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*BIOSYNTHESIS OF GLYCOPROTEINS OF THE EHRLICH ASCITES CARCINOMA CELL MEMBRANES**

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In mammalian systems, the biosynthesis of complex macromolecular structures containing protein, carbohydrate, and lipid is little understood. The present work was initiated to elucidate the biosynthesis of glycoprotein associated with cellular membranes of tumor cells. The microsomal fraction obtained from cellular homogenates is a rich source of membranes of the smooth and rough endoplasmic reticulum.[1] It has been demonstrated[2] in the case of the Ehrlich ascites carcinoma that membranes of the microsomal fraction contain large quantities of sialic acid which has been shown to be present in glycoprotein in this tumor.[3]

In this report the biosynthesis of membrane glycoprotein and protein has been studied *in vitro* using glucosamine-C^{14}, serine-C^{14}, and leucine-C^{14}, respectively, together with various inhibitors. Our data suggest that the carbohydrate moieties of membranes are added to completed polypeptide chains within the membrane complex of the rough endoplasmic reticulum.

Materials and Methods.—Preparation of tumor cell suspensions: A hyperdiploid strain of Ehrlich ascites carcinoma cells (originally provided by Dr. R. McKee, University of California at Los Angeles) was grown intraperitoneally in 7–8-week-old male Swiss white mice following inoculation of 0.1 ml of tumor-bearing peritoneal fluid. The tumor was harvested on the seventh or eighth day following inoculation and the cells were washed free of ascitic fluid as described previously,[4] except that Krebs III medium[5] was used in place of physiological saline. For incorporation studies *in vitro*, 1 vol of washed cells was incubated with 1.5 vol of Krebs III medium and 0.5 vol of an amino acid mixture[6] to which ovalbumin (2 mg/ml), L-glutamine (1 mg/ml), and D-glucose (2 mg/ml) had been added. When L-serine-C^{14} or DL-leucine-C^{14} incorporation was being studied, that amino acid was omitted. The final pH of the medium was 7.0 and the incubation was carried out in flasks open to the air at 37°, rocked at 60 cycles/min in a Dubnoff metabolic shaker. When puromycin (Nutritional Biochemicals Corp.) or tenuazonic acid (gift of Dr. H. T. Shigeura) were used, 2 ml of cells were incubated for 15 min; and with 6-azauridine (Calbiochem), for 40 min, before addition of 15 μc amino acid (DL-leucine-C^{14}, New England Nuclear, 104 dps/mg; L-serine-

C^{14}, Volk Radiochemical Co., 90 mc/mM, U.L.) or 12.5 μc D-glucosamine-C^{14} (Volk, 180 mc/mM, U.L.).

Preparation of smooth- and rough-surfaced membranes: Following incorporation of labeled compound, intact cells were washed twice in physiological saline,[4] resuspended in an equal volume of saline, and broken at 1500 psi in an Aminco-French pressure cell, previously cooled to 4°, using a Wabash hydraulic press. The homogenate was centrifuged at 20,200 *g* for 20 min at 0°. The supernatant fluid (1 ml from 1–5 ml of packed cells) was layered onto linear sucrose density gradients of density 1.10–1.20 and pH 7.5 (maintained with 0.5 *M* Tris buffer). The gradients were centrifuged at 105,000 *g* for 16 hr in the 40 rotor of the model L Spinco ultracentrifuge. The results from 32 separate runs have consistently shown a white semiopaque band of material (density 1.12–1.14) corresponding to smooth endoplasmic reticulum,[7, 8] and a milky aqueous layer at the top and a clear pellet at the bottom of the tube. The latter pellet corresponds to rough endoplasmic reticulum.[7, 8] The aqueous layer and smooth-surfaced membrane fractions were removed by aspiration, while the pellet was resuspended in 0.5 ml of the denser sucrose. All fractions were dialyzed exhaustively against distilled water at 4°, precipitated with 10% TCA, washed 3 times with cold 5% TCA, and then twice with ethanol-diethyl ether (1:1). The ethanol was added first and the sample thoroughly suspended before adding diethyl ether to the mixture. After drying at 80°, the precipitates were dissolved in 1 *N* NaOH solution, with warming, and applied to no. 3MM Whatman filter paper squares. The radioactivity was measured in a Nuclear-Chicago scintillation counter.

