(1964), *Zh. Obshch. Biol. 25*, 321.
Sporn, M. B., Wanko, T., and Dingman, W. (1962), *J. Cell Biol. 15*, 109.
Werbin, H., Chaikoff, I. L., and Imada, M. R. (1959), *Proc. Soc. Exptl. Biol. Med. 102*, 8.
Wyatt, G. R. (1951), *Biochem. J. 48*, 584.

Attachment of Glucosamine to Protein at the Ribosomal Site of Rat Liver*

Janos Molnar† and Dalisay Sy

ABSTRACT: Rats were treated first with glucosamine-^{14}C and then with puromycin and the effects on the bound glucosamine-^{14}C and sialic acid-^{14}C of microsomal subfractions of liver and plasma proteins were measured. A rapid decrease of protein-bound glucosamine-^{14}C in ribosomes, rough microsomal membranes, and smooth microsomes occurred and the appearance of glucosamine-^{14}C-labeled plasma protein was inhibited. The incorporation of sialic acid-^{14}C, derived from glucosamine-^{14}C *in vivo*, was inhibited only in the smooth microsomal fraction and plasma proteins. Ribosomes labeled with glucosamine-^{14}C *in vivo* were isolated and treated with puromycin; 18–34% of the ribosomal radioactivity was released due to puromycin. Most of the released material could be precipitated by 5% trichloroacetic acid. Factors (salts, temperature, and mercaptoethanol and puromycin concentration) enhancing the puromycin-dependent release of nascent polypeptides had parallel effects on the release of glucosamine-^{14}C-labeled proteins. The specific activity (disintegrations per minute per micromole of glucosamine) of the released polypeptides was ten times higher than that of the residual ribosomes. The experiments suggest that glucosamine attachment to glycoproteins begins while the polypeptides are still associated with tRNA at the ribosomal site.

Some of the basic problems in the synthesis of glycoproteins are the course and subcellular site of attachment of the connecting monosaccharide to the polypeptide backbone and the assembly of the other carbohydrates forming an oligosaccharide unit. A number of studies led to the conclusion that the oligosaccharide chains are put together on the polypeptide within the membranes of the endoplasmic reticulum after the polypeptide has been completed and separated from the ribosomes (Sarcione, 1964; Sarcione *et al.*, 1964; Molnar *et al.*, 1965a; Cook *et al.*, 1965; Spiro and Spiro, 1966; Bouchilloux and Cheftel, 1966; Lawford and Schachter, 1966). The appearance of protein-bound labeling of ribosomes isolated from livers of glucosamine-^{14}C-treated rats (Helgeland, 1965) and a kinetic analysis of this labeling (Molnar *et al.*, 1965a) have implicated, however, the ribosomes as a site of attachment of glucosamine to protein. This suggestion has been supported recently by Lawford and Schachter (1966) who observed that puromycin treatment of glucosamine-^{14}C-labeled liver homogenate yielded ribosomes with diminished radioactivity. In this paper we present further evidences for the involvement of ribosomes in the attachment of glucosamine to polypeptide.

Experimental Procedure

Materials and Methods

Holtzman strain rats weighing 300–400 g were starved overnight. Each rat received 4 μc of glucosamine-1-^{14}C (12 mc/mmole, New England Nuclear Corp.). After 30 min, 15 mg of puromycin dihydrochloride (Nutritional Biochemicals Corp.) in 0.2 ml of distilled water was also injected by the same route (all injections were made intraperitoneally). Control animals were treated in the same manner except puromycin was omitted. At given time intervals the animals were killed by decapitation; plasma proteins, liver ribosomes, rough microsomal membranes, and smooth microsomes were isolated as described previously (Molnar *et al.*, 1965a).

