Inhibitors of Protein Synthesis in Rat Liver Microsome Fractions*

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SUMMARY

Rat liver microsome fractions contain two general kinds of inhibitors of amino acid incorporation in vitro. The first is derived from lysosomes contaminating the microsome fractions. Lysosomes may be removed from rough vesicles (ribosomes plus endoplasmic membranes) by a density centrifugation technique.

Lysosomal inhibition of incorporation in vitro is much more prominent in normal than in regenerating liver microsomes even though the lysosome content is the same, suggesting that normal lysosomes release their degradative enzymes more readily during incubation. Rough vesicles from normal and regenerating livers are alike in activity, as are the polyribosomes derived therefrom.

The second inhibitor is a heat-labile factor associated with the endoplasmic reticular membrane, which requires oxidized glutathione under certain conditions and which can be reversed by an excess of guanosine triphosphate or sulfhydryl reagents. The inhibitor-sensitive step is the transfer of amino acids from aminoacyl soluble ribonucleic acid to protein.

Enzymes catalyzing the transfer of amino acids from soluble ribonucleic acid to protein are less than half as active (per mg of supernatant protein) in normal as in regenerating liver when tested at limiting concentration and in the absence of GTP. Their activity is equalized in the presence of an excess of GTP. The enzymes from normal liver also increase in activity upon aging at 0°C, to become more nearly equal in activity to the enzymes from regenerating liver.

Mechanisms controlling the rate of protein synthesis in animal cells are poorly understood. In growing bacteria it is known that the rate of protein synthesis depends to a large extent on the concentration of ribosomes in the cell (1). The problem is more difficult to study in animal cells and the mechanisms may well be more complex. A comparison of the rate of protein synthesis in normal and regenerating rat livers offers a model for the study of this problem. Higher levels of incorporation of radioactive amino acids have been reported in regenerating livers, both in vivo and by liver slices (2). Cell-free systems prepared from regenerating livers have also been found to incorporate amino acids more actively than those from normal liver, and differences in the activity of the two kinds of microsomes have been consistently reported (3–8).

In a previous publication (6) we described conditions for obtaining a large difference in the incorporating capacity of microsomes from normal and from regenerating liver when these were supplied with supernatant factors, adenosine triphosphate, and an ATP-generating system. Among these conditions, the use of “heavy microsomes” (sedimented from a 15,000 x g supernatant at 60,000 x g for 15 min) at a relatively high concentration and the avoidance of the usual addition of an excess of GTP to the incubation mixture were found to be important. When microsomes from normal and regenerating liver were mixed the incorporation was lower than could be expected from their separate activities, and this effect was more pronounced when GTP was absent. We postulated that an inhibitor of protein synthesis was associated with the microsomes, was more active in microsomes from normal liver, and was antagonized by GTP. Extracts obtained by ultrasonic treatment of microsome suspensions were indeed inhibitory, and inhibition was reduced when GTP was added in excess to the incubation mixture (6).

In this paper we present further evidence for the presence of inhibitory factors in microsomes. They can be accounted for, in part, by the presence in this fraction of contaminating amounts of lysosomes. We will describe a method for the preparation of rough vesicles largely free of lysosomes, and will show that the properties of these “pure microsomes” are quite different from those of crude microsomes. With the contaminating lysosomes eliminated, the reticuloendoplasmic membranes have been found to carry an inhibitor of the transfer of amino acids from aminoacyl-sRNA1 to ribosomes, the action of which is reversed by GTP and favored by oxidized glutathione.

EXPERIMENTAL PROCEDURE AND RESULTS

Methods

Animals and surgical procedures were the same as those previously described (6). Regenerating livers were obtained

1 The abbreviation used is: sRNA, soluble ribonucleic acid.
20 to 24 hours after partial hepatectomy. The animals were fasted during this time. (This period of fasting did not affect the activity of liver microsomes or ribosomes. Livers obtained 48 hours after partial hepatectomy gave essentially the same results.)

Incorporation studies with microsomes, pH 5 fraction (0.2 g eq per ml), ATP (1 mm), phosphoenolpyruvate (10 mm), P-enolpyruvate kinase (0.05 mg per ml), and 14C-leucine (0.02 mM, 0.1 μC) were identical with those previously described (6). GTP was used at a concentration of 0.4 mm unless otherwise stated. In the experiments described in “Section II” microsomes were replaced by polysomes prepared according to method of Wilson and Hoagland (9) at a final concentration of 2 mg of RNA per ml. In that case, liver supernatant was added together with the other components (0.1 ml/1.0 ml of incubation mixture). Liver supernatant was obtained from a homogenate of liver in 2 volumes of medium which was centrifuged first for 10 min at 15,000 × g and then for 2 hours at 100,000 × g. Supernatant from regenerating liver was used unless otherwise stated. It was used either directly or after passage through a column of Sephadex G-25 to remove small molecules (10 ml were placed on a column, 20 × 1.8 cm, and the first 10 ml of the eluted protein solution were collected). Supernatant was also used in the experiments of “Section III.” Unless otherwise stated it was used at a concentration of 0.1 ml per ml of incubation mixture (about 3 mg of protein).

