A Cytochemical Study on the Pancreas of the Guinea Pig

IV. Chemical and Metabolic Investigation of the Ribonucleoprotein Particles*

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ABSTRACT

Five ribonucleoprotein (RNP) fractions were isolated from the postmitochondrial supernatant of the pancreas of the guinea pig. Two were obtained from the microsomes which, by deoxycholate (DOC) treatment, were subdivided into a DOC-soluble and a DOC-insoluble fraction. The latter was taken to represent attached RNP particles. Two other fractions obtained from the microsomal supernatant supposedly represent free RNP particles existing as such in the cytoplasm, while a third fraction resisted sedimentation for 20 hours at 105,000 *g* and is considered to be a soluble nucleoprotein. These fractions exhibited different RNA/protein ratios and also different RNA turnover patterns, as determined after *in vivo* labelling with adenine-8-C^{14}. However, little discernible differences could be detected in the nucleotide composition of the RNA moieties of these RNP fractions.

Amino acid-"activating" enzymes were found to occur in the fraction containing the soluble nucleoproteins.

The discussion focuses on the relationships between these fractions and protein synthesis in the pancreas, using data given in this and a previous paper, and data contained in the literature.

In a detailed cytochemical investigation of the pancreas of the guinea pig (1–5), we have found that it is possible to separate several cytologically distinct types of nucleoprotein particles (2, 5) derived mostly from the cytoplasm of the exocrine cells of this organ. Since we have already studied (5) the *in vivo* turnover of the proteins of these particles, we have decided to determine, also *in vivo*, the turnover of their nucleic acid moiety. In this paper we present our findings on this point together with additional data pertaining to the chemical composition of the particles.

The metabolic heterogeneity of the ribonucleic acid (RNA) at cellular and subcellular levels has been noted many times in the past, in experiments using either phosphorus, glycine, or purine bases as RNA precursors. This heterogeneity reveals itself either as differences among various fractions of the whole cellular RNA (6), or as differences between nuclear and cytoplasmic RNA (7–9), or as differences among the RNA's of various cytoplasmic fractions, *e.g.* the mitochondrial, microsomal, and final supernatant fractions (10–21). Our work extends this line of inquiry towards recently recognized cell fractions and subfractions, many of which consist of ribonucleoprotein (RNP) particles.

Finally, in view of the connections of RNA with the process of protein synthesis, we have studied the distribution of "amino acid-activating enzymes" among these newly defined, RNA-containing cell fractions. As is known, these enzymes are located in the microsomal supernatant and are assumed to be involved in the initial steps of the process of protein synthesis (22–28).

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Experimental

Guinea pigs, starved for 40 to 48 hours, received 0.5 ml. of adenine-8-C^{14} (containing 0.43 mg. and 12.3 μc.) by intravenous injection. At various times after the injection, the pancreases were removed under ether anesthesia, homogenized in 0.88 M sucrose, and fractionated by differential centrifugation as described earlier (3). The isolated microsomes were treated with 0.3 per cent deoxycholate (DOC) at pH 7.5–7.8, to obtain their attached RNP particles in one fraction and the solubilized microsomal contents and membranes in another (2). This treatment gives about equal amounts of RNA in each of these fractions. The supernatant of the original microsome fraction was centrifuged at 105,000 *g* for 3 hours to give a first postmicrosomal fraction (PM_1), and the supernatant from this spin was centrifuged for 16 to 17 hours at 105,000 *g* to give a pellet, the second postmicrosomal fraction (PM_2), and a final supernatant (FS). The loose superficial layer of all these pellets was removed by pouring over a small amount of 0.88 M sucrose, then swirling and decanting.

The various fractions were treated with trichloroacetic acid (TCA) at a final concentration of 10 per cent. The precipitated proteins and nucleoproteins were washed three times with cold 5 per cent TCA and three times with cold distilled water, centrifuging each time between the washes. The final pellets were resuspended in 3 to 4 volumes of 10 per cent NaCl in 0.1 M acetate buffer, pH 5, and heated for 20 minutes at 85°C. (*cf.* 29). This treatment has been found by us to extract all the RNA from the TCA precipitate of the microsomes. Three volumes of 95 per cent ethanol were added to the salt extract and the solution kept in the deep freeze (−20°C.) overnight. The precipitated nucleic acids were then plated on paper following a procedure previously described (30) and counted with a Nuclear-of-Chicago gas-flow counter provided with "micromil" window. After counting, the plated papers were placed in a suitable amount of 0.5 N KOH and the absorbance of the extract read at 260 mμ, to determine the amount of nucleic acid in each sample. The absorbance was converted to weight and, after corrections were made for the absorption of counts (30), the specific activity was obtained as counts per minute per mg. RNA.