Isolation of Labeled Ribosomes. Rats were given 4 μc of glucosamine-^{14}C or 4 μc of L-leucine-U-^{14}C (210 mc/mmole, Volk Radiochemical Co.). The glucosamine-treated rats were killed 40 min later, while the leucine-^{14}C-treated rats were killed 20 min after injection. The

* From the Department of Biological Chemistry, College of Medicine, University of Illinois, Chicago, Illinois. *Received February 13, 1967.* This investigation was supported in parts by U. S. Public Health Service Grants USPHS 2591 and GRSG 153 and also by a grant from the American Cancer Society (ACS P-406). We are indebted to Dr. Richard J. Winzler for his initial support of this work by his Research Grant USPHS CA2951, and also for his helpful criticism of this paper.

† U. S. Public Health Service Career Development Award No. 5-K3-CA-11, 158-03.

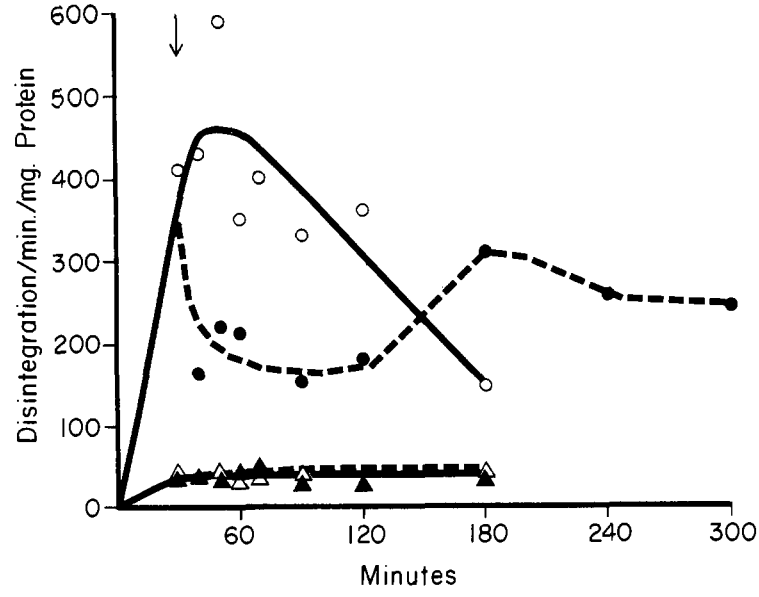

FIGURE 1: Appearance of radioactive glucosamine and sialic acid in ribosomes. Control: glucosamine (○——○) and sialic acid (△——△). Puromycin treated: glucosamine (●---●) and sialic acid (▲---▲). Each point in this and subsequent figures represents the average of at least two experimental results up to 180 min.

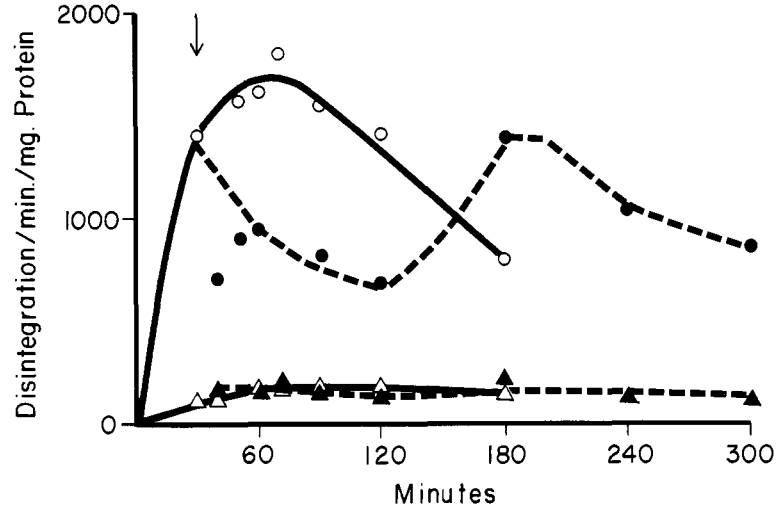

FIGURE 2: Incorporation of glucosamine and sialic acid into rough microsomal membranes. Control: glucosamine (○——○) and sialic acid (△——△). Puromycin treated: glucosamine (●- - -●) and sialic acid (▲- - -▲).