In some experiments (cf. Fig. 6) pH 5 supernatant was used. This was prepared from the supernatant of the pH 5 precipitation step (6) by adjusting its pH to 7.4, lyophilizing, dissolving, in a small quantity of medium, and passing the concentrated supernatant through a Sephadex G-25 column as indicated above. The final concentration of protein was approximately 80 mg per ml.

Transfer RNA labeled with 14C-leucine was prepared according to method of Hoagland and Comly (10).

Protein was precipitated with hot trichloroacetic acid, washed with lipid solvents, and counted as previously described (6).

Inhibitory extracts were obtained by treatment of the different fractions with ultrasonic vibrations for 5 min (6). Generally these suspensions were centrifuged for 1 hour at 100,000 × g and the supernatant was used. In the experiments where it was desirable to determine total inhibitory capacity of normal and regenerating microsomes (as described in “Section III” and also in the experiment shown in Fig. 2) the sonically treated suspension was added directly to the assay mixture, without prior centrifugation.

In expressing the inhibitory activity a unit is defined as the amount of the extract reducing activity of the assay system (with microsomes, in a final volume of 0.5 ml) by 50%. This was calculated by interpolation in a graph similar to those of Fig. 2B. As described at the end of “Section I,” it was found that the lysosomal inhibitor (not reversed by GTP) and the reticuloendothelial inhibitor (completely reversed by GTP) act independently when present together. Accordingly, the relative participation of each component in extracts was calculated by difference, after assaying extracts simultaneously with or without GTP.

Succinic dehydrogenase activity was used as a marker for mitochondria, and was assayed according to method of Hirsch et al. (11). Results are expressed in micromoles of 2-iodophenyl-3-nitrophenyl-5-phenyltetrazolium chloride reduced by sodium succinate per min per mg of protein. Acid phosphatase was used as a marker for lysosomes, and was assayed according to Shibko and Tappel (12). Results are expressed as millimicromoles of phosphate released from β-glycerophosphate per min per mg of protein. Glucose 6-phosphatase, as a marker for reticuloendothelial membranes, was assayed according to De Duve et al. (13). Results are expressed as for acid phosphatase. RNA of ribosomes was measured by direct reading at 260 μm with a correction for ferritin contamination according to Wilson and Hoagland (9). RNA in microsomes was assayed according to method of Scott, Fraccastoro, and Taft (14). Protein was measured by the method of Lowry et al. (15) with bovine serum albumin as a standard. Phospholipid was extracted according to Marinetti, Erbland, and Stotz (16), digested with concentrated H2SO4 at 130° overnight. The residues were bleached with H2O2 for 30 min at 110°. Pyrophosphate was then hydrolyzed in boiling water and the phosphorus was determined by the method of Fiske and SubbaRow (17). Results are expressed as micromoles of phospholipid phosphorus.

I. Inhibitory Factors in Microsomes

Inhibition by Lysosomal Factors—It was previously found that ultrasonic treatment of crude microsomal preparations yielded extracts inhibitory to protein synthesis (6). It was important to ascertain if this property was truly characteristic of the microsomes (envisioned as endoplasmic reticular membrane with associated ribosomes, i.e. rough vesicles), as opposed to other particulate fractions. To determine this, a homogenate of normal liver was centrifuged at 750 × g for 10 min. The pellet, presumably containing nuclei, unbroken cells, and cell debris, was discarded. From the supernatant five fractions were arbitrarily obtained by differential centrifugation. Succinic dehydrogenase and acid phosphatase activities (as markers for mitochondria and lysosomes, respectively), RNA to protein ratios, and the ability of ultrasonic extracts of the fractions to inhibit protein synthesis were measured in each of the fractions. The results are shown in Fig. 1A. As expected, fractions sedimenting at lower speeds were rich in mitochondria and lysosomes, while subsequently sedimenting fractions showed higher RNA to protein ratios. Surprisingly, however, total inhibitory activity was associated with the heavier fractions. An aliquot of the heaviest of these fractions (Fraction 1) was subjected to further analysis by density equilibrium centrifugation in a sucrose gradient. The results are shown in Fig. 1B. Clearly the distribution of the inhibitory activity followed the distribution of the lysosomes and not that of the mitochondria. Further evidence on the association of inhibitory activity and lysosomes will be presented below.

Separation of Lysosomes and Rough Vesicles—Resolution of the inhibitory activity of crude microsomes was accomplished by experiments of the kind shown in Fig. 2A. In this experiment a suspension of crude microsomes was subjected to density equilibration on a sucrose gradient. As expected, fractions nearer the bottom of the tube have a high RNA to protein ratio due to the presence of the dense ribosomes. When the ability of each fraction to inhibit protein synthesis in vitro was tested in the presence of GTP the distribution followed that of the acid phosphatase activity. In the absence of GTP, however, the distribution was quite different (Fig. 1A, the unshaded area, as compared to the total area). In the fractions collected from the dense region of the gradient, enriched for rough vesicles, in-
most of the lysosomes were sedimented at 15,000 $\times$ g for 10 min.

2 volumes of 0.25 $\text{M}$ sucrose medium. Nuclei and cell debris were sedimented at 750 $\times$ g for 10 min. The pellet was discarded. The supernatant solution was centrifuged twice for 10 min at 5,000 $\times$ g, the pellets were pooled, suspended in a full tube volume of 0.25 $\text{M}$ sucrose medium, and resedimented at the same speed. This was designated Fraction 1. Fractions 2 to 5 were successively obtained at increasing speeds in the same way: for 10 min at 15,000 $\times$ g; 20 min at 27,000 $\times$ g; 15 min at 60,000 $\times$ g; and 1 hour at 100,000 $\times$ g. The two last fractions were centrifuged in the Spinco rotor No. 40; the others in the SS-34 rotor of the Servall RC2 refrigerated centrifuge. In a preparation from 56 g of liver the fractions contained the following amounts of protein: 1, 99 mg; 2, 31 mg; 3, 80 mg; 4, 114 mg; and 5, 50 mg.

Supernatant fractions of sonic extracts, assayed in the absence of GTP, were the source of inhibitor. B, distribution of succinic dehydrogenase, acid phosphatase, and total inhibitory activity (inhib.) in a density equilibrium centrifugation as described in Fig. 1B. Inhibitory activity was assayed in the absence of GTP (total area) or in its presence (shaded area). Black area therefore represents distribution of the GTP-reversible inhibition. B, inhibitory activity of Fractions 1 (upper) and 8 (lower) (indicated in the gradient of A with arrows) in the absence ($\bullet$) and presence (○) of GTP. Assay was with regenerating microsomes, in 0.5-ml incubation mixture, as described in "Methods." The incorporation of the system in the absence of added inhibitors is 100%.

Solid sucrose was dissolved in the supernatant (on ice and with continuous stirring) up to a final concentration of 1.53 M (density, 1.20 at room temperature). The suspension was then centrifuged at 160,000 $\times$ g for 1 hour. Material with a density lower than 1.20 rose to the surface forming a well packed pellicle that could easily be removed with a spatula. The suspension of rough vesicles, the density of which is that of the sucrose, was then diluted with 1 volume of sucrose-free medium, and the particles were sedimented from it at 160,000 $\times$ g for 1 hour. The RNA, protein, and acid phosphatase content of these particles are compared in Table I with those of the usual preparation of crude microsomes sedimented for 1 hour at 160,000 $\times$ g from the original 15,000 $\times$ g supernatant. The recovery was usually from 75 to 90% of the RNA (crude microsomes considered as 100%) in this purified fraction, with an RNA to protein ratio of 0.18 to 0.22.

The action of inhibitor prepared by sonic disruption of this purified fraction is more readily reversed by GTP than the activity of that prepared from crude microsomes (similar to Fig. 1B). As will be shown in a later section of this paper, the ability of this preparation to incorporate amino acids into protein is strikingly different from that of the crude microsomes.

Membrane Localization of GTP-reversible Inhibitor--We wanted to establish whether inhibitor in these purified microsomes (rough vesicles) was associated with the ribosomes or...
TABLE I
Recovery of RNA, protein, and acid phosphatase in crude and purified microsomes from normal and regenerating livers

Explanations in the text.

<table>
<thead>
<tr>
<th></th>
<th>RNA (mg/g liver)</th>
<th>Protein (mg/g liver)</th>
<th>Acid phosphatase (mole P/min/g liver)</th>
<th>Ratio of RNA to Protein</th>
<th>Ratio of Acid Phosphatase to Protein</th>
<th>Ratio of Acid Phosphatase to RNA</th>
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</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
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<tr>
<td>Crude</td>
<td>2.60</td>
<td>22.8</td>
<td>820</td>
<td>0.115</td>
<td>36.2</td>
<td>316</td>
</tr>
<tr>
<td>Purified</td>
<td>2.16</td>
<td>11.6</td>
<td>154</td>
<td>0.185</td>
<td>11.0</td>
<td>60</td>
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<td>Regenerating</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Crude</td>
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<td>18.6</td>
<td>670</td>
<td>0.14</td>
<td>36.2</td>
<td>264</td>
</tr>
<tr>
<td>Purified</td>
<td>2.26</td>
<td>10.3</td>
<td>129</td>
<td>0.22</td>
<td>14.9</td>
<td>68</td>
</tr>
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</table>

**TABLE II**
Evidence for association of GTP-reversible inhibitor with membranes, but not ribosomes, of purified rough vesicles

In Experiment 1, microsomes (6 mg of RNA per ml) were suspended in 1.3% deoxycholate. Ribosomes were sedimented for 2½ hours at 105,000 X g. Aliquots of the original suspension, the deoxycholate supernatant, and the resuspended ribosomes were dialyzed against Tris buffer (0.1 M, pH 7.7, at 0°) for 36 hours. Each fraction was then sonically disrupted and assayed for the components indicated.

In Experiment 2, a suspension of rough vesicles was sedimented in a 10 to 35% sucrose gradient for 2½ hours in an SW-25 Spinco rotor. Tubes containing the small amount of material (about 0.15 mg of RNA per g of liver) that sedimented in a position corresponding to free ribosomes and dimers were pooled and sedimented for 2 hours at 105,000 X g. The pellet was resuspended and sonically disrupted.

In Experiment 3, 0.5 ml of a water suspension of purified microsomes (12 mg of protein, 2.6 mg of RNA per ml) was exposed to 0.5 M EDTA (pH 7.0) at 0° for 1 hour. The suspension was then diluted 3-fold, layered on 20 ml of 0.5 M sucrose, and centrifuged for 2 hours at 25,000 rpm in a Spinco rotor SW-25. The pellet containing membranes and dissociated ribosomes was suspended in water, layered on 1.5 M sucrose, and centrifuged for 2 hours at 39,000 rpm in a Spinco rotor SW-39. Material accumulated at the interface (purified membranes) was collected and sonically disrupted.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>RNA</th>
<th>Phospholipid phosphorus</th>
<th>Inhibitor</th>
</tr>
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<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Original suspension</td>
<td>16.2</td>
<td>6.0</td>
<td>5.1</td>
<td>42</td>
</tr>
<tr>
<td>Ribosomes</td>
<td>6</td>
<td>5</td>
<td>0.51</td>
<td>8.5</td>
</tr>
<tr>
<td>Deoxycholate supernatant</td>
<td>8.5</td>
<td>5.1</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribosomes</td>
<td></td>
<td></td>
<td>0.22</td>
<td>0.27</td>
</tr>
<tr>
<td>Rough vesicles</td>
<td></td>
<td></td>
<td>0.98</td>
<td>3.42</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Original rough vesicles</td>
<td>0.22</td>
<td>0.28</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>EDTA membranes</td>
<td>0.044</td>
<td>0.25</td>
<td>4.2</td>
<td></td>
</tr>
</tbody>
</table>

* For Experiment 1, results are given in milligrams per ml.
* For Experiment 1, results are given in milligrams per ml; for Experiment 3, in milligrams per mg of protein.
* For Experiment 1, results are given in micromoles per ml; for Experiment 2, in micromoles per mg of RNA; for Experiment 3, in micromoles per mg of protein.
* For Experiment 1, results are given in units per ml; for Experiment 2, in units per mg of RNA; for Experiment 3, in units per μmole of phospholipid phosphorus.

with the membrane. For this purpose three separate kinds of experiments, shown in Table II, were performed. In the first, membranes and ribosomes were separated by deoxycholate treatment. While most of the RNA of the original suspension was recovered in the ribosomes, the bulk of the phospholipid and inhibitor appeared in the deoxycholate supernatant (Table II, Experiment 1).

In the second approach a suspension of rough vesicles was sedimented in a conventional sucrose gradient thereby separating free ribosomes from membrane-associated ribosomes. The latter are sedimented to the bottom of the tube. Fractions containing the smaller components were pooled and sedimented for 2 hours at 105,000 X g. The RNA to protein ratio of the free ribosomes was 1.06. Table II, Experiment 2, shows that the phospholipid and inhibitor content of these ribosomes was low compared to the rough vesicle fraction.

Lastly, membranes relatively free from ribosomes were prepared from purified microsomes (rough vesicles) by the action of EDTA, which at relatively high concentrations is known to detach ribosomes from the membrane and dissociate them into subunits (19). The distribution of inhibitor and phospholipid in these purified membranes is shown in Table II, Experiment 3. It is seen that all of the inhibitory activity is retained in the purified membranes.

From these three independent lines of evidence it was concluded that the inhibitor is associated with the membranes rather than with the ribosomes of the rough vesicles.

**Some Properties of GTP-reversible Inhibitor**—The inhibitory activity seems to be very firmly associated with the membranes. Attempts to release it with the following various treatments failed: sodium citrate, 0.025 M, pH 3 and pH 5; sodium phosphate buffer, 0.025 M, pH 7; sodium bicarbonate, 0.025 M, pH 9; NaCl, 0.15 M, alone or in combination with each of the previous buffers, or with CaCl₂, 0.01 M; and EDTA, pH 7.0, at various concentrations between 0.005 and 0.5 M. With carbonate buffer, 0.025 M, pH 10.7, about half of the protein and inhibitory activity of the purified microsomes became nonsedimentable (100,000 X g, 1 hour) but both reprecipitated upon dialysis against 0.1 M Tris, pH 7.5.