For each cellular fraction, samples of nucleic acids dissolved in 0.5 N KOH were pooled and incubated at 37°C. for about 40 hours (*cf.* 31). The alkaline digest was then neutralized by adding enough 1.5 N $HClO_4$ and the preparation was left overnight in the cold room to precipitate all the $KClO_4$. After spinning down the precipitate, the supernatant fluid was brought to pH 8.0–8.5, and placed on a Dowex-1 column (200 to 400 mesh; 0.9 sq. cm. x 6 cm.) prepared in the formate form. The various nucleotides were eluted from the columns as described by Herbert *et al.* (29). The recoveries of material placed on the columns were uniformly around 95 per cent. The identity of the bases was checked by spectral analyses of the various peaks, and the results were found in agreement with the sequence in which the nucleotides are known to come off the Dowex-1 column. At acid pH values, the average absorbance ratios for E_{280}/E_{260} were as follows: cytidylic acid, 1.72; adenylic acid, 0.33; uridylic acid, 0.37; and guanylic acid, 0.68. The following extinction coefficients were used for absorbances at 260 mμ and at acid pH values (32): adenylic acid, 14.2×10^{-3}, cytidylic acid, 6.8×10^{-3}; uridylic acid, 10.0×10^{-3}; and guanylic acid, 11.8×10^{-3}.

For the amino acid–activating enzymes (*cf.* Table III), the hydroxamic acid assay system of Davie *et al.* (23) was employed. Salt-free hydroxylamine was prepared by a modification of the method of Beinert *et al.* (33) and its concentration was determined colorimetrically (34). Hydroxamic acid formation was measured by a modification of Lipmann and Tuttle's procedure (35), using leucine hydroxamate as a standard. The standard and information about the modified procedure were generously provided by Dr. F. Lipmann's laboratory. Inorganic phosphate was determined by the Soyenkoff method (36).

The amounts of RNA and protein in the various cell fractions (Table I) were determined as described previously (2). The deoxycholic acid was obtained from the Wilson Co., Chicago, and the chromatographically pure adenine-8-C^{14} (3.82 mc./mM) from the New England Nuclear Corporation, Boston.

RESULTS

Morphology

A brief morphological description of the isolated fractions will first be given (*cf.* 2).

The microsomal fraction is primarily made up of pinched-off fragments of the endoplasmic reticulum of the exocrine cells. It consists of closed vesicles, 50 to 200 mμ in diameter, bound by a thin ($\sim$7 mμ) membrane which bears dense particles, $\sim$15 mμ in diameter, attached to its outer surface. In the case of starved animals, the content of the microsomal vesicles is apparently amorphous and generally of moderate density. After treatment with 0.3 per cent DOC, the microsomal material left in sedimentable form consists of dense particles, 10 to 15 mμ in diameter, generally similar to those originally attached to the microsomal membranes. The finding indicates that the membrane and content of the microsomes is solubilized by DOC and suggests that the attached particles, or at least part of them, survive the treatment. As we have previously found (2), these detached particles contain only 50 to 60

per cent of the microsomal RNA, the balance being present in the supernatant as "soluble" nucleoprotein. It is unknown whether the DOC-soluble RNA preexists in a truly soluble state in the microsomes (*cf.* 37) or represents a part of the population of attached RNP particles more susceptible to DOC treatment than the rest. Whatever the reasons, the microsomal RNA of the pancreas is more extensively affected by DOC than its counterpart in rat liver microsomes; only ~10 per cent of the latter is solubilized by 0.3 per cent DOC (38).

