*ASPECTS OF CONTROL OF PROTEIN SYNTHESIS IN NORMAL AND REGENERATING RAT LIVER, I. A CYTOPLASMIC RNA-CONTAINING FRACTION THAT STIMULATES AMINO ACID INCORPORATION**

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The discovery of messenger RNA and the concept of control of protein synthesis by repression have laid the groundwork for understanding the chemistry of information transfer and its control in bacteria.[1, 2] The present work was initiated to determine whether such mechanisms operated in higher organisms and whether they could be studied in cell-free preparations.

We chose to examine certain properties of the cell-free protein-synthesizing system of rat liver as an approach to these problems. The ability of the liver to regenerate in response to partial hepatectomy supplies a valuable comparative system: the heightened protein synthetic activity of the regenerating liver may be compared with that characteristic of the normal adult state with respect to the occurrence of messenger RNA and possible cytoplasmic and nuclear control mechanisms.

The present paper is a more definitive account of work previously reported.[3] A number of reports have since appeared of the occurrence in animal cells of a nuclear RNA fraction having turnover and sedimentation properties similar to those of bacterial messenger RNA (see *Discussion*). The studies recounted here indicate that liver cytoplasm contains a fraction having certain properties in common with messenger RNA including the ability to stimulate the *in vitro* amino acid incorporation system. The paper to follow presents evidence that microsomes from normal liver are intrinsically less responsive to the messenger-like fraction (and to other manipulations of their *in vitro* environment) than are those from regenerating liver. This difference in microsomal responsiveness appears to be due to the presence in the normal system of a dissociable inhibitor which renders microsomes from either normal or regenerating liver less active *in vitro*.

Materials and Methods.—Female Sprague-Dawley rats (Charles River Breeding Company), weighing 250–300 gm, were used for the experiments. Partial hepatectomies, removing approximately 65 per cent of the liver, were performed according to the technique of Higgins and Anderson.[4] The animals were killed by decapitation 20 to 24 hr later, and the livers were excised and immediately chilled. Normal rats of the same stock and weight were used in comparative studies. All animals were deprived of food for 20 to 24 hr before killing.

Preparation of cytoplasmic fractions: The livers were homogenized in two volumes of a medium containing: tris (hydroxymethyl) amino methane (Tris) buffer (pH 7.3) 0.1 *M* · $MgCl_2$ 0.005 *M;* sucrose 0.15 *M;* KCl 0.0025 *M;* and 3-mercaptoethanol 0.005 *M*. Microsomes (M) were prepared from a 15,000 gm supernatant fraction by centrifugation at 100,000 *g* for 1 hr. The microsomal supernatant fraction was then further centrifuged at 105,000 *g* in the Spinco No. 40 rotor for 12–13 hr. The resulting pellet, after suspension in medium, is referred to as the X fraction. The supernatant solution from this centrifugation was used to prepare a pH 5 fraction by diluting it with an equal volume of water, adding 1 *N* acetic acid to pH 5.2, collecting the resultant precipitate, suspending it in distilled water, and, after recentrifugation, dissolving it in medium. This fraction is referred to as the pH 5(−X) fraction; it differs from the usual pH 5 fraction in that it lacks X. All operations were performed in the cold. All three fractions were stored at −70°C, the M and X fractions as pellets and the pH 5(−X) redissolved in medium. Under these con-

ditions, they retained their activity for at least 2–3 weeks. All quantities of fractions are expressed as gram equivalents (gm eq): i.e., the wet weight of liver from which the particular fraction was derived. In our hands, fractions from equal weights of normal and regenerating liver were equal in RNA content (within 15 per cent). With all fractions, the subscripts N and R, following their abbreviated designation, refer to the source—normal or regenerating liver. The standard assay system used throughout these experiments contained half the concentration of the medium components given above except for KCl, whose final concentration was 0.07 *M*. The following added components were present in a total volume of 1.0 ml: 0.15 gm eq *M;* 0.2 gm eq pH 5(−X); 10 μmoles of phosphoenolpyruvate; 0.5 μmoles of GTP; 0.5 μmoles of ATP; 50 μgrams of pyruvate kinase; and 0.025 μmoles of C^{14}-leucine containing from 8 to 10 × 10^4 cpm. The excess gm eq of pH 5(−X) fraction was found to be adequate to insure that this fraction was not limiting. Incubations were carried out for 30 min at 37°C, and the reaction was stopped by addition of 5 ml of 4 per cent trichloracetic acid. Proteins were washed with hot trichloracetic acid and lipid solvents,[5] plated on aluminum planchets from ether, dried, and counted in a Nuclear Micromil window gas flow counter. In experiments where *in vivo* P^{32} labeling of the RNA of the above fractions was determined, the P^{32} (200 μc) was injected intraperitoneally at various times before killing. The RNA from the fractions obtained was extracted and counted essentially as described by Hecht *et al.*[6]

Fractionating and extraction techniques: A DEAE-Sephadex column was used to remove soluble RNA from the X fraction. DEAE-Sephadex (grade A-25, coarse) from Pharmacia, Sweden, was washed briefly with 0.1 *N* HCl and distilled water, treated for 10 min with 0.5 *N* NaOH, and then washed to neutrality with water containing several drops of dilute HCl. The Sephadex was equilibrated with 0.05 *M* Tris buffer, pH 6.9, and columns were prepared by permitting the Sephadex to settle by gravity. The buffer used for the final column contained 0.05 *M* Tris pH 6.9, 0.001 *M* $MgCl_2$, and 0.001 *M* 3-mercaptoethanol, and columns were washed with cold buffer under slight air pressure before use. Concentrated X fraction (from 3 to 4 gm of liver, suspended in 0.5 ml of the above buffer) was loaded onto a column (1 × 8 cm), permitted to sink in, and washed through with the above cold buffer. A large part of the protein and the stimulatory activity were not adsorbed onto the column—and were eluted with the buffer front which was easily recognized by its yellowish color. About 90 per cent of the material passing through the column could be collected in a volume of 2 ml (as verified by absorption density of the collected fractions at 260 and 280 mμ). Although the column was run at room temperature, the X material was kept as cold as possible by the use of ice-cold buffers and cold collecting tubes.

RNA was extracted from X fraction by the following modification of the phenol method. The X pellet from 4 gm of liver was dissolved in 1 ml of medium and extracted with an equal volume of redistilled phenol previously saturated with the medium. The emulsion was stirred for 10 min at 15°C, and all subsequent handling was done at +3°C. The emulsion was centrifuged for 10 min at 5,000 *g*, the aqueous layer removed, and extracted five times with several volumes of ether. Ether was removed from the solution by bubbling nitrogen through it for 10 min. The absorption density (per ml) of the resulting RNA solution was about 14–20 at 260 mμ, with a 260/280 ratio of 2. This procedure recovers about 80 per cent of the RNA present in the X pellet. Crystalline ribonuclease, deoxyribonuclease (highest grade), and trypsin were Worthington Chemical Company products. Adenosine and guanosine triphosphates were obtained from Sigma Chemical Company, phosphoenolpyruvate and pyruvate kinase from California Biochemical Corporation, and L-leucine-1-C^{11} (~25 mc/m mole) from New England Nuclear Corporation. RNA was determined spectrophotometrically after partial alkaline hydrolysis, using an extinction coefficient at 260 mμ of 31.8 cm^2/mg (cf. ref. 6). Sucrose gradients were prepared and used according to the method of Britten and Roberts.[7]

Results.—It has previously been noted[8, 9] that the soluble components of the *in vitro* incorporation system from regenerating liver are more active than those from normal liver. It has been suggested that the difference is due in part to levels of activating enzymes.[9] In our hands, however, pH 5(−X) fractions which contain most of the soluble RNA and activating enzymes exhibited essentially the same activity whether prepared from normal or regenerating livers. The activity was measured both by over-all incorporation of amino acids into microsomal protein and

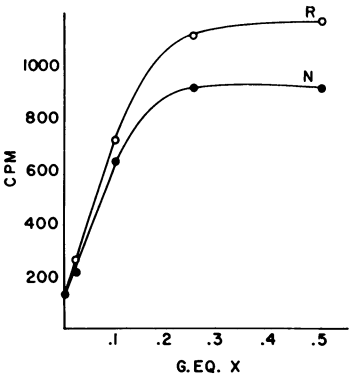

FIG. 1.—Effect of X fractions from normal (N) and regenerating (*R*) liver on microsomal protein synthesis. Assay conditions were as described in the text, using 0.2 gm eq pH $5(-X)_{R,N}$ and 0.15 gm eq M_R. Radioactivity in counts per min represents the C^{14}-leucine incorporated into the total protein of the system after correction for self-absorption of samples.

by adenosine triphosphate-dependent attachment of amino acids to soluble RNA. Hence, in the following experiments we have simply pooled the pH $5(-X)$ fractions from normal and from regenerating livers (designated pH $5(-X)_{R,N}$).