Isolation of hexosamine-C^{14}: A quantity of cells (4.0 ml) were incubated with 25 μc of glucosamine-C^{14}, and the processed smooth- and rough-surfaced membranes obtained as above. The residues were hydrolyzed at 80° for 2 hr in 0.03 *N* H_2SO_4 to remove sialic acid. The remaining residues were hydrolyzed with 2 *N* HCl at 100° for 6 hr to remove hexosamine. The H_2SO_4 hydrolysates were neutralized with saturated $Ba(OH)_2$ solution, the HCl hydrolysate was passed through Dowex-1-carbonate X2 columns, and the resulting solutions were assayed for sialic acid and hexosamine before lyophilization. The sugars were chromatographed on Whatman no. 3MM paper using *n*-butanol:*n*-propanol:0.1 *N* HCl (1:2:1 by volume) and pyridine:water:ethyl-acetate:acetic acid (5:3:5:1 by volume). Standard compounds were also chromatographed and sialic acids detected with orcinol reagent[9] and ninhydrin for hexosamines.[10]

Association of glucosamine-C^{14} and leucine-C^{14} with polysomal RNA: Cells (2 ml) were incubated 4 min, 20 min, and 2 hr with 30 μc D-glucosamine-C^{14} or 30 μc L-leucine-C^{14} (222 mc/mM U.L.). The rough endoplasmic reticulum was isolated and dissociated with a 1.3% w/v desoxycholate solution in 0.001 $MgCl_2$, 0.05 *M* KCl at pH 7.3 (maintained with 0.05 *M* Tris). Examination on linear sucrose density gradients of density 1.02–1.08 in the ultracentrifuge at 54,000 *g* for 5 hr in the SW 25.1 rotor was made.

Electron microscopy: Materials obtained from the sucrose gradient (density 1.10–1.20) fractionation of microsomal material, were kindly examined by Dr. R. F. Baker and Dr. B. Kramarsky, Department of Medical Microbiology. The specimens were fixed in buffered osmic acid (pH 7.4) for 1 hr, dehydrated in ethanol, embedded in Vestopal resin, and thin sections cut and examined at 16,000 magnification in the RCA EMU-3F electron microscope.

Assays: Sialic acid (as N-acetylneuraminic acid) was determined by the Warren technique[11] following neuraminidase (Behringwerke, Germany) release. RNA was assayed by the orcinol method,[12] using yeast RNA as standard, and protein by the microbiuret technique[13] with bovine serum albumin as standard. Hexosamine, as glucosamine-hydrochloride, was estimated by the modified Elson-Morgan method.[14]

Results and Discussion.—Cells were ruptured at 1500 psi in order to achieve optimal breakage while leaving the nuclei intact as judged by optical microscopy using an oil immersion lens.

The smooth-surfaced membrane fraction contained 0.04–0.10 per cent sialic acid (dry weight), and the RNA was beyond the limit of detection (~0.018% with quantities used). However, the rough-surfaced membranes contained 3.40–10.50 per cent RNA (dry weight), while the sialic acid content was beyond the limit of detection (0.008%). The range of sialic acid and RNA values probably reflects the efficiency

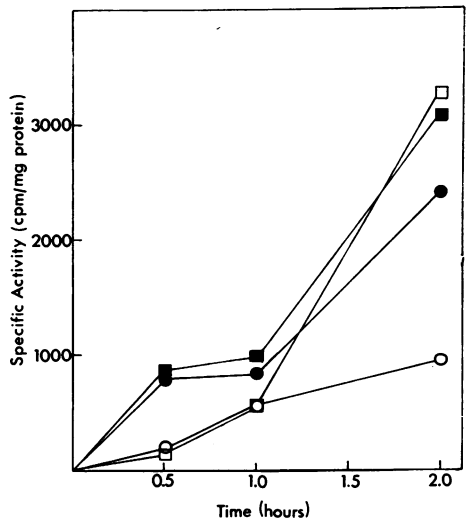

FIG. 1.—Incorporation of glucosamine-C^{14} into smooth membranes (■) and rough membranes (□), and leucine-C^{14} into smooth membranes (●) and rough membranes (○), as a function of hours of incubation *in vitro* of intact cells. Incubation and isolation techniques are as described in the text, except that the concentration of radioactive components was reduced by 75%.

of cellular breakage since younger cells probably show more resistance to rupture. In view of the almost identical ratios of smooth membrane sialic acid to rough membrane RNA in every case, the influence of mechanical disintegration is probably of secondary importance. The chemical data correlate well with the electron microscopy, where the smooth-surfaced membrane fraction appeared as a lacy membranous network devoid of nuclei, mitochondria, and microsomes. Ribosomes were, however, located in the rough endoplasmic reticulum. The RNA content of this fraction is close to the 7.4–10.4 per cent RNA reported for mouse liver microsomes.[15]

A typical incorporation of labeled glucosamine and amino acid into smooth- and rough-surfaced membranes is illustrated as a function of time in Figure 1. The data show a similarity in the incorporation of glucosamine and leucine into the smooth endoplasmic reticulum. In the rough membranes, there is a progressive increase in both glucosamine and leucine incorporation up to 1 hr, after which glucosamine incorporation is greatly enhanced. It appears that carbohydrate and protein are incorporated into the smooth membrane at the same rate.

The lag between 0.5 and 1 hr in glucosamine and leucine incorporation into the smooth membranes terminates with the enhanced incorporation of glucosamine into the rough membranes. This suggests that glycoprotein biosynthesis in the smooth membranes is limited by the carbohydrate synthesis.