Both postmicrosomal fractions (PM_1 and PM_2) consist primarily of dense particles, ~15 mμ in diameter, disposed either in clusters or in chains, and are assumed to represent particles of similar appearance which *in situ* occur freely scattered in the cytoplasmic matrix of the exocrine cells (*cf.* 2). Since in density, size, and general shape these free particles appear to be similar to those attached to the membrane of the endoplasmic reticulum, it can be argued that the postmicrosomal fractions may consist in part or in whole of particles detached from the membrane of the reticulum during tissue homogenization and fractionation. Data presented in this paper and in a previous one (5) indicate, however, that there are metabolic differences between the RNP particles detached from microsomes by DOC treatment and the RNP particles of the postmicrosomal fractions. These differences, which concern the protein as well as the RNA moieties of the particles, strongly suggest that PM_1 and PM_2 are not grossly contaminated by detached particles and should be considered as truly representative of the free RNP particles of the cytoplasmic matrix.

As is known (2), 10 per cent of the RNA of the cell remains in the supernatant of PM_2 as ribonucleoprotein. The form taken by this nucleoprotein is unknown but, in view of the preparative procedure involved (still unsedimented after 19 to 20 hours at 105,000 *g*), and in view of its metabolic properties (*cf.* below), it can be assumed that the nucleoprotein of the final supernatant occurs in non-particulate form and is different from that of the RNP particles, free or attached.

Chemistry

RNA/Protein Ratios:

Table I gives some chemical data of the nucleoprotein fractions under consideration. It can be seen that 0.3 per cent DOC solubilizes more than half of the microsomal RNA but over 90 per cent of the microsomal protein, so that the detached particles have a high RNA/protein ratio. That there is some meaning in separating the postmicrosomal particle population into two fractions, PM_1 and PM_2, can be seen not only in the higher RNA/protein ratio consistently obtained in PM_1 (Table I), but also in their metabolic differences (Fig. 1). In a previous paper (2) we obtained PM_1 by centrifuging the microsomal supernatant for only 2 hours at 105,000 *g*, and separated PM_2 by spinning the supernatant of PM_1 for 15 to 16 hours at the same speed. In that case, PM_2 had a significantly higher RNA/protein ratio than did PM_1 (2). Therefore, by simply centrifuging the microsomal supernatant for 1 more hour (3 hours all told) to obtain the PM_1 of the experiments here reported, we centrifuged down from this supernatant a variety of particles having a higher RNA/protein ratio than that of the particles still remaining in the supernatant. This sharp break in the RNA/protein ratio revealed by the centrifugation procedure makes us believe that the particles sedimented during the first 3 hours (PM_1) differ in nature from those coming down thereafter. However, at the present level of resolution the electron microscope shows no discernible differences, in size, shape, or density, between PM_1 and PM_2.

TABLE I

Some Chemical Characteristics of Various Cytoplasmic Nucleoprotein Fractions Separated from the Pancreas of Starved Guinea Pigs

Fraction	RNA/gm.*	Protein-N/gm.*	Mg. RNA/mg. Protein-N	Mg. RNA/mg. Protein‡
	mg.	*mg.*		
Attached particles	0.85	0.25	3.40	0.54
Microsomal contents	0.95	3.04	0.31	0.05
First postmicrosomal fraction (free RNP particles)	1.22	0.62	1.97	0.32
Second postmicrosomal fraction (free RNP particles)	1.03	1.36	0.76	0.12
Final supernatant	0.40	4.95	0.08	0.01

* Per gram wet weight pancreas.

‡ Calculated by dividing the previous column by 6.25.

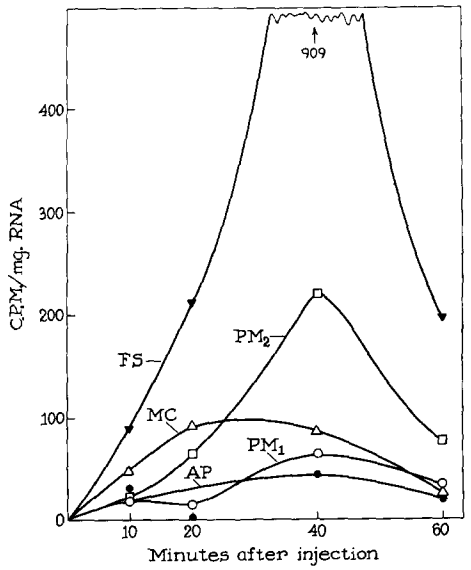

FIG. 1. Rate of incorporation of adenine-8-C^{14} into the RNA of various cytoplasmic nucleoprotein fractions. Techniques described in the Experimental section. *AP*, attached nucleoprotein particles; *MC*, microsomal contents; *PM_1*, first postmicrosomal fraction; *PM_2*, second postmicrosomal fraction; *FS*, final supernatant.

Incorporation of Adenine-8-C^{14} into RNA:

Metabolic differences among the RNA's of the various pancreatic RNP particles can be revealed by using the incorporation of radioactive adenine into RNA as a specific measure of RNA turnover, since it has been shown that adenine is a precursor of both adenine and guanine in the RNA of the internal organs of the rat (*cf.* 39). Fig. 1 is an example of the differences found among the rates of adenine incorporation into the RNA of the various RNP fractions studied. The type of incorporation was determined in one experiment for the RNA's of PM_1, PM_2, and final supernatant fraction ("soluble RNA"). In all cases, after hydrolysis and chromatography, ~86 per cent of the incorporated counts were recovered as the 2′- and 3′-adenylic acids and ~8 per cent as the isomeric guanylic acids, while the rest were not retained by the column. Accordingly, the incorporation of the labelled precursor can be considered as a meaningful turnover of the whole RNA molecule. A similar conclusion can be reached by an inspection of the high turnover data which has been obtained by other workers (*cf.* 39). The adenine incorporation found *in vivo* appears to be different from the terminal incorporation recently studied *in vitro* (40–45) in the final supernatant of rat liver and ascites tumor cells. In that case the RNA becomes radioactive after incubation with purine-labelled ATP or CTP (but not with AMP or CMP) and much of the label can be recovered after alkaline hydrolysis as adenosine, indicating that some of the labelled purine was attached at the end of the RNA molecule. From the protocol of these experiments it appears that the RNA which becomes terminally labelled *in vitro* may be the same RNA that shows maximal "internal" labelling *in vivo*.

Fig. 1 shows that the RNA of the nucleoprotein in the final supernatant has by far the highest specific activity among the fractions tested. PM_2, the most active of the particulate fractions, incorporates at a rate 3 to 5 times higher than PM_1. Between the other fractions with low activity, namely the attached particles and the microsomal contents, a difference has consistently been noted, suggesting that there is also a metabolic difference between the DOC-soluble and insoluble nucleoproteins of the microsomes. Bhargava *et al.* (46) have also found a difference in incorporative rates of P^{32} into the RNA's of various subfractions of liver microsomes, with the ribonucleoprotein having the highest rate of amino acid incorporation into its protein having the lowest rate of P^{32} incorporation into the RNA moiety. Although the PM_2 curve crossed in all experiments the curve given by the microsomal contents, it is not possible to determine with the information at hand any precursor-product relationship among the fractions tested. In three experiments the initial rates of adenine incorporation followed the same decreasing order, namely: final supernatant > microsomal contents > PM_2 > PM_1 > attached particles, with the maximal specific activities decreasing in the sequence: final supernatant > PM_2 > microsomal contents > PM_1 > attached particles. The latter two fractions showed similarly low incorporative rates in all experiments. If we calculate the total number of counts incorporated into the RNA's of the cellular fractions, we obtain an equally high total incorporation into PM_2 and the final supernatant, followed by ⅛ to ¼ as much into PM_1 with the microsomal contents and the attached particles RNA having still lower total counts.

Our findings agree in general with already published results (11–15, 17–19) which indicate that various RNA precursors are more actively incorporated into the "soluble" RNA of the final

supernatant than into the microsomal RNA. We have carried out, however, a morphologically controlled separation of microsomal, submicrosomal, and postmicrosomal fractions and we have found that certain fractions are not only cytologically, but also metabolically different. The finding applies primarily to the attached and free RNP particles of the cytoplasm. This conclusion is further strengthened by a comparison of the curves in Fig. 1 with those previously published (5) for the rates of leucine-1-C^{14} incorporation into the proteins of the same fractions. The attached particles, which are distinguished by the highest initial rate of leucine incorporation into their proteins, show a very low uptake of adenine into their RNA. Conversely, some of the fractions which are sluggish in leucine incorporation, *e.g.*, the final supernatant and PM_2, are most active in adenine incorporation into their RNA moieties. Partly similar results have been reported by Shigeura and Chargaff (19) for liver fractions.

The peak of adenine incorporation occurs at ~40 minutes, whereas that of initial leucine incorporation comes much earlier at ~3 minutes. The finding may reflect differences in the availability of the corresponding blood precursors or real differences in turnover rates between the most reactive RNA's and proteins. The last alternative appears more probable in the light of the work of Balis *et al.* (47) who have found a similar situation in rat liver, using labelled glycine as a common precursor for both RNA and protein. There again the protein was labelled more rapidly than the RNA.

Nucleotide Composition of the RNA in the RNP Fractions:

Since we have shown that the RNP fractions of the cytoplasm are metabolically heterogeneous, we tried to find out whether this functional diversity is underlined by certain chemical differences. To this intent we determined the nucleotide composition of the RNA's extracted from the nucleoprotein fractions listed in Table I. Because of the small amounts involved, the determinations were carried out on pooled fractions obtained from 4 to 6 animals. The pooling was advisable in the case of the RNA's of the attached particles and microsomal contents, and necessary in the case of the RNA's of the postmicrosomal and final supernatant fractions. Table II shows that there are only small differences among the ratios from one RNA to another

TABLE II

Nucleotide Composition of the RNA in the Cytoplasmic Nucleoprotein Fractions of the Pancreas (Starved Guinea Pig)

The results are expressed as moles per hundred moles of total nucleotides. Each figure represents one determination; the figures in parentheses represent averages.

Fraction	Cytidylic acid	Adenylic acid	Uridylic acid	Guanylic acid
Attached particles	15	15	25	45
	13 (14)	17 (16)	14 (20)	56 (51)
Microsomal content	15	14	20	51
	13 (14)	16 (15)	16 (18)	55 (53)
Whole microsomes	15	14	21	50
	15 (15)	14 (14)	21 (21)	50 (50)
First postmicrosomal fraction (free particles)	20	19	17	44
	17	16	19	48
	19	14	17	50
	14 (18)	17 (16)	16 (17)	53 (49)
Second postmicrosomal fraction (free particles)	15	15	21	49
	20	17	20	43
	14	17	17	52
	18 (18)	16 (16)	24 (20)	42 (47)
Final supernatant	17	15	15	53
	17	15	15	53
	17	13	13	57
	18 (17)	15 (15)	21 (16)	46 (52)

and, as these differences are within the range of experimental error, it can be concluded that the RNA's of the various fractions are similar in their gross chemistry. If differences exist among the RNA moieties of various nucleoproteins studied, they either are too small to be resolved by present analytical methods or concern nucleotide sequence, rather than nucleotide ratios.[1]

In agreement with earlier findings on pancreas tissue from various sources (48–56), we uniformly found a high amount of guanylic acid in all the fractions isolated from guinea pig pancreas. A relatively large amount of guanylic acid (35 per cent of the total) has also been reported in the

[1] The only apparently discernible and reproducible difference concerns the uridylic and cytidylic acids: they occur in equal amounts in the postmicrosomal fractions, whereas in the microsomes and their subfractions there is a slight excess of uridylic acid.

microsomal RNA of rat liver (31), and has been confirmed in the case of human hepatic microsomes (57), but not in the case of microsomes isolated from rat liver (57). In addition, it has been shown (58) that the total cytoplasmic RNA extracted from calf liver, thymus, kidney, and heart contains more guanylic acid than any other nucleotide. However, only in the pancreas, and for all animals tested, does the amount of guanylic acid account for as much as 50 per cent of the total nucleotides. It is possible that this high content is, at least in part, an artefact due to the high ribonuclease (RNase) activity of the pancreas (*cf.* 55, 56, 59) since it has been shown that yeast RNA yields upon hydrolysis with pancreatic RNase a nondialyzable "core" characterized by a high guanylic acid content (60). A similar explanation is suggested by the recent work of Kemp and Allen (61) who, by minimizing RNase activity, have obtained from dog pancreas RNA preparations containing 30 per cent guanylic acid, 32 per cent cytidylic acid, 18 per cent adenylic acid, and 15 per cent uridylic acid. It should be noted, however, that Kirby (62) obtained from calf pancreas RNA low in pyrimidines and with a 50 per cent guanylic acid content. He used a phenol extraction method which he found to inhibit pancreatic RNase. There are, moreover, a number of points which cannot be explained by the assumption that the RNA's studied are extensively degraded by RNase. For instance, we have found that the incubation of pancreatic microsomes at 0°C. for 30 minutes causes no change in the microsomal RNA/protein ratio (2). The "core" obtained by the RNase treatment of yeast RNA has a characteristically low content of uridylic acid (60), much lower than that of the RNA we extracted from any pancreatic fraction. Finally, a high level of RNase activity is necessary (*cf.* 2) to decrease the relative amounts of cytidylic and adenylic acid (60) and it is unlikely that such levels are reached in our experiments in which all operations were carried out below 4°C.