The present paper deals with the properties of the X fraction. The X pellet is a brownish-red, hard-packed, translucent sediment which contains both RNA and protein. In a typical preparation the RNA content of various fractions (in mg per gm eq of liver) was: 15,000 *g* supernatant, 2.24; microsomes, 2.09; X, 0.16; and pH $5(-X)$, 0.09. The quantity of RNA in equivalent normal and regenerating fractions is approximately equal (± 15 per cent).

Figure 1 shows the effect of adding increasing amounts of X_N and X_R to the assay system. Maximal stimulation was generally achieved when X was present in a 1.5- to 2-fold tissue equivalent excess. We have consistently found that X_R has more activity (20 to 30 per cent in this figure) than X_N, on the basis of either tissue equivalence of RNA content. It should also be noted that the curves attain and maintain different maxima, a finding inconsistent with the postulate that there is simply a different quantity of stimulatory component(s) in X fractions from the two sources. It suggests that X_N may contain in addition a factor limiting its stimulatory capacity. The nature of this inhibitory activity is the subject of a paper to follow.

In the absence of microsomes, the X fractions have no intrinsic incorporating ability when supplemented with pH $5(-X)$ and cofactors. X activity is, as mentioned previously,[3] precipitable at pH 5. Owing to the prolonged centrifugation required to clear the supernatant of X, amino acid-activating enzymes and soluble RNA are present in this fraction (as measured by adenosine triphosphate-dependent amino acid labeling of RNA). It is for this reason that X is assayed in the presence of nonlimiting levels of pH $5(-X)$ fraction. Under the conditions described in the *Methods* section, DEAE Sephadex adsorbs about 85 per cent of the RNA in the X fraction. However, the material passing through the column at neutrality gives the same degree of stimulation of microsomes (per gm eq) as the original X fraction. The presence of pH $5(-X)$ fraction is required for this stimulation, indicating that the bulk of the soluble RNA has been removed by the column. The use of buffered concentrated KCl (0.5–0.8 *M*) elutes a large part of the RNA from the column.[10] No stimulatory activity could be demonstrated when this RNA (following dialysis) was added to the assay system consisting of microsomes and pH $5(-X)$. Finally, it was ascertained that following passage through the column the residual RNA is unable to bind amino acids in the presence of ATP, though added transfer RNA was rapidly labeled by the preparation. It would thus appear that transfer RNA is retained by the column, while amino acid-activating enzymes and the material responsible for X activity pass through.

RNA extracted directly from X by the phenol method has stimulatory activity (Fig. 2, curve labeled X). Attempts to extract active RNA from the DEAE Sephadex column effluent of X were unsuccessful, but the amount of RNA was very small. These RNA derivatives of X, however, never give the degree of stimulation of the system obtained with the native X or the column effluent of X.

Figure 2 also shows the stimulatory effects of RNA preparations from other sources: bacterial (B), tumor (T), and liver nuclear (N). The results show that heterologous RNA also is able to affect the assay system, although bacterial RNA appears to be less effective than RNA from animal sources. Soluble RNA was essentially inactive in the system, and in concentrations above 100 μg/ml it was inhibitory. Whole ribosomal RNA gave the same degree of stimulation as X-RNA if supplied in high enough concentration (150 μg/ml).

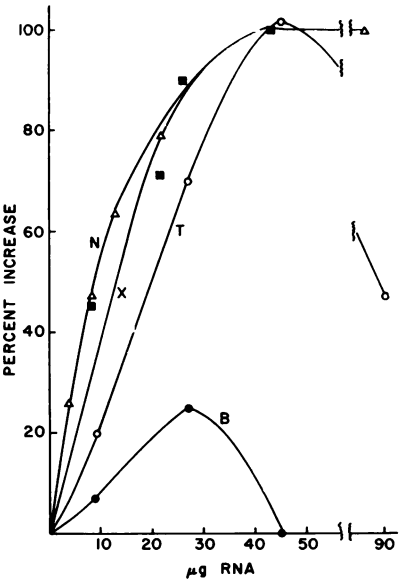

FIG. 2.—Stimulation of amino acid incorporation into protein by phenol-extracted RNA from X and from other tissue fractions. RNA samples were assayed using 0.15 gm eq M_R and 0.2 gm eq pH $5(-X)_{R,N}$ as described in the text. The data are plotted as per cent increase in radioactivity (C^{14}-leucine incorporated into total protein in the system).

RNA was extracted as described in the text from: ■ X fraction from regenerating liver; △ the 1,000 *g* sediment of liver homogenate (containing mainly nuclei and whole cells); ○ a homogenate of mouse plasma cell tumor RPC-5 obtained from M. Potter, National Cancer Institute, Bethesda; and ● bacteria (*E. coli*).

The stimulatory effect of the RNA extracted from X is labile to low concentrations of ribonuclease, as shown in Figure 3. It is of considerable interest that native X, or the column effluent of X, is remarkably insensitive to ribonuclease. Thus, treatment of the native X suspension with as much as 40 μg of ribonuclease per ml (for 5 min at 37°C) has no observable effect on the stimulatory activity of the RNA subsequently extracted from it by the phenol method. This is in marked contrast to the extremely high sensitivity of the microsomes to RNase, which will be discussed in a subsequent publication. DNase has essentially no effect on the activity of the complete system at levels of 0.5 μg/ml, and small variable effects ($\pm$ 5–20 per cent) at levels of 5.0 μg/ml (30 min at 37°C). (There is no detectable DNA in the fractions used in these experiments.) Trypsin (for 30 min at 37°C) has no effect on the complete system at 0.5 μg/ml, but at 5.0 μg/ml it causes 64 per cent inhibition. This latter effect, taken together with the failure of the active material to be adsorbed by the column, its resistance in the native state to ribonuclease, and the failure of the RNA to account for all the X activity, suggests that the RNA is not free but complexed and that there may be an additional protein component important for its biological action.

We next undertook to determine whether the RNA of the X fraction became labeled more rapidly *in vivo* than other cytoplasmic RNA fractions. At intervals preceding the killing of animals whose livers were regenerating, they were exposed

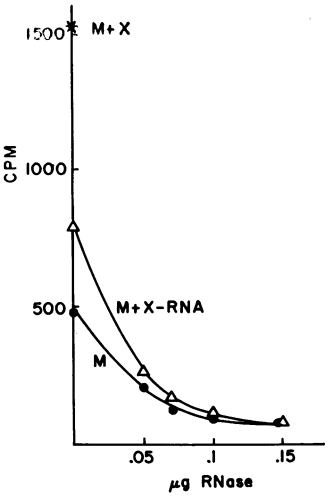

FIG. 3.—Ribonuclease sensitivity of X-RNA stimulation of amino acid incorporation into protein. Varying amounts of ribonuclease (RNase) alone (●) and with 40 μg of RNA extracted by phenol from X (△) were preincubated for 8 min at 37°C. The usual assay system was then added; radioactivity as cpm represents the C^{14}-leucine incorporated into protein. The point marked "$M + X$" is the stimulatory effect of the native X fraction from which the RNA was derived.

to P^{32} (see *Materials and Methods*) and the rate of uptake of P^{32} into the three RNA-containing fractions was measured. Figure 4 depicts the results of such an experiment. In spite of the crude nature of the X fraction it does appear to incorporate P^{32} into its RNA at a more rapid rate than does the soluble RNA of the pH 5(−X) fraction or the ribosomal RNA.

If the microsomes from such P^{32}-labeled livers are suspended in a small volume of water, incubated at 37° for 5 min, and resedimented for an hr at 100,000 *g*, the resulting supernatant contains RNA that is also labeled more highly than microsomal or soluble RNA (Fig. 4, curve labeled M_s). RNA derived from this (M_s) fraction by the phenol method has stimulatory activity comparable to X-RNA in the M, pH 5(−X) assay system. Equal amounts of this active material are obtained from normal and regenerating liver microsomes.

X fractions from such P^{32}-labeled animals have been passed through DEAE-Sephadex columns to reduce their soluble RNA content and then centrifuged through sucrose gradients in attempts to locate a P^{32}-labeled peak coinciding with the peak of biological activity. As previously reported,[3] sucrose gradients of X do show peaks of stimulatory activity in regions corresponding roughly to S values below 20. Figure 5 shows the outcome of two such experiments. It is clear that there is considerable stimulatory activity over the upper third of the gradient under the conditions employed and that its distribution does not conform to the distribution of the bulk of the 260 mμ absorption (a part of which, according to 280/260 ratios, is due to protein). The distribution of P^{32}, furthermore, follows the 260 mμ absorption, indicating that in spite of the removal of most of the transfer RNA by the column, some RNA (perhaps protein bound) remains and contributes largely to the P^{32} peak. However, there is a shoulder of P^{32}-labeled material that appears to coincide with the major areas of stimulatory activity. Attempts to increase the amount of this peak relative to the larger peak by shorter periods of exposure to P^{32} have thus far been unsuccessful. The activity would appear, then, to be associated with material larger than soluble RNA, but generally of lower sedimentation coefficient than messenger RNA in bacteria.

Gradients performed on phenol-extracted X-RNA gave broad bands of activity extending from the 15S region to material smaller than 4S. The 260 mμ-absorbing material in such gradients in the 4S region could not be labeled with amino acids. Hence, the active material in this fraction does not appear to include transfer RNA.

Discussion.—The X fraction appears to contain important components of the amino acid incorporation system of rat liver. It stimulates incorporation *in vitro*, part of its activity being in a fraction with higher sedimentation rate than transfer

RNA. Phenol extraction yields an RNA moiety that is labeled more rapidly *in vivo* than transfer or ribosomal RNA and that retains some of the *in vitro* stimulatory activity of the native X fraction. A fraction having similar properties may be obtained by water extraction of microsomes.

Though the active material in X resembles bacterial messenger RNA in several respects, it is insensitive to ribonuclease in the native state, and it does not behave like free RNA or DEAE-Sephadex columns. The failure of the RNA alone to give as high a stimulation as native X suggests either that more than one factor accounts for the X effect or that the RNA is damaged in the process of isolation. The native fraction may contain transfer enzymes such as those described by Nathans and Lipmann[11] and Grossi and Moldave.[12]

As mentioned earlier, numerous recent studies suggest that there is a nuclear RNA fraction in animal cells having turnover and sedimentation properties similar to those of bacterial messenger RNA. Thus, Cheng has found such a fraction in human amniotic cells;[13] Sibatani *et al.*, in thymus nuclei;[14] Hiatt, in rat liver nuclei;[15] Marks *et al.*, in white blood cells;[16] and Scherrer and Darnell, in HeLa cells.[17] These fractions have not been found to have biological activity. Amos and Kearns have reported that whole bacterial RNA stimulates the production of protein, immunologically identified as bacterial, by chick fibroblasts in tissue culture.[18] Niu *et al.*[19] have observed the synthesis of liver enzymes by ascites tumor cells exposed to whole liver RNA. It has also been reported that RNA polymerase activity increases in the rat liver following the induction of regeneration.[20] We have found that the high-turnover nuclear RNA fractions obtained by Hiatt (and supplied to us by him) are inactive in our system. (This might be due to the use of sodium dodecyl sulfate in their isolation.) As noted in Figure 2, a phenol-extracted RNA from crude liver nuclei gives as good stimulation as X-RNA and is, in fact, obtained in larger amounts from nuclei than from cytoplasm.

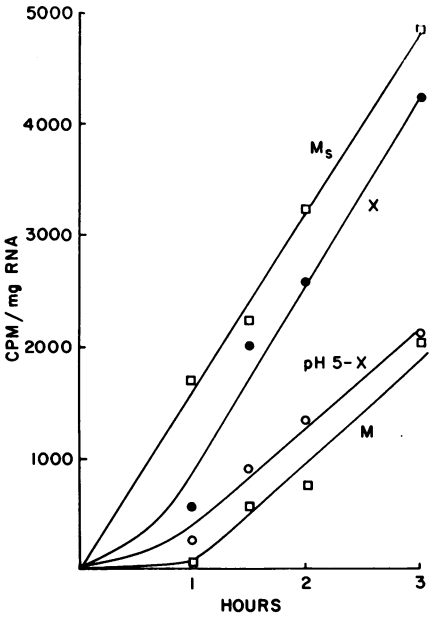

FIG. 4.—Rate of incorporation of P^{32} into liver cytoplasmic RNA fractions *in vivo*. Rats were killed 24 hr after partial hepatectomy. A pair of animals was used for each time point, being exposed to 200 μc of P^{32} for the time indicated before killing. Cytoplasmic fractions, i.e., microsomes, 13 hr X pellet, and pH 5(−X), were prepared. A portion of the microsomes (corresponding to about 2 gm of liver) was washed in the buffered medium and then treated with 5 ml. of water for 5 min at 37°C. The microsomes were then reisolated by spinning for 2 hr at 100,000 *g*. RNA was salt-extracted (as described in the *Methods* section) from the microsomal water extract (M_s), the stripped microsomes (*M*), X pellet, and pH 5(−X).