The results of various inhibitors are shown in Table 1. Puromycin and tenuazonic acid, which both inhibit protein biosynthesis, but by different mechanisms,[16, 17] inhibit glucosamine incorporation into each fraction far less. This suggests that a sizeable pool of membrane protein exists in the cell and that the carbohydrate components turn over relatively slowly. This is in agreement with the results in Figure 1. It is possible that puromycin, which can release polypeptide from ribosomes,

TABLE 1

EFFECT OF INHIBITORS ON THE INCORPORATION OF C^{14}-LABELED GLUCOSAMINE AND AMINO ACIDS INTO VARIOUS CELLULAR FRACTIONS

Fractions	Inhibitor	% Inhibition* D-glucosamine-C^{14}	% Inhibition* L-serine-C^{14}	No. experiments
Smooth membranes	1.9×10^{-4} *M* puromycin	7–27	53–78	5
Rough membranes		28–29	71–82	
Extracellular protein		(+)2–14	45–89	
Smooth membranes	4.0×10^{-3} *M* tenuazonic acid	(+)20–(+)22	68 (L-leucine-C^{14}, ref. 17)	2
Rough membranes		(+)20		
Extracellular protein		17		
Smooth membranes	4.5×10^{-2} *M* 6-azauridine	80–96	50–96	3
Rough membranes		89–92	57–94	
Extracellular protein		84–94	87–112	

* Activity calculated as cpm/mg protein, except extracellular protein expressed as cpm/ml packed cells. Extracellular protein represents TCA-insoluble material secreted by the cells into the medium during the incubation period. The cells were incubated for 2 hr as described in text. With 6-azauridine, the inhibition is complete within 1 hr.

releases nearly completed polypeptide which can act as a receptor for carbohydrate. The possibility that carbohydrate is able to exchange independently of the polypeptide, though remote, is not excluded. The inhibition of *in vivo* biosynthesis of glycoproteins in liver by puromycin has been measured by glucosamine and leucine incorporation,[18] and is greatly in excess of that reported here for ascites tumor cells. The effect appears to vary considerably with time. In fact, when ascites tumor cells were incubated for 4 hr with puromycin, the inhibition of glucosamine incorporation into the various fractions rose to 50–66 per cent.

Azauridine, however, greatly inhibits both glucosamine and amino acid incorporation. It is likely that glucosamine is converted into a uridine diphosphate derivative before being incorporated into glycoprotein,[19] and that azauridine blocks synthesis at some point in this pathway. Also, a nonfunctional azauridine diphosphate glucosamine derivative might be formed. The fact that protein synthesis is also inhibited suggests that protein of the membranes may not be synthesized independently of the carbohydrate. In the case of pancreatic cells and liver, azauridine had no effect on protein biosynthesis.[20] However, in the case of Ehrlich ascites cells, the possible conversion of azauridine to phosphorylated derivatives could influence protein synthesis indirectly through inhibition of mRNA biosynthesis.[21]

We demonstrated by paper chromatography that glucosamine-C^{14} is incorporated into the smooth-surfaced membranes as N-acetylneuraminic acid (0.6% total activity), galactosamine (56.0%), and glucosamine (34.0%); in the case of rough-surfaced membranes, as galactosamine (57.0%) and glucosamine (34.0%). In neither case was any activity found in hexoses or protein (acid-insoluble residue or amino acids). The rest of the radioactivity, about 10 per cent, was recovered in compounds with low R_f values. These latter compounds probably represent oligosaccharides of aminosugars. In the case of the smooth endoplasmic reticulum, 47 μg hexosamine per 4.0 ml packed cells were found, but in the rough endoplasmic reticulum, hexosamine content was below the level of detection. The fact that glycoprotein, as judged by sialic acid and hexosamine content, is not detectable chemically in rough membranes, while labeled galactosamine and glucosamine are present in identical ratios in both membrane fractions, suggests strongly that the rough endoplasmic reticulum is a site of glycoprotein synthesis.

The glycolipids were excluded as sites of glucosamine incorporation by extraction before counting. The same results were obtained when two additional washes in

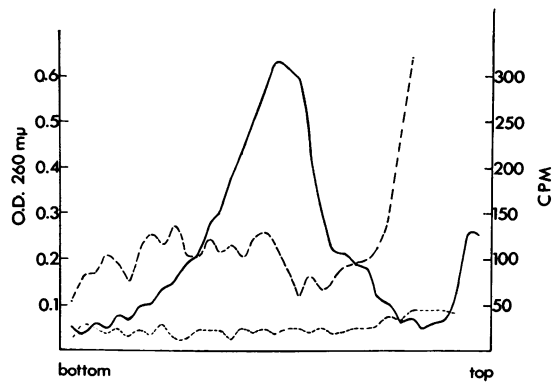

FIG. 2.—Sedimentation analysis in 5.0–20.0% (density 1.02–1.08) sucrose gradients of desoxycholate-dissociated rough-surfaced membranes which were obtained from cells incubated for 4 min at 37°. ————————, Absorption at 260 mμ (RNA). ———— ————, Incorporