSPHATE*

BY MAHLON B. HOAGLAND, OSCAR A. SCORNIK, AND LORRAINE C. PFEFFERKORN

DEPARTMENT OF BACTERIOLOGY AND IMMUNOLOGY, HARVARD MEDICAL SCHOOL, BOSTON

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The present work was born of the thought that certain aspects of regulation of protein synthesis might be studied in cell-free systems. The burst of vigorous protein synthesis accompanying the regeneration of rat liver offered a dramatic alteration of an established control pattern amenable to such a study, provided one could reproduce in vitro what one saw in vivo. A comparative examination of cell-free preparations from normal and regenerating liver permitted the control of such variables as ribosome number, availability of messenger, and other soluble factors, thereby opening to closer examination the intrinsic properties of the microsomes themselves.

An earlier report from this laboratory focused attention on the striking difference in activity of cell-free amino acid incorporation systems from normal and regenerating rat liver.1 This has in small part been accounted for by the greater activity in regenerating liver cytoplasm of a fraction having certain properties in common with messenger RNA.2 A much larger contribution to the difference in activity, however, was made by the microsome fractions. An analysis of this aspect of the rat liver system is the subject of the present paper. It will show that normal adult rat liver microsomes contain, in excess over the amount found in regenerating microsomes, a heat-labile factor that inhibits amino acid incorporation in regenerating liver microsomes or ribosomes. The action of inhibitor fraction is antagonized by
guanosine triphosphate, there being a direct proportionality between inhibitor concentration and degree of GTP stimulation. It will be suggested that the role of GTP in protein synthesis may be related to this phenomenon.

Materials and Methods.—Preparation of tissue fractions: Female rats (Charles River Breeding Co.) weighing 250–350 gm were partially hepatectomized 22 hr before killing, as described previously. They and their unoperated sisters were starved for this 22-hr period, were then killed by decapitation, and their livers immediately chilled and minced. The livers were homogenized in 2 vol of cold medium (0.15 M sucrose, 0.025 M KCl, 0.1 M Tris buffer pH 7.6 at 4°C, and 0.005 M MgCl₂), and the homogenate was centrifuged at 15,000 g for 10 min.

Microsomes were prepared from the 15,000-g supernatant fraction by centrifuging at 59,000 g for 15 min in the no. 40 rotor of the Spinco Model L preparative ultracentrifuge. The resulting loose pellet was gently resuspended in medium, using a small glass tube and Teflon pestle by hand, at a concentration of 4 gm eq/ml (equal to about 8 mg RNA/ml). (As in the previous paper, quantities of fractions are usually expressed as gram equivalents: the wet weight of liver from which the fraction was derived.) There was generally about one sixth more RNA per gm eq in the regenerating liver microsome fraction. (This pellet contained about 60% of the RNA that would ordinarily be obtained by centrifuging the original 15,000 g supernatant for 1 hr at 40,000 rpm.) In certain experiments, the supernatant of this first centrifugation was centrifuged for an additional 45 min at 40,000 rpm, and the pellet similarly suspended. (These are referred to as "light" microsomes in Table 1.) Microsomes were washed for certain experiments by resuspension in medium at a concentration of 0.25 gm eq/ml and recentrifuged for the same period used in their initial isolation. In all comparative studies microsomes from normal and regenerating liver were adjusted to the same RNA concentration (about 6 mg RNA/ml) before each experiment.

Microsomes from regenerating liver are designated MR and normal microsomes MN. Ribosomes were prepared by the method of Kornberg and were stored frozen. The pH 5 fraction from regenerating liver was prepared as described in the first paper.

Incubations: Standard incubations were carried out at 37°C with the following final concentrations of components: microsomes 0.3 gm eq/ml (about 0.8 mg RNA/ml), pH 5 fraction 0.2 gm eq/ml; PEP kinase 0.05 mg/ml; PEP 0.01 M; ATP 1.0 mM; tris buffer 0.05 M, pH 7.0 at 37°C; KCl 0.045 M; MgCl₂ 0.0025 M; sucrose 0.075 M; C¹⁴-leucine 0.02 mM (0.1 μc). GTP was added where indicated at a final concentration of 0.5 mM, optimal for the system. Other variations in the composition of the incubations will be described in the captions under the pertinent figures and tables. Reactions were stopped with trichloroacetic acid, and protein was washed, plated, and counted as previously described. Activity is expressed as total cpm of C¹⁴-leucine incorporated in 30 min of incubation.

Orthophosphate, RNA, and protein were determined by standard procedures. The materials used in these experiments were obtained from the following sources: nucleotides from Sigma Chemical Company; polyuridylic acid from Miles Laboratories; C¹⁴-labeled amino acids from Schwarz BioResearch, Inc., and New England Nuclear Corp. C¹⁴-labeled poly U was synthesized and generously supplied by Dr. Julian Davies.

Results.—We were early led to suspect that the activity of ribosomes associated with membranes might be controlled by factors other than simple availability of messenger. When microsomes were incubated with C¹⁴-leucine and a fraction (X) which was enriched for endogenous messenger-like RNA, MR were found to be about three times as responsive to X as were MN. Ribosomes from the two sources, though readily stimulated by X, showed no such differences. Furthermore, a simple water extraction of washed MN and MR yielded equal quantities of an RNA-containing fraction having all the properties of the X fraction: i.e., it stimulated amino acid incorporation in vitro; in vivo its RNA incorporated labeled ribonucleotide precursors more rapidly than either sRNA or ribosomal RNA; and it had similar sedimentation characteristics.

Relative activity of MR and MN and apparent inhibitory activity of MN: Our earlier studies had shown that regenerating rat liver microsomes could be as much as
fifteen times as active as those of normal adult rat liver in incorporating amino acids into protein in vitro. They incorporated at a higher initial rate and for a longer period. Furthermore, mixtures of equal quantities of MR and MN consistently incorporated at a substantially lower rate than would be predicted were their activities simply additive. This suggested the possibility that MN might be inhibiting MR. The demonstration of this pattern of microsome behavior was made possible by several changes in our usual methods for preparing and incubating them.

Tab. 1

Table 1. Relative Activity of "Heavy" and "Light" Microsomes

<table>
<thead>
<tr>
<th></th>
<th>Total Cpm in Protein</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Heavy&quot;</td>
<td>MR</td>
<td>1640</td>
<td>910</td>
</tr>
<tr>
<td></td>
<td>M_N</td>
<td>123</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>M_RM_N</td>
<td>318 (26)</td>
<td>232 (30)</td>
</tr>
<tr>
<td>&quot;Light&quot;</td>
<td>MR</td>
<td>1220</td>
<td>1280</td>
</tr>
<tr>
<td></td>
<td>M_N</td>
<td>228</td>
<td>431</td>
</tr>
<tr>
<td></td>
<td>M_RM_N</td>
<td>555 (66)</td>
<td>788 (85)</td>
</tr>
</tbody>
</table>

The fractions were prepared and incubated without GTP as described in the text. The figures in parentheses are per cent of additivity: [Activity of MN-MR mixture] minus MN activity]/[Activity of MR and MN activities] minus MN activity X 100. Thus, if the observed activity of the mixture were the same as MN, the per cent of additivity would be 0. Two experiments, involving different fresh preparations of liver fractions, are shown. In these experiments the concentration of microsomes was 0.8 gm eq/ml and of pH 5 fraction 0.4 gm eq/ml.

The activity of microsomes sedimented in the first 15 min at 30,000 rpm ("heavy" M) is compared, in Table 1, with the activity of those sedimented in the ensuing 45 min at 40,000 rpm ("light" M). Although the total activity of the two fractions is not greatly different, the heavier fraction shows the greater MR/MN activity differential and the characteristic less-than-additive incorporation by the mixture. The heavier fraction is relatively richer in protein (presumably endoplasmic membrane components): its RNA to protein ratio is 0.18, while that of the lighter fraction is 0.24.

2. Concentration of microsomes: The critical role of microsome concentration during the incubation is apparent in Figure 1. Total counts incorporated are generally found to be linearly proportional to microsome concentration in a range up to 0.8–1.0 gm eq/ml for MR but only up to 0.4–0.5 gm eq/ml for MN. Below these concentrations additive incorporation with the MR, MN mixture is obtained, while above them incorporation is less than additive. (The fall in activity at high levels of MR is not a constant finding.)

3. Effect of GTP: The total amino acid incorporation by MN is stimulated 3.5-fold by GTP, while incorporation by MR is increased only 1.5-fold (average of eight experiments). Thus, the relative difference in activity between MR and MN is reduced by GTP. In these experiments incorporation by the MR, MN mixture became more nearly additive with GTP (Fig. 2): in the absence of GTP the mixture gave 58 (27–82) per cent of additive incorporation, in its presence 79 (66–103) per cent. Furthermore, although washing of microsomes decreased their baseline activity and increased their GTP requirement, the relative difference in re-
sponse to GTP was maintained: washed \( M_R \) were stimulated 5.8-fold, washed \( M_N \) 11.0-fold (four consecutive experiments).

Direct assay of an inhibitor extracted from microsomes: In order to ascertain more directly the nature of the microsomal interaction, microsomes were incubated at 37°C long enough to destroy 90 per cent or more of their protein synthetic capacity, and then their effect upon fresh, active regenerating microsomes was assessed. It was found that these preincubated \( M_R \) and \( M_N \) preparations both inhibited the active \( M_R \) system and that \( M_N \) were more inhibitory than \( M_R \); in three experiments the average inhibition by preincubated \( M_N \) was 76 per cent, by preincubated \( M_R \), 37 per cent—at a concentration of 0.5 gm eq/ml in the standard system. These inhibitions were reversed by GTP.

This direct evidence that the microsome fractions did indeed contain an inhibitory component prompted an attempt to obtain the active fraction in soluble form. Suspensions of fresh, washed microsomes were sonically disrupted for five min, and then centrifuged for one hr at 100,000 \( g \) to remove particulate material. The resulting clear supernatants were found to be strongly inhibitory as shown in Figure 3. The figure also shows that the inhibition is antagonized by GTP and that the per cent stimulation by GTP is linearly proportional to the quantity of inhibitor present. Also note that ATP, UTP, and CTP are completely without such effect. In an average of ten experiments sonic extracts of \( M_N \) gave 68 per cent inhibition, \( M_R \) 36 per cent, at 0.8 gm eq/ml.

As a further step toward isolating and identifying the inhibitor(s), portions of dialyzed sonic extracts, as prepared above, were centrifuged through sucrose density gradients, and the collected fractions assayed separately with an active

![Figure 2](image1.png)

**Fig. 2.**—Activity of normal (●), regenerating (●), and mixed microsomes (○) in presence and absence of GTP. Incubations were carried out as described in the text.

![Figure 3](image2.png)

**Fig. 3.**—The effect of sonic extracts of washed \( M_N \) on incorporation by active microsomes in presence and absence of GTP. Extracts were prepared from once-washed microsomes by sonic disruption of approximately 4 gm eq/ml suspensions (adjusted to equal RNA concentrations) in sealed cellulose tubes in a 10-ke Raytheon oscillator for 5 min at +4°C. The suspensions were then centrifuged at 105,000 \( g \) for 1 hr, and the pellets discarded. The clear supernatants were added in the quantities indicated to the standard assay system. Controls, at one concentration of inhibitor fraction, are shown in which 0.5 mM ATP (△), UTP (▲), and CTP (○) replace GTP (●).
regenerating liver incorporation system. As shown in Figure 4 the inhibitory activity is distributed rather widely over the gradient, but there tends to be a concentration of activity in the region of 4–10 S. Maximum inhibitory activity was consistently found in this region, but the degree of polydispersity and the location of the troughs varied from one sonic extract to another, as shown. This may be due either to multiple inhibitors or to differences in extent to which a single species of inhibitory element remains attached to fragments of membrane.

Note again that all inhibition disappears when GTP is added to the assay tubes (Fig. 4, uppermost profile). GTP must be present in the assay incubation in order to obtain this effect, for if it is added to the sonic extract at the top of the gradient before centrifugation, the usual inhibition profile is subsequently observed.

The incorporations of serine, isoleucine, lysine, valine, threonine, and the amino acids of an algal hydrolysate are also inhibited. The inhibitory activity is heat-labile, being 60 per cent reduced by heating at 70° for five min. (It is unaffected by heating at 55° for five min.) The sonic extracts may be stored at −10°C for several weeks.

**Negative experiments on the nature of inhibition:** (1) Inhibition is not due to depletion of GTP during the course of the incubation. The rate of release of the terminal phosphate of GTP is identical in the M_N and M_R sys-
tems (Fig. 5). (The γ-P₃²-labeled GTP was generously supplied by Dr. U. Littauer.) Since, however, there might be a difference in the extent to which the nucleotide was rephosphorylated, the fate of C¹⁴-GTP in the presence of inhibitor fractions was measured directly. Figure 6 shows clearly that GTP levels are maintained in spite of the strong inhibition of protein synthesis.

(2) Inhibition is not due to impaired binding of messenger to ribosomes. It was found that poly U-stimulated phenylalanine incorporation by ribosomes was readily inhibited by the sonic extracts. We therefore determined the extent to which C¹⁴-labeled poly U was bound to ribosomes in the presence of normal and

![Fig. 6.—The fate of C¹⁴-GTP in presence of inhibitor fractions. Two standard incubation systems (lacking C¹⁴-leucine) were made up in a volume of 4.0 ml. One contained M₄ plus sonic extract of re-generating microsomes, the other M₄ plus sonic extract of normal microsomes (each in a final concentration of 0.8 mg of original microsomal RNA per ml). Two 0.5-ml aliquots were taken from each tube, and incubated with C¹⁴-leucine for 6 and 12 min, respectively [upper graph: M₄(C): M₄ plus regenerating sonic extract (Δ); M₄ plus normal sonic extract (♦)]. The activity of the microsomes alone was determined in a separate incubation and is shown in the upper-most curve. To the remaining 3.0 ml was added 0.06 ml of 0.8 mM C¹⁴-GTP containing 4.7 × 10⁶ cpm (the same concentration of C¹⁴-GTP was added to the C¹⁴-leucine incubations above). Incuba-tion was then started, 0.5-ml aliquots being taken into 0.1 ml of 2 N perchloric acid at the times indicated. Carrier GTP, GDP, and GMP were added to these extracts, they were neutralized, the perchlorate was centrifuged off, and an aliquot chromatographed on Whatman 3MM paper by the method of Palade. The GTP spots were eluted with water and plated for counting. The cpm are those actually recorded.

![Fig. 7.—The effect of microsomal extracts on the attachment of polyuridylic acid to ribosomes and the incorporation of phenylalanine into protein. Two parallel experiments are shown in which the attachment of C¹⁴-poly U to ribosomes (full line) and the incorporation of C¹⁴-phenylalanine into hot TCA-precipitable material (dotted lines) were measured at different times of incubation in the absence (Δ) or presence of sonic extracts of regenerating (●) and normal (○) microsomes. Incubations were carried out at 37°C in a final volume of 0.275 ml in the presence of the following components: ribosomes from normal liver, 0.1 mg of RNA/ml; poly U 180 μg/ml; 105,000 γ supernatant from regenerating liver (dialyzed 24 hr), 0.4 gm eq/ml; KCl 80 mM; MgCl₂ 7.2 mM; tris-HCl (pH 7.2 at 37°C) 15 mM; 2-mercapto-ethanol 15 mM; ATP 1.0 mM; GTP 0.5 mM; PEP 10 mM; PEPkinase 0.05 mg/ml; phenylalanine 0.04 mM; microsomal sonic extract (when present) 0.7 gm eq/ml. Ribosomes (0.2 mg of RNA) and poly U (50 μg) were pre-incubated for 30 min at 0°C in a volume of 0.05 ml in the presence of MgCl₂ (17 mM) and KCl (80 mM) and then mixed with the other components. Leloir. The GTP spots were eluted with water and plated for counting. The cpm are those actually recorded.

In one set of experiments C¹⁴-poly U (344 cpm/μg) and C¹⁴-phenylalanine were used. At the end of the incubation samples were chilled, layered over 7 ml of a solution containing sucrose (0.25 M), KCl (80 mM), and MgCl₂ (5 mM), and the ribosomes were spun down at 105,000 γ for 6 hr at 0°C. The pellet was rinsed several times with the same solution, resuspended in water, and plated directly. (Control experiments with radioactive compounds done for other purposes have shown no appreciable contamination of the pellet from supernatant material with this procedure.) In the other set of experiments C¹⁴-poly U and C¹⁴-phenylalanine (5 μc/μmole) were used, and TCA-insoluble material was obtained as described in Methods.

Incorporation of C¹⁴-phenylalanine in the absence of poly U (not shown) yielded at 20 min 150 cpm in the absence, and 130 and 115 cpm in the presence of regenerating or normal microsomal extracts, respectively. Although greater stimulation could be obtained with larger amounts of poly U the amount used in this experiment was limited by the availability of the radioactive material.)
regenerating sonic extracts. Figure 7 shows that the inhibition by either extract is not reflected in differences in the extent of poly U binding or the rate at which it disappears from the ribosomes during the incubation.

(3) The inhibitory factor would not appear to be derived from the blood contained in the liver since perfusion of the organ with cold isotonic medium through the portal vein before preparation of the factor does not alter its inhibitory activity. Also whole blood, dialyzed serum, and purified rat serum albumin lack inhibitory activity.

(4) Inhibition cannot be due to reduced release of nascent protein since 90 per cent of counts remain associated with ribosomes in these systems.

Discussion.—The existence of inhibitory elements in the membrane-ribosome complex strongly suggests a role in regulation of protein synthesis. The microsome fraction used probably represents a more physiological entity than isolated ribosomes. It is not only rich in membranes, but the method by which it is prepared minimizes damage. The high concentration of microsomes in the incubations also simulates conditions one would expect intracellularly.

The preparation of ribosomes from microsomes by the use of detergents is generally accompanied by an increase of activity. Hawtrey and Schirren, for example, have reported a trebling of activity on preparing ribosomes from microsomes by isooctane. They also noted that the membrane fraction so obtained inhibited the ribosomes, an observation perhaps related to those we report here.

We have shown that inhibition is due neither to reduced availability of GTP nor to altered binding of messenger (at least in the case of poly U). As previously reported, there are few or no differences between normal and regenerating systems in activity of amino acid activating enzymes, sRNA levels, and messenger-like RNA content.

As a working hypothesis, we favor the concept that the low rate of general protein synthesis in adult rat liver is maintained by inhibition and that regeneration involves at least in part the temporary and incomplete loss of this primary negative control.

Ever since the discovery that GTP played a role in protein synthesis in vitro, there has been a tacit assumption that it supplied energy for the process. There has never been a satisfactory theoretical or experimental justification for this assumption. We have presented evidence that GTP antagonizes the action of an inhibitor of protein synthesis in vitro. In the absence of any evidence for a role of GTP vis-à-vis protein synthesis in vivo, we suggest that it may have a role in regulating protein synthesis through its interaction with inhibitor. This might be accomplished by altering the molecular configuration of a protein, or class of proteins, in such a way as to inactivate their inhibitory function. Precedent for such a mechanism is provided by Frieden's observation that GTP and/or GDP dissociate glutamic dehydrogenase.7 (The possible relevance of this observation to our findings was first called to our attention by Dr. Mildred Cohn.) Implicit in this hypothesis is the notion that GTP is not involved on the direct path of protein synthesis, and that the extent to which GTP is required in various protein synthesizing systems in vitro is determined by the degree to which they are "contaminated" by natural inhibitory elements. The hypothesis is at least refreshing and should be tested experimentally. It is also to be noted that the experience of many workers in protein synthesis in vitro has brought no unanimity about the conditions necessary
to elicit a GTP effect; there seems to be no correlation between the degree of "purity" of a system and its GTP requirement. In this respect it is worth noting that Griffin\(^8\) has developed an incorporation system, highly active \textit{in vitro}, from a rapidly growing tumor of liver origin. He uses ribosomes twice cycled through deoxycholate and a pH 5 fraction twice reprecipitated and extensively dialyzed—and this system has little or no GTP requirement. Our experience with liver ribosomes is similar: more often than not GTP fails to stimulate their activity.

There is a growing body of indirect evidence that at least in some cases repressive control of protein synthesis may actually be exerted at the level of the ribosome.\(^*\)\(^{-11}\) The possibility that our inhibitor fraction may be a mixture of repressor substances is therefore to be considered. On the other hand, it would not be surprising if protein synthesis were subject to a separate general rate control, unrelated to specific repressive control of individual enzyme synthesis.

\textit{Summary.}—We have presented evidence that normal adult rat liver microsomes contain, in excess over their regenerating liver counterparts, a heat-labile factor(s) that inhibits amino acid incorporation on ribosomes. The factor may be isolated in soluble form by sonic disruption of microsomes and the bulk of its activity sediments in a polydisperse fashion on sucrose gradients. Its action is antagonized by GTP. It does not function by depleting GTP in the assay system, by destroying messenger, or interfering with its binding. It is suggested that protein synthesis in normal adult tissues may be under control of an inhibitory element(s), and that GTP may play a role in regulatory processes.

Abbreviations: PEP, phosphoenolpyruvate; ATP and GTP, the triphosphates of adenosine and guanosine, respectively; RNA, ribonucleic acid; poly U, polyuridylic acid; tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid.

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A preliminary report of this work was made at the International Symposium on the Mechanisms of Regulation of Cellular Activities in Microorganisms, Marseilles, 1963 (report now in press).


4 Hoagland, M. B., unpublished data.


8 Griffin, A. C., personal communication.