livers were perfused to be free of blood *in situ* with 0.9% NaCl, weighed, and homogenized with two volumes of 0.25 M sucrose containing 5 mM $MgSO_4$, 20 mM Tris-HCl (pH 7.8), 40 mM NaCl, 100 mM KCl, and 6 mM mercaptoethanol (medium M of Munro *et al.*, 1964). The homogenate was centrifuged at 15,000*g* for 10 min, the supernatant fluid was decanted, and the pellet was suspended and homogenized with the same volume of medium as used before. The centrifugation was repeated and the combined supernatants were treated with one-ninth volume of ice-cold 10% desoxycholate, introduced slowly while stirring the suspension. After 10 min more stirring at 0° 15 ml of suspension was layered over 20 ml of 1 M sucrose containing medium M per tube and centrifuged for 4 hr at 78,000*g* (average) in a Beckman L-2 ultracentrifuge (Munro *et al.*, 1964). The supernatant was carefully aspirated, and the ribosomal pellet was surface washed and suspended in medium M.

Preparation of Cell Sap. The procedure described by Munro *et al.* (1964) was used.

Treatment of Ribosomes with Puromycin. The condi-

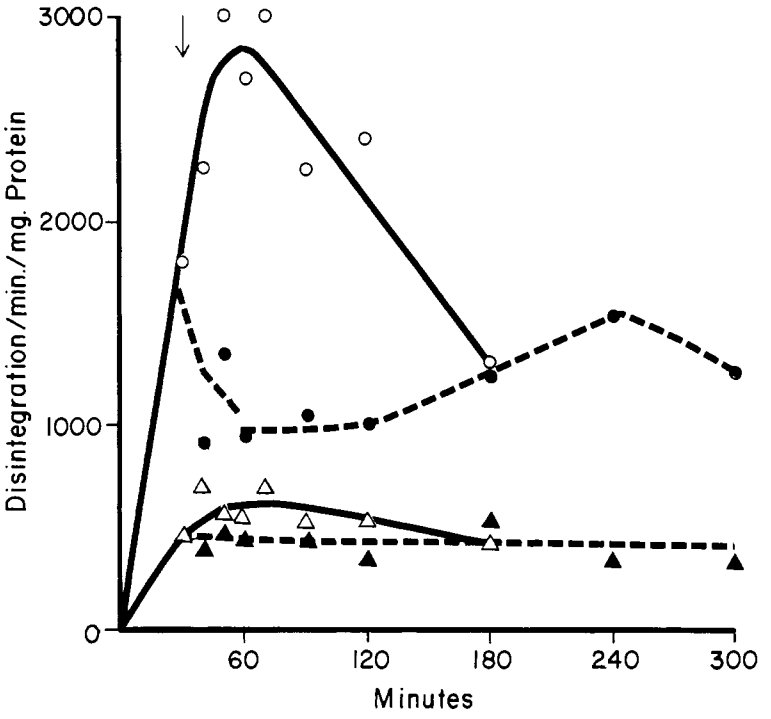

FIGURE 3: Incorporation of glucosamine and sialic acid into smooth microsomes. Control: glucosamine (O——O) and sialic acid (△——△). Puromycin treated: glucosamine (●- - -●) and sialic acid (▲- - -▲).

tions described for the release of nascent polypeptides by Hultin (1966) was adopted as specified in the text. After reaction, the mixtures were diluted to 11 ml with medium M and centrifuged at 105,000*g* for 1 hr; the supernatants were decanted and mixed with equal volumes of 10% trichloroacetic acid. If the incubation was carried out without cell sap 5 mg of bovine serum albumin was admixed to this supernatant prior to addition of trichloroacetic acid. The ribosomal pellets were suspended in distilled water and treated with equal volumes of 10% trichloroacetic acid. The mixtures were stored at 3° overnight, then centrifuged at about 1000*g* (low speed). The sediments were washed twice with 95% ethanol, dissolved in NaOH, and counted as described below.

Preparation of Other Samples for Chemical Analysis and Counting. Pellets were suspended in cold distilled water, and equal volumes of 10% trichloroacetic acid were added to these, to given supernatants, and to plasma. The precipitates were collected by low-speed centrifugation and washed with acid and organic solvents as described previously (Molnar *et al.*, 1965a).

Sialic acid was released by hydrolysis with 3 ml of 5% trichloroacetic acid at 80° for 1 hr. The mixtures were kept in ice for several hours after hydrolysis in order to aid coagulation of very fine particles. The precipitates were collected by low-speed centrifugation and washed with 1 ml of 5% trichloroacetic acid. The washing solutions were combined with the first supernatants and extracted with ether to remove trichloroacetic acid. The solutions were adjusted to pH 8 and chromatographed on a Dowex-1-Cl column to purify sialic acid (Molnar *et al.*, 1965a). Sialic acid and radioactivity were determined in both the hot acid extracts and purified samples. Because some of the bound glucosamine became solubilized by the hydrolysis, the portion of radioactivity not associated with sialic acid was added to the protein-bound glucosamine value as a correction factor.

The hot trichloroacetic acid insoluble residues were dissolved in 1–2 ml of 0.2 M NaOH and aliquots of these were used for measuring radioactivity, protein, and glucosamine contents. Glucosamine was released by hydrolysis with 3 N HCl at 100° for 4 hr.

Chemical Determinations. Sialic acid was measured according to Warren (1959), protein by the biuret reaction (Gornall *et al.*, 1949); glucosamine was purified and determined as described by Boas (1953); and radioactivity was measured as reported previously (Molnar *et al.*, 1964).

Results

Experiments in Vivo. When rats were treated with puromycin 0.5 hr after the administration of glucosamine-^{14}C a very rapid decrease of protein-bound glucosamine-^{14}C activity of ribosomes, rough microsomal membranes, and smooth microsomes of liver was observed (Figures 1–3). The appearance of protein-bound glucosamine-^{14}C in plasma proteins was diminished by the treatment (Figure 4).

Since glucosamine is converted partially to sialic

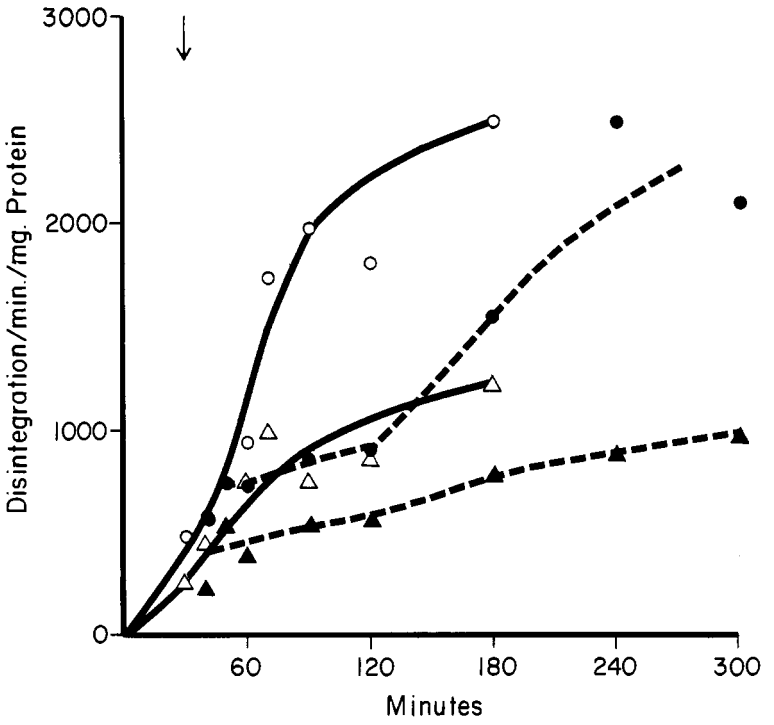

FIGURE 4: Incorporation of glucosamine and sialic acid into plasma proteins. Control: glucosamine (○——○) and sialic acid (△——△). Puromycin treated: glucosamine (●- - -●) and sialic acid (▲- - -▲).

TABLE I: Release of Glucosamine-^{14}C- and Leucine-^{14}C-Labeled Polypeptides by Puromycin.[a]

Additions (mM)				Acid-Insoluble Activity (cpm)					
				Glucosamine-^{14}C			Leucine-^{14}C		
KCl	Puromycin	Thiolethanol	Omission (°C)	Ribosomes	% Decrease	Released	Ribosomes	% Decrease	Released
100	1		0	566	53.0	484	1250	50	855
100	0		0	780	35.0	221	2000	20	210
600	1		0	400	65.7	650	1100	56	1350
600	0		Cell sap 0	690	42.5	265	2100	16	250
600	1		Cell sap 0	304	74.7	560	940	62.4	1350
600	0		0	624	48.0	221	2060	17.6	245
600	1	6	0	345	71.3	664	951	62.0	1450
600	0	6	0	660	45.0	320	2200	12.0	300
600	1		25	300	75.0	610	770	69.2	1340
600	0		25	710	41.0	300	1800	28.0	350

[a] Isolation of labeled ribosomes is described in Materials and Methods. Each reaction tube contained in addition to the specified reagents 5 mM $MgSO_4$, 40 mM Tris-HCl, (pH 7.8), 14 mg of protein, cell sap, and ribosomes in a total volume of 10 ml. The glucosamine-^{14}C-labeled ribosomes contained 6 mg of protein and 1200 cpm, while the leucine-^{14}C-labeled ribosomes had 8.8 mg of protein and 2500 cpm. The mixtures were incubated for 20 min at temperatures defined above and then treated as described in Materials and Methods.

acid *in vivo*, the effect of puromycin was also measured on the incorporation of sialic acid-^{14}C. In contrast to glucosamine, the incorporation of sialic acid into proteins of ribosomes and rough microsomal membranes was not affected markedly (Figures 1 and 2). These fractions contained very little radioactivity associated with sialic acid, as noted before (Molnar *et al.*, 1965a). Incorporation of sialic acid-^{14}C into

proteins of smooth microsomes and plasma proteins was inhibited by puromycin (Figures 3 and 4). The uptake of glucosamine by all liver fractions and plasma resumed about 90 min after the administration of puromycin.

Puromycin Treatment of ^{14}C-Labeled Ribosomes in Vitro. The experiments described in the former section showed that a sudden decrease of protein-bound glucosamine-^{14}C of ribosomes takes place after puromycin treatment of animals. This decrease may be attributed either to the lack of acceptor molecules and discharge of completed polypeptides containing some glucosamine labeling into the membranes of the endoplasmic reticulum, or to a release into the cytoplasm or into the channels of the endoplasmic reticulum of incomplete polypeptides containing glucosamine-^{14}C and puromycin due to reaction of polypeptidyl-tRNA with puromycin (Nathans, 1964). To test these possibilities, glucosamine-^{14}C- and leucine-^{14}C-labeled ribosome were isolated and treated with puromycin in test tubes. The reactions were performed as described recently by Hultin (1966) for the release of nascent polypeptides of liver. Table I shows the results of a typical experiment; 35–45% of glucosamine-^{14}C activity originally present in ribosomes was released by the salts alone. Puromycin, however, had an additional effect, releasing 18–34% of the original activity. This release was somewhat enhanced by increasing KCl concentration. Addition of thioethanol, omission of cell sap, and increasing the temperature of treatment had very little effects. Most of the released material was precipitable with 5% trichloroacetic acid. Puromycin had very similar effects on the release of nascent polypeptides labeled with leucine-^{14}C under the above conditions. Treatment of ribosomes with salts released 12–28% of the original activity and puromycin caused an additional discharge of radioactive molecules amounting to 35–44% of the initial activity present in ribosomes.

The effect was independent of puromycin concentration between 0.1 and 1 mM under the conditions of Table I using 0.6 M KCl. Treatment of ribosomes a second time with puromycin in the presence of 0.6 M KCl, under the conditions described in Table I, caused no change of the residual glucosamine-^{14}C activity of puromycin-treated ribosomes.

The unspecific release of radioactivity of ribosomes, observed in the control experiments, could be avoided by washing the ribosomes with the salt mixture used in the release experiments. Such ribosomes when treated with puromycin released about 50% of their glucosamine-^{14}C content, again in a form precipitable with trichloroacetic acid (Table II). This puromycin-dependent release occurred without the addition of cell sap. It appears that the enzyme(s) catalyzing this reaction are still present in the washed ribosomes in adequate quantities (see Hultin, 1966). The specific activity (disintegrations per minute per micromole of glucosamine) of the released polypeptides was ten times higher than that of the treated ribosomes (Table III).

In one experiment smooth microsomes labeled with glucosamine-^{14}C were isolated and treated with 1 mM puromycin in the presence of 0.1 and 0.6 M KCl and cell sap, under the conditions of Table I there was no measurable decrease of radioactivity of these microsomes. This experiment shows that the effect of

TABLE II: Release of Glucosamine-^{14}C-Labeled Polypeptides of Washed Ribosomes by Puromycin.[a]

Treatment of Ribosomes	Total TCA[b] Insoluble Activity (cpm) (related to 43 mg of protein of ribosomes) Ribosomes	Released
No	10,000	
KCl (1st)	6,400	1,400
KCl (2nd)	5,600	400
KCl (1st), puromycin	3,700	3,000

[a] Four rats were treated, each with 4 μc of glucosamine-^{14}C; after 40 min they were killed and ribosomes were isolated as described in Materials and Methods. First KCl treatment: 35 mg of protein of ribosomes was suspended in 20 ml of 0.6 M KCl containing 5 mM $MgCl_2$ and 50 mM Tris-HCl (pH 7.8). After incubation at 0° for 20 min the suspension was centrifuged at 105,000*g* for 90 min. Second KCl and puromycin treatments: the washed ribosomal pellet was suspended in 25 ml of salt mixture, as above; to 10 ml of this suspension puromycin was added to obtain 0.25 mM concentration. After 30 min at 0° this suspension and 10 ml of the remaining ribosomal suspension were centrifuged for 120 min at 105,000*g*. Aliquots of the described ribosomal and supernatant fractions were treated with trichloroacetic acid and washed as described in Materials and Methods. The final residues were hydrolyzed with 3 N HCl and glucosamine, and radioactivity were measured after purification by Dowex 50 resin. [b] TCA = trichloroacetic acid.

TABLE III: Glucosamine Specific Activities of Ribosomes and Released Polypeptides (described in Table II).

Treatment of Ribosomes	mμmole of Glucosamine/mg of Protein Ribosomes	dpm/μmole of Glucosamine Ribosomes	Released
No	1.5	21,500	
KCl (1st)	1.1	18,100	71,000
KCl (2nd)	1.04	19,000	
KCl (1st), puromycin	1.02	12,000	100,000

puromycin on the release of glucosamine-^{14}C-containing molecules is specific for the ribosomal particles.

Discussion

Most of the plasma glycoproteins studied so far have their oligosaccharide side chains linked to the polypeptide backbone through an asparaginyl-*N*-acetylglucosamine unit (core glucosamine). The outer region of the side chains may contain more *N*-acetylglucosamine, galactose, mannose, sialic acid, and fucose; the latter two are always in the terminal position (Sharon, 1966). Glucosamine-^{14}C administered to rats is taken up mainly by the liver where it is readily incorporated into glycoproteins, which are discharged to the blood (Shetlar, 1961; Kohn *et al.*, 1962; Robinson *et al.*, 1964). This incorporation occurs through the utilization of UDP[1]-*N*-acetylglucosamine (McGarrahan and Maley, 1962; DelGiacco and Maley, 1964; Molnar *et al.*, 1964). A part of glucosamine is converted to sialic acid which is also incorporated into glycoproteins. From these considerations it follows that we may expect radioactive labeling from glucosamine in three distinct positions of the oligosaccharide units, namely the core glucosamine, the outer region glucosamine, and the terminal sialic acid. A few experiments indicate that these three distinct "glucosamine" positions may be completed at different sites of the endoplasmic reticulum (Molnar *et al.*, 1965a; Lawford and Schachter, 1966). If so, the core glucosamine is expected to be attached close to or at the ribosomal site of protein synthesis. The involvement of ribosomes in such a reaction has been suggested by the observations that the specific activity–time curves of *in vivo* labeled ribosomes behaved as expected from a pool having a fast turnover. A kinetic analysis of ribosomal labeling on the other hand excluded the ribosomes as a site of attachment of most of the incorporated glucosamine, and the suggestion was made that the core glucosamine was the one most likely added at the ribosomes and the outer region glucosamine and sialic acid at different sites of the membranes of the endoplasmic reticulum. The present experiments revealed that a very rapid decrease of ribosomal glucosamine-^{14}C activity occurred by treatment of rats with puromycin. These experiments support the earlier ones in showing that the ribosomal glucosamine pool has a rapid turnover rate. This puromycin-induced decrease of ribosomal labeling may be attributed to a reaction of glucosamine-^{14}C-labeled polypeptidyl-tRNA with puromycin. In this reaction puromycin would replace the tRNA and concomitantly the affinity of the product to the ribosomes would be lost. A demonstration for the possibility of this reaction has been presented by Lawford and Schachter (1966). They treated glucosamine-^{14}C-labeled liver homogenates with puromycin and then isolated ribosomes. The protein-bound radioactivity of the treated ribosomes was considerably lower than that of the control ribosomes. Our experiments presented here correspond to the above. We have found that glucosamine-^{14}C-labeled ribosomes when treated with puromycin discharged 18–34% of their radioactivity.

Under similar conditions puromycin caused a 35–44% release of leucine-^{14}C-labeled polypeptides. Most of the released radioactive molecules (both glucosamine-^{14}C and leucine-^{14}C labeled) could be precipitated with 5% trichloroacetic acid, indicating that these molecules are fairly large.

Ribosomes labeled with glucosamine-^{14}C and treated previously with puromycin did not lose additional radioactivity when subjected to a second puromycin treatment. This and data shown in Table II suggested that the ribosomal radioactivity was due to three distinct components: the first could be released by washing the ribosomes with salts; the second required puromycin for release; and the third remained tightly bound to the ribosomes. The difference between these components was also evident in their respective specific activities: the salt-released component had 71,000 dpm/μmole of glucosamine; the puromycin-released material had 100,000 dpm/μmole and the residual ribosomes had 12,000 dpm/μmole. The high specific activities of the released materials indicate that these ribosomal components turn over rather rapidly. The precursor, UDP-*N*-acetylglucosamine, should have had about 300,000 dpm/μmole of activity at the time the animals were sacrificed (Molnar *et al.*, 1964, 1965a). The very low specific activity of the residual ribosomes suggests a slow turnover rate and may represent structural glucosamine, or some kind of tightly bound cell component.

The experiment showing that puromycin had no effect on the protein-bound glucosamine-^{14}C activity of smooth microsomes *in vitro* demonstrated that the release by puromycin was specific to the ribosomal particles. This means that the decrease of glucosamine-^{14}C activity of the rough microsomal membranes and smooth microsomes caused by puromycin treatment *in vivo* was due to completion of glycosylation of carbohydrate acceptors and discharge to the plasma of glycoproteins without replacing these with new acceptors. This can be realized in Figure 4 where it is shown that the appearance of radioactivity in the plasma proteins persists for a short period after puromycin administration and then declines.

These experiments altogether support the idea that a part of glucosamine-^{14}C of ribosomes is associated with polypeptides combined with tRNA. This then means that the attachment of the core glucosamine begins during the growth of polypeptide and thus defines the location of oligosaccharide units. How big this polypeptide has to be for such a reaction, what kind of intermediates are involved, and whether other types of linkages between carbohydrate and peptide are formed at the ribosomal site have to be investigated.

Previous experiments (Molnar *et al.*, 1965a); Lawford and Schachter, 1966) revealed that ribosomes and rough microsomal membranes contained, relative to glucosamine, very little radioactivity in sialic acid.

[1] Abbreviation used: UDP, uridine diphosphate.

These sialic acid activities varied very little in time, and puromycin had no measurable effects on them (Figures 1 and 2). These observations suggested that these particles are not a site of attachment of sialic acid. The smooth microsomes on the other hand contained radioactivity in sialic acid approaching that of plasma proteins, and the time course of labeling was suggestive as being the site of attachment of this carbohydrate. Puromycin caused measurable inhibition of sialic acid incorporation into this fraction as well as into plasma proteins. These findings support the idea that carbohydrates are added to the growing oligosaccharide chain at various parts of the endoplasmic reticulum, and sialic acid terminating this unit is attached at structures isolated with the smooth microsomes.

The rapid cessation of production and discharge to the plasma of glucosamine-^{14}C- and sialic acid-^{14}C-labeled proteins by puromycin indicates that the concentration of carbohydrate acceptors in the liver is rather limited and puromycin does not interfere with the transfer to the plasma. This is in contrast to the observations with Ehrlich ascites carcinoma cells (Cook *et al.*, 1965; and Molnar *et al.*, 1965b) and that of thyroid gland (Spiro and Spiro, 1966); in these tissues carbohydrate incorporation persists for longer times after puromycin treatment.

References

Boas, N. F. (1953), *J. Biol. Chem. 204*, 533.

Bouchilloux, S., and Cheftel, C. (1966), *Biochem. Biophys. Res. Commun. 23*, 305.

Cook, G. M. W., Laico, M. T., and Eylar, E. H. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 247.

DelGiacco, R., and Maley, F. (1964), *J. Biol. Chem. 239*, PC2400.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.

Helgeland, L. (1965), *Biochim. Biophys. Acta 101*, 106.

Hultin, T. (1966), *Biochim. Biophys. Acta 123*, 561.

Kohn, P., Winzler, R. J., and Hoffman, R. C. (1962), *J. Biol. Chem. 237*, 304.

Lawford, C. R., and Schachter, H. (1966), *J. Biol. Chem. 241*, 5408.

McGarrahan, J. F., and Maley, F. (1962), *J. Biol. Chem. 237*, 2458.

Molnar, J., Robinson, G. B., and Winzler, R. J. (1964), *J. Biol. Chem. 239*, 3157.

Molnar, J., Robinson, G. B., and Winzler, R. J. (1965a), *J. Biol. Chem. 240*, 1882.

Molnar, J., Lutes, R. A., and Winzler, R. J. (1965b), *Cancer Res. 25*, 1438.

Munro, A. J., Jackson, R. J., and Korner, A. (1964), *Biochem. J. 92*, 289.

Nathans, D. (1964), *Federation Proc. 23*, 984.

Robinson, G. B., Molnar, J., and Winzler, R. J. (1964), *J. Biol. Chem. 239*, 1134.

Sarcione, E. J. (1964), *J. Biol. Chem. 239*, 1686.

Sarcione, E. J., Bohne, M., and Leahy, M. (1964), *Biochemistry 3*, 1973.

Sharon, N. (1966), *Ann. Rev. Biochem. 35*, 623.

Shetlar, M. R. (1961), *Ann. N. Y. Acad. Sci. 94*, 44.

Spiro, R. G., and Spiro, M. J. (1966), *J. Biol. Chem. 241*, 1271.

Warren, L. (1959), *J. Biol. Chem. 234*, 1971.