Inhibitory activity was heat-labile; 50% inhibition was reached in 10 min at 60°. Inhibitor preparations were quite stable after long storage at -20°.

Preparations of red cell membranes (devoid of reticuloendothelial components), obtained by lysis of red cells in diluted phosphate buffer as described by Dodge, Mitchell, and Hanahan (20) followed by sonic disruption, were not inhibitory.
II. Mode of Action of GTP-reversible Inhibitor

In an attempt to elucidate the mode of action of the GTP-reversible inhibitor an assay system consisting of liver polysomes, pH 5 fraction, and crude supernatant was used as indicated in "Methods." Supernatant from sonically treated purified microsomes was used as a source of inhibitor.

Effect of Oxidized Glutathione—It was first found that the addition of 2-mercaptoethanol or reduced glutathione at concentrations higher than 1 mM prevented the inhibition. When they were added to the incubation mixture after inhibition was established the system was fully reactivated (Fig. 3).

When the supernatant used in these experiments had been dialyzed or passed through a Sephadex G-25 column (see "Methods"), the system still incorporated amino acids actively but was no longer inhibitable. Response to inhibitor was restored by addition of an equivalent amount of boiled supernatant. It was concluded that a small thermostable substance was associated with an oxidative process, because of the dramatic effect of 2-mercaptoethanol referred to above. Attempts to replace the boiled supernatant with various concentrations of DPN, DPNH, TPN, or TPNH were unsuccessful. Restoration of the inhibition could be obtained, however, with low concentrations of GSH, but at concentrations higher than 0.05 mM, GSH tended to counteract the inhibition. Oxidized glutathione on the other hand, was as effective as GSH at low concentrations but supported the inhibition at higher concentrations with an optimum about 0.1 mM. Also, when crude supernatant or supernatant passed through Sephadex G-25 was used (together with a low concentration of GSH), inhibition was attained with a lag (see Fig. 3, Curve 3). When GSSG was used no lag occurred, as will be seen in Fig. 4A, Curve 4.

It was thus speculated that GSSG was the active adjuvant for inhibitor action and that GSH functioned after being oxidized to GSSG. This view was supported by experiments of the kind shown in Table III. It can be seen in this table that GSH ceased to be effective when air was replaced by nitrogen in the system. GSSG was just as effective in nitrogen as in air.

Inhibition of Transfer Reaction—The experiment shown in Fig. 4 clearly establishes that the step in protein synthesis affected by the GTP-reversible inhibitor is the transfer of amino acids from adaptor to peptide linkage. In Fig. 4A, the kinetics of inhibition of the over-all system (free amino acid to protein) are shown. Inhibition is linear from time zero, dependent on GSSG, and (not shown) completely reversed by GTP. In Fig. 4B, the kinetics of incorporation from prelabeled pH 5 fraction (transfer RNA) to protein are shown in an otherwise identical system. The absence of a lag is, of course, characteristic of this system, since the delay in aminoacylation of transfer RNA has been circumvented, but inhibition shows the same kinetics as in the over-all system. As a control to show that only the transfer reaction is being measured, Fig. 4B, Curve 5 shows that an excess of unlabeled amino acid has no effect on incorporation, in contrast to its effect when the reaction starts with free amino acid and ATP (Fig. 4A, Curve 5).

In an attempt to delineate further the inhibitable system, purified 3H-aminoacyl transfer RNA, pH 5 supernatant fraction, and polynucleosomes were used. It was found that about the same degree of inhibition was obtained with this system as with cruder systems. A peculiarity of the inhibition of the transfer system became obvious when the concentration of pH 5 supernatant fraction was varied (Fig. 5). At relatively high concentration of enzyme extract, inhibition was, as expected, GSSG-dependent. As the amount of enzyme extract was reduced, however, substantial inhibition could be obtained in the absence of GSSG. Inhibition remained fully reversible by GTP, regardless of the GSEC requirement. This apparently gratuitous nature of the GSSG effect remains to be explained, and is a subject of current study.

The role of GTP in the reversal of inhibition has attracted our attention for some time. We early considered the possibility that inhibitor might destroy GTP, thereby increasing the

### Table III

<table>
<thead>
<tr>
<th>Addition</th>
<th>Activity in</th>
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<td></td>
<td>Air</td>
</tr>
<tr>
<td>None</td>
<td>80</td>
</tr>
<tr>
<td>GSH, 0.05 mM</td>
<td>49</td>
</tr>
<tr>
<td>GSSG, 0.10 mM</td>
<td>24</td>
</tr>
</tbody>
</table>
FIG. 4. Comparison of the effects of inhibitor and GSSG upon the over-all incorporation system (A), and the transfer reaction (B), with the use of polysomes, pH 5 fraction, and Sephadex-treated supernatant. The two systems are identical in all components, except that in A pH 5 fraction was preincubated with 1C-leucine, 1C-valine, and ATP, and the final incubation was carried out with 1C-leucine and 1C-valine, while in B pH 5 fraction was preincubated with 1C-leucine, 1C-valine, and ATP and the final incubation was carried out with 1C-leucine and 1C-valine. In both cases, the preincubated pH 5 fraction was reprecipitated twice before use as previously described (4). Curves 1, controls; Curves 2 and 3 with either GSSG (0.1 mM) or inhibitor (0.1 ml/1.0 ml of incubation mixture); Curves 4 with both GSSG and inhibitor; and Curves 5, controls plus an excess of 1C-valine (0.4 mM). The final concentrations of all components of the reaction mixture were the same as indicated in "Methods."

requirement for it (4). A more recent experiment, with 1C-labeled GTP (0.004 mM, a concentration that does not reverse inhibition) inhibitor derived from purified microsomes, and more precise techniques for isolating labeled GTP (thin layer chromatography), confirmed our earlier observation that GTP destruction is not a feature of inhibitor action.

Another possibility is that inhibitor induces an irreversible binding of GTP to some component of the reaction mixture, thereby decreasing its availability. This might not be detected by an experiment like the above since bound GTP might be released by the trichloracetic acid treatment preceding the chromatographic isolation of the nucleotide. Therefore, an experiment was performed in which the complete incorporation system, after incubation with 1C-GTP, was passed through a column of Sephadex G-25. Any GTP bound to protein (or nucleic acid) would be expected to appear in the front. It was clear that the presence of inhibitor, or GSSG, or both did not influence the extent of binding of GTP, which in any case was of slight degree (~5%).

It would appear that inhibitor interferes directly with an enzymatic step in the transfer of amino acid from sRNA to peptide linkage. One of these steps is known to require GTP (21–24). The limitation of a transfer enzyme utilizing GTP might be expected to increase the requirement for GTP. This situation may be readily simulated in the transfer system by reducing the quantity of enzyme extract and determining the effect on the requirement for GTP. Fig. 6 shows that the degree of stimulation of the system by GTP does indeed rise sharply as the quantity of enzyme is reduced. The enzyme extract used in this particular experiment was passed twice through Sephadex G-25 to ensure complete removal of traces of GTP. We therefore assume that GTP allows the inhibited system more effectively to utilize its residual enzymic capacity. Consistent with this interpretation is the fact that, after inhibition has been established, the addition of either fresh supernatant or 2-mercaptoethanol fully restores the activity of the system; a similar late addition of GTP is progressively less effective with time.

III. Differences between Incorporation Systems from Normal and Regenerating Livers

Microsomes As previously described, crude preparations of microsomes from regenerating liver incorporate at a higher rate and for a longer period than normal ones. These differences in activity are exaggerated in the absence of added GTP (Fig. 7A). We have already presented evidence for the existence of a GTP-reversible inhibitor associated with the microsomal membrane. This could account for differences in GTP stimulation if we further postulated that normal membranes contained more inhibitory capacity. The following experiment showed this not to be so. Three preparations of microsomes from normal liver and three from regenerating liver were suspended at a...
concentration of 10 mg of protein per ml and sonically disrupted for 6 min. This treatment abolished the ability of microsomes to incorporate amino acids and they could thus be added directly to the assay system to measure their inhibitor content in the presence and absence of GTP.2 The inhibitory capacity of the reticuloendoplasmic membranes was calculated by difference to be (in units per reticuloendoplasmic membranes was calculated by difference to be (in units per

3In order to calculate the relative contribution of the lysosomal and reticuloendoplasmic components on the basis of the differential GTP effect it was necessary first to know if, when present simultaneously, they did not interfere with or potentiate each other. Such noninterference or potentiation could be shown by assaying a sonic extract of a pellet, rich in lysosomes but poor in microsomes, as in Fig. 1B, Fraction 1, and a similar extract from rough vesicles, in the presence and absence of GTP, separately and mixed in the same assay tube. The inhibitory activity of the mixture in the absence and presence of GTP was calculated to be 96 and 92%, respectively, of the sum of their activities when assayed separately.

in “Section I,” and the suspension of rough vesicles was diluted with 1 volume of sucrose-free medium, 5 ml of this diluted suspension was mixed with 0.75 ml of sodium deoxycholate (10%), layered on 5 ml of 1.0 M sucrose medium, and sedimented for 3 hours at 160,000 × g. The pellet contained 80 to 100% of the RNA of the original rough vesicles. Its RNA to protein ratio varied between 0.65 and 0.75. The capacities to incorporate amino acids of these ribosomes from normal and regenerating livers were equal as in the case of the purified rough vesicles (Fig. 7C).

Purification of the rough vesicles consists in the removal of the pellicle, rich in lysosomes, which rises to the surface in 1.53 M sucrose. The effect of this treatment on the incorporating ability of the vesicles is, most strikingly, that microsomes from normal liver continue to incorporate for a longer time, more like microsomes from regenerating liver. If the particles constituting the pellicle are added back to the rough vesicles, the original properties of the crude microsomes of normal liver are restored.

Particles removed in the pellicle seem then to offer an explanation for a good part of the differences between normal and regenerating crude microsomes.

Supernatant Fraction—It was considered possible that some of the differences in activity of normal and regenerating crude unwashed microsomes might also be due to differences in the activity of contaminating soluble enzymes. Crude microsomes preparations washed by resuspension and resedimentation, tested in the presence of an excess of pH 5 fraction, did indeed have an additional requirement for whole supernatant, indicating that transfer enzymes were limiting in the system. When freshly prepared whole supernatant fractions were assayed with washed microsomes and pH 5 fraction in the over-all incorporation system, the fractions from regenerating liver were consistently about twice as active as those from normal liver. This

4The effect of supernatant concentration upon the requirement for GTP in the transfer of amino acid from sRNA to polysome. Conditions of the incubation were similar to those in Fig. 5. Curve 1, no added GTP; Curve 2, 0.005 mM GTP; Curve 3, 0.01 mM GTP.

5Finally, and more important, when purified microsomes from normal and regenerating livers were prepared as described in “Section I,” their ability to incorporate amino acids was very similar in several experiments, one of which is shown in Fig. 7B. Ribosomes were then prepared from these purified microsomes in the following way. After the pellicle was removed, as described

Fig. 6. The effect of supernatant concentration upon the requirement for GTP in the transfer of amino acid from sRNA to polysome. Conditions of the incubation were similar to those in Fig. 5. Curve 1, no added GTP; Curve 2, 0.005 mM GTP; Curve 3, 0.01 mM GTP.

Fig. 7. Time course of the incorporation of leucine, in the absence (solid line) and presence (dashed line) of GTP in the absence of pH 5 fraction, ATP, and an ATP-generating system of A, crude normal (N) and regenerating (R) microsomes; B, purified rough vesicles from normal and regenerating livers; and C, ribosomes prepared by deoxycholate treatment of the same preparations as described in the text. In B and C, crude regenerating supernatant (0.1 ml per ml of incubation mixture) was added. Microsomes, rough vesicles, and ribosomes were incubated at a concentration of 0.6 mg of RNA per ml. Results are expressed in counts per min incorporated per mg of (microsomal or ribosomal) RNA. A is a representative single experiment while B and C are the average and range (bars) of a representative experiment done in triplicate, i.e., purified microsomes and ribosomes were prepared simultaneously from three different livers and assayed independently at the same time.
Fig. 8. Comparison of the activity of normal and regenerating liver supernatant fractions in the transfer of amino acids from sRNA to protein. Incubation conditions were as described in Fig. 5 except that fresh supernatant solutions replaced the pH 5 supernatant. The supernatant fractions were prepared, as described in “Methods,” just before the experiment, from the livers of three normal rats △—△; three sham-operated rats, △—△; and three partially hepatectomized rats, O—O. Each point represents the averaged activity of supernatants from three animals assayed separately, and the range in activity is indicated by the bars. Incubations were for a period of 5 min at 37º. The average activity of three supernatant solutions from the livers of normal rats (V), sham-operated rats (V), and partially hepatectomized rats, O—O. Each point represents the averaged activity of supernatants from three animals assayed separately, and the range in activity is indicated by the bars. Incubations were for a period of 5 min at 37º. The average activity of three supernatant solutions from the livers of normal rats (V), sham-operated rats (V), and partially hepatectomized rats (●) in the presence of GTP is also shown.

encouraged us to examine the effect of similarly freshly prepared supernatant fractions upon the transfer of amino acids from sRNA to ribosomes. A large number of supernatant fractions, separately prepared from normal animals, sham-operated animals, and animals with regenerating livers, were assayed with polysomes and leucyl-sRNA in a transfer system similar to that described in Fig. 5. Short incubations were used to ensure linear kinetics. Also in each assay concentration curves of supernatant fractions were made to ensure that transfer enzymes were limiting. A typical experiment is shown in Fig. 8. In four such experiments, the activity of supernatants from the sham operated animals exceeded that of normal supernatants by 10% (12 animals, range -14 to 32%). There were no differences in activity, however, in the presence of excess supernatant or in the presence of limiting supernatant plus GTP, as shown in Fig. 8.