Localization of Amino Acid–Activating Enzymes:

For obvious reasons we thought it worthwhile to determine the distribution of amino acid–activating enzymes (22–28) among the fractions described in this paper. Because of the low activity of the enzymes involved and because of the small protein content of certain fractions (*cf.* Table I), we were obliged to pool pancreatic tissue from 4 animals in order to complete an experiment like the one shown in Table III. It is clear from the representative results tabulated therein that the bulk of the activity resides in the final supernatant fraction. Indeed, the latter accounts for 44 to 54 per cent of the tyrosine activation, 68 to 83 per cent of the tryptophane activation, and 67 to 69 per cent of the casein hydrolysate activation obtained with our original, cytoplasmic preparation.[2] It is unlikely that the pattern of distribution illustrated by Table III is due to the fact that ATPase activity is lower in the final supernatant than in all the other fractions, for only half of the added ATP was hydrolyzed in the microsomal assays and in these same assays there was good stoichiometry between the amounts of fraction added and the quantities of hydroxamate produced. It should be also noted that the two activities vary independently from one fraction to another: for instance, the ATPase activity of PM_1 is twice that of the final supernatant fraction but hydroxamate formation by PM_1 is only one-eighth of that obtained with the final supernatant.

The large amount of enzymatic activity of the final supernatant is not reflected in the figures which give the specific activity of the various fractions, but the comparison is not valid because it is made between well differentiated cell fractions, such as the attached particles, PM_1 and PM_2, which contain only a small part of the proteins of the cell, and a fraction containing a much larger and undoubtedly more heterogeneous protein population. Washing by resuspension in 0.88 M sucrose followed by centrifugation for 3 hours at 105,000 *g* resulted in a 70 per cent loss of activity from the microsomal fraction and 89 per cent loss from PM_1, the corresponding specific activities being diminished by 52 and 83 per cent, respectively. Such results suggest that the activating enzymes are either truly soluble or only loosely bound to cytoplasmic structures. However, it is not precluded that some, though small, activity does reside in the particulate fractions, and that different amino acids may be "activated" at different sites.

To find out whether the activating enzymes in the final supernatant fraction are attached to, or

[2] The preparation is a mitochondrial supernatant and as such contains all the components of the cytoplasm with the exclusion of mitochondria and zymogen granules.

TABLE III

Amino Acid Activation by Various Cytoplasmic Fractions from Guinea Pig Pancreas

Additions: 10 μm. ATP, 1,000 μm. salt-free hydroxylamine, 10 μm. $MgCl_2$, 100 μm. tris (hydroxymethyl) aminomethane buffer, pH 7.8, 10 γ pyrophosphatase, H_2O or amino acids as given, and 1.3 and 2.6 mg. N of microsomes (M_1) or 0.83 mg. N of first postmicrosomal fraction (PM_1) or 1.8 mg. N of second postmicrosomal fraction (PM_2), or 0.46 and 0.92 mg. N of final supernatant (FS), all in final volume of 1.0 ml. Incubation at 35°C. for 60 minutes. Without the addition of amino acids the following values (μm. hydroxamate/gm./hr.) were obtained: Mi_1 = 0.50; PM_1 = 0.52; PM_2 = 0.41; FS = 5.81. The figures in the tables represent increments in hydroxamate formation above these blank values. The ATPase activity was determined by measuring the amount of IP accumulated during the experiment, and then subtracting the theoretical amount of IP due to hydroxamate formation (1 μm. hydroxamate = 2 μm. IP).

Amino acids added		Mi	PM_1	PM_2	FS	Total
Tyrosine (5 μm. L-)	μm. hydroxamate/gm./hr.	0.35	0.40	0.50	1.00	2.25
	per cent	16	18	22	44	100
	μm. hydroxamate/mg. protein-N/hr.	0.11	0.64	0.37	0.20	0.23
Tryptophane (15 μm. DL-)	μm. hydroxamate/gm./hr.	0.37	0.42	0.42	2.52	3.73
	per cent	10	11	11	68	100
	μm. hydroxamate/mg. protein-N/hr.	0.11	0.68	0.31	0.51	0.38
Casein hydrolysate (15 per cent, neutralized, 9 μm. DL-tryptophane added)	μm. hydroxamate/gm./hr.	1.80	1.62	3.15	13.40	19.97
	per cent	9	8	16	67	100
	μm. hydroxamate/mg. protein-N/hr.	0.55	2.61	2.32	2.71	2.03
ATPase	μm. IP/reaction vessel/hr.	4.6	4.1	2.9	1.9	—
	μm. IP/gm./hr.	11.6	3.2	2.4	15.2	—

are part of, its nucleoprotein, we added $MgCl_2$ to this fraction to a final concentration of 0.01 M. The cloudy suspension thereby obtained was centrifuged and the resulting pellet tested for RNA content and enzymatic activity. The Mg-precipitated material contained about 25 per cent of the RNA of the final supernatant, but only 5 per cent of the activity of the tyrosine-activating enzyme and none of the activity of the corresponding tryptophane enzyme. It seems therefore unlikely that the enzymes in the final supernatant are connected with the "soluble" nucleoprotein there present. It is also noteworthy that the addition of $CaCl_2$ (final concentration: 6×10^{-3} M) to the final supernatant, a procedure sometimes used to precipitate cellular nucleoprotein, brought down material which contained no RNA and about 10 per cent of the activity of the enzymes involved in tyrosine-, tryptophane-, and casein hydrolysate-activation.

DISCUSSION

The results obtained in this study and in previous ones indicate that the postmitochondrial fractions of the pancreas of the guinea pig contain at least five varieties of nucleoproteins, two of which can be identified cytologically by virtue of their characteristic relations *in situ*. They correspond respectively to the particles attached to the membrane of the endoplasmic reticulum, and to the free particles of the cytoplasmic matrix. Data presented in this paper indicate that this morphological difference is underlined by metabolic heterogeneity and suggest that functional categories are actually more numerous than the morphological types that can be differentiated at present. It appears, for instance, that among the free particles we may distinguish a fraction (PM_2) which incorporates actively labelled adenine into RNA, presumably by RNA synthesis, and another fraction (PM_1) which is considerably less active in the same process. It is also likely that in addition to the RNP of their particles, the microsomes contain another type of RNP distinguished by its greater sensitivity to DOC treatment and possibly by some metabolic properties: this microsomal DOC-soluble fraction seems to be more active in the incorporation of adenine

into RNA than the attached particles. Finally, the high incorporative ability for RNA precursors shown by the ribonucleoprotein of the final supernatant, together with its "solubility" and its apparent lack of structural connections set it apart as a distinct type of RNP and minimize the possibility that in its case we might deal with incomplete sedimentation of free RNP particles.

Metabolic differences among these various nucleoproteins increase in scope when the incorporation of labelled amino acids into proteins is considered conjointly with the results here reported. The attached particles, for instance, are found to differ sharply from PM_2 in two metabolic properties, and a clear cut metabolic difference also appears between the former and PM_1. In the tentative picture that emerges from these observations, there appear to be three relatively well defined types of RNP namely: (*a*) those of the attached particles active in protein synthesis but practically inactive in RNA turnover; (*b*) the particles of PM_2 which seem to have complementary abilities; and finally (*c*) the soluble RNP of the final supernatant distinguished by its high RNA turnover. The two other types, *i.e.*, the DOC-soluble RNP of the microsomes and the particulate RNP of PM_1 are less clearly defined and seem to possess intermediary metabolic abilities.

At present, nothing is known about the interrelations of these various types of RNP's. Restricting the topic to the two types which have some structural similarity, *i.e.*, the attached and the free particles, three possibilities can be considered: (1) the two types of particles are distinct in origin, function, and fate from one another; (2) both types of particles have a common origin, the free particles being on their way to become attached to the membrane of the endoplasmic reticulum; (3) the two types are functionally identical, the free particles having been detached either *in vivo* (metabolically) or *in vitro* (mechanically or otherwise) from their supporting membrane. The adenine turnover data seem to rule out the third of these possibilities. The second is not excluded, but if it operates, then the process involved is slower than the RNA turnover, since no indication of radioactive label transfer from free to attached particles was found within the time period explored in our experiments.

Assuming that at least some of the RNP preparations we studied are involved in protein synthesis, it becomes of interest to try to correlate our findings with the wealth of data recently obtained from studies concerned with this process in *in vitro* systems. The enzymes which activate various amino acids, to form amino acyl–adenylates, were already known to remain in the supernatant fluid after the sedimentation of the microsomes (1 hour at 105,000 *g*) (22–28, 63, 64). Our results indicate that they are still in the supernatant after 19 to 20 hours centrifugation at 105,000 *g*. They do not sediment with the particles of the postmicrosomal fractions and, moreover, do not seem to be associated with the soluble RNP of the final supernatant. These observations indicate that the enzymes in question are either truly soluble or only loosely associated with particulate or soluble RNP's.

It has been proposed that after activation, the amino acids are attached to the end of the chain of the soluble, non-microsomal RNA of the final supernatant which acts as a carrier and transfers them to the microsomal RNA, presumably the RNA of the attached RNP particles (43, 65–70). At this latter level it is assumed that the amino acids are linked in proper sequence into newly synthesized proteins. It is possible that the soluble RNA which supposedly acts as a carrier, by binding terminally CMP and AMP and the amino acids (41–45), is part of the RNP found in the final supernatant after 19 to 20 hours centrifugation at 105,000. As already mentioned, this RNP is characterized by the highest internal turnover of RNA among all the fractions we studied.

The binding of amino acids might be connected with internal RNA turnover, since it has been shown in *Escherichia coli* that in the absence of amino acids there is no synthesis of RNA (71). Thus it might be inferred that the presence of amino acids, or perhaps "activated" amino acids, is necessary for RNA synthesis to occur in the final supernatant fraction of the cell, while the presence of the already finished polynucleotide molecule is necessary for protein synthesis to occur in the attached nucleoprotein particle fraction of the microsomes. More work will have to be done to make even the cytological inference more of a certainty.

In a number of exploratory experiments we have tried to find out whether the RNA–amino acid intermediates detected and described *in vitro* (43, 65–70), occur also *in vivo*. To this intent 5 minutes after the intravenous injection of DL-leucine-1-C^{14}, we have removed the liver and pancreas from both young and adult guinea pigs and isolated the whole proteins and RNA's of these organs. The total counts in the RNA extracted either by the salt or phenol procedure (62) amounted to only 10

per cent of the total counts in the respective proteins. In other experiments, 3 minutes after the injection of labelled leucine the pancreas was removed and subsequently fractionated into microsomal, postmicrosomal, and final supernatant fractions. The RNA extracted from each of these fractions contained, again, less than 10 per cent of the counts in the proteins of the same fractions, and the counts in the most radioactive RNA were much less than the counts in the lowest radioactive protein. In none of these cases was the RNA reprecipitated to see whether the radioactivity could be removed. The low and uniform labelling is in apparent disagreement with the results obtained by incubating whole ascites cells *in vitro* (*cf.* 70). We admit, however, that the concentration of any highly reactive intermediate should be low *in vivo* and we also realize that during the long time required by our fractionation and extraction procedures the intermediates or their label may have been removed or relocated. An additional difficulty in correlating results obtained *in vitro* and *in vivo* was encountered recently in the final step of the process of protein synthesis, namely in the incorporation of amino acids into microsomal proteins. In liver microsomes the protein fractions with a high radioactivity post *in vivo* labelling turned out to have low radioactivity postlabelling *in vitro*, and vice versa (72). Recently a certain amount of uncertainty was introduced in the entire problem by the finding that the amino acyl-adenylates are highly chemically reactive compounds that can label non-enzymatically both proteins (73, 74) and nucleic acids (73) in *in vitro* systems in which relatively high concentrations of radioactive amino acyl-adenylates are present.

It appears from the preceding that a satisfactory correlation between results *in vivo* and *in vitro* cannot yet be established primarily because of insufficient information concerning initial synthetic steps *in vivo*. It is evident, however, that such a correlation is highly desirable since it can validate the remarkable scheme of the process of protein synthesis proposed by Zamecnik and his collaborators (42) on the basis of their results with *in vitro* systems.

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