The paucity of evidence for a rapidly labeled *cytoplasmic* RNA fraction (excepting the data shown in Fig. 4) may be explained by assuming that in animal cells there is a small cytoplasmic pool that is replenished with a lag. It may be that in cells of higher organisms, with a nuclear membrane, messenger RNA accumulates in the

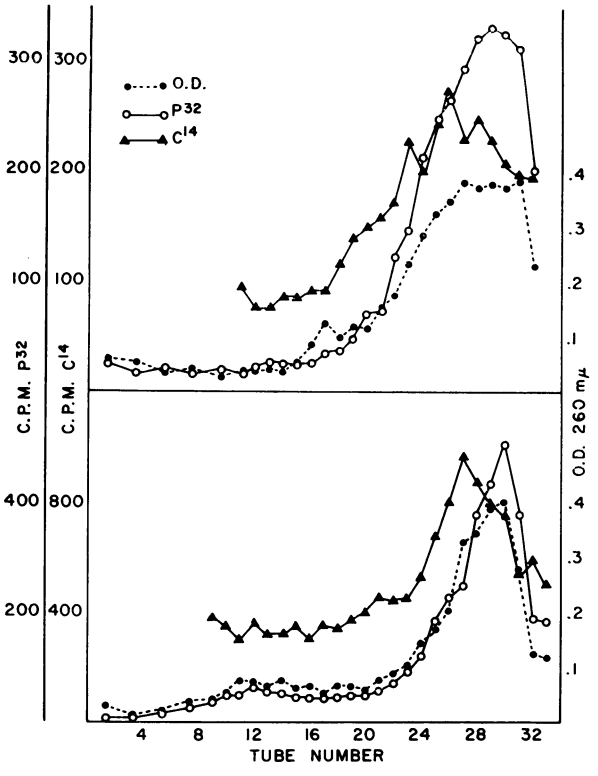

FIG. 5.—Distribution of P^{32} and stimulatory activity of X on sucrose gradients. X was prepared from regenerating livers from rats injected 1.5 hr previously with 200 μc P^{32} intraperitoneally. X was treated with DEAE Sephadex as described in the *Methods* section and 1.2 ml, equivalent to 3 gm of liver, was layered onto a sucrose gradient. A linear gradient of 5 to 20% sucrose, containing the buffered medium, was used. The gradient tube was centrifuged for 5 hr at 25,000 rpm in the swinging bucket rotor Spinco type SW-25. 33 fractions of 0.71 ml each were collected, tube #1 representing the bottom of the gradient. 0.2 ml of each was used for determining absorption at 260 mμ (●) and cpm due to P^{32} (○). 0.3 ml of each gradient fraction was assayed for stimulatory effect on protein synthesis using regenerating microsomes (0.15 gm eq) and pH 5(−X) (0.2 gm eq). ▲ shows incorporation of C^{14}-leucine into protein. Two separate experiments are shown.

nucleus and is supplied to the cytoplasm only after a delay. Our results further suggest the possibility that outside the nucleus the messenger may be bound to protein. It is of interest that an RNA with turnover and specific stimulatory activity very similar to X may be extracted from the microsomes.

Since the RNA in X is only about 5 per cent of the total cytoplasmic RNA and the active material of X is present in at most 15 per cent of its RNA, there is probably very little of this material normally present in the cytoplasm.

It would appear that the X fraction accounts in part for the enhanced activity of soluble fractions from regenerating, as compared with normal, liver.

Summary.—Evidence is presented for the occurrence in rat liver cytoplasm of an RNA-containing fraction having certain properties in common with bacterial messenger RNA. The material stimulates amino acid incorporation in microsomes, is labeled more rapidly *in vivo* with P^{32} than are other cytoplasmic RNA fractions, and sediments in a sucrose gradient more rapidly than soluble RNA and

more slowly than ribosomes. Preparations of this material from regenerating liver are more active than those from normal liver. Phenol-extracted RNA from the preparation is also active in the *in vitro* system. It is emphasized, however, that the material appears to differ from bacterial messenger RNA in certain other properties. Notable among these is the insensitivity of the RNA, in the native state, to ribonuclease.

Note added in proof: After this paper was completed, two further reports appeared on biologically active RNA fractions from liver: E. S. Maxwell, these PROCEEDINGS, **48**, 1639 (1962), and Barondes, S. H., C. W. Dingman, and M. B. Sporn, *Nature*, **196**, 145 (1962).

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