During the course of these experiments occasional supernatant fractions showed no such differences. This apparently anomalous result was traced to the fact that these preparations had invariably stood on ice several hours before they had been assayed. The possibility that aging might reduce differences in enzyme activity was tested more directly by performing assays as described above on fresh supernatants, allowing them to stand for 3 to 4 hours, and reassaying them. As anticipated the activities had approached equality after aging; in six separately assayed supernatant fractions those from the sham-operated animals exceeded the normal by 20% (range 16 to 25%), while the regenerating exceeded the normal by 63% (range 59 to 68%). In all cases the reduction of the difference was due to an increase in the activity of supernatants from normal and sham-operated animals while supernatants from regenerating livers retained their original activity.

We conclude that transfer enzyme capacity is more limited in normal than in regenerating liver and that this might account for some of the differences in activity of crude microsome preparations and for part of the differential GTP effect.

Thus it would appear that differences in activity of fresh preparations of microsomes from normal and regenerating livers in vitro can largely be accounted for by (a) release of lysosomal components and (b) altered transfer enzyme capacity. The latter may be due to the GTP-reversible inhibitor.

DISCUSSION

Our earlier studies on the differential incorporating capacity, GTP requirement, and behavior of mixtures of normal and regenerating rat liver microsomes that led us to postulate the existence of a GTP reversible, membrane-associated inhibitor have been amply confirmed, and some of the properties of the inhibitor elucidated. We have shown that the inhibitor is firmly associated with endoplasmic membranes, that it inhibits the transfer of amino acids from aminocacyl-sRNA to nascent protein on polysomes, that it requires, under certain conditions, oxidized glutathione, and that its action can be completely reversed by reduced glutathione, mercaptoethanol, or GTP. Inhibition does not seem to be a property of all membranes, since red cell ghosts were devoid of such activity. Finally, inhibition does not involve destruction of GTP or binding of GTP to macromolecular components of the system in vitro.

The only evidence we can offer at this time that the GTP-reversible inhibitor plays a role in regeneration is circumstantial; the reactions catalyzing the transfer of amino acid from sRNA to protein are substantially less active in fresh extracts of normal liver than in those of regenerating liver; and normal transfer capacity is less than its potential (i.e. inhibited) because it increases on aging. GTP stimulates the normal transfer reaction so that it is nearly equal to the regenerating.

Numerous recent reports in the literature offer evidence for mechanisms controlling the rate of protein synthesis at the polysome level itself (cf. review by Spirin (25)). Frequently a protein...
factor has been implicated, the intimate association of which with the lysosome suggests a regulatory role. Whether our GTP-reversible inhibitor is involved in such a protein synthetic rate-controlling process remains a subject for further study.

In addition to the GTP-reversible inhibitor of the reticuloendothelial lysosome-rich pellicle, another inhibitory activity, not affected by GTP, was found associated with the lysosomes contaminating the crude microsome fraction. We know nothing about the nature of this inhibition (except that it is largely thermolabile), but we assume that it is the result of the activity of one or more of the many destructive enzymes known to reside in these particles (26).

Rough vesicles largely freed of lysosomes, from either normal or regenerating livers, incorporate equally well, as do the polyribosomes extracted therefrom by deoxycholate. The early shutoff of incorporation characteristic of crude microsomes from normal liver is not observed with purified microsomes but can be restored by adding back the lysosome-rich pellicle.

We do not know in what way contaminating lysosomes in normal and regenerating microsomes differ. The total concentration of lysosomes, as measured by acid phosphatase, seems essentially the same (Table 1). But differences could be the result of activity or fragility. The finding by McCorquodale, Veach, and Mueller (27) that the ability of the 15,000 × g supernatant from normal liver to incorporate amino acids is more sensitive to low pH values than that from regenerating liver, fits with the present assumption, in that lysosomal enzymes are known to have an acid optimum pH (26).

If the difference in crude microsomes from normal and regenerating livers is largely accounted for by lysosomes, the differential effect of GTP requires further explanation. Part of the explanation would appear to lie in the fact that crude microsomes contain whole supernatant which, as we have shown, differs in activity in normal and regenerating liver; and this difference is abolished by GTP (Fig. 8). Another reason is intrinsic in the kinetics of the incorporation reaction. GTP stimulates the initial rate of incorporation until the system shuts off earlier because of other factors (most likely completion of chains, since we estimate that these systems incorporate 30 to 60 amino acids per ribosome, and there is negligible release of nascent protein). Lysosomes cause an early shutoff of synthesis well before the synthetic potential of the polysomes in the preparation is realized. Thus the regenerating system is able to reach the maximum permissible level of incorporation, in the absence of GTP, more readily than the normal, thereby making the degree of stimulation by GTP appear smaller. This is shown schematically in Fig. 9 (Curves 1 and 2). Lysosomes cause an early shutoff in either the absence or the presence of GTP (Curves 3 and 4); but because of the kinetics of the reaction, the effect of this early shutoff on the final incorporation is more dramatic in the absence (Curve 3) than in the presence (Curve 4) of GTP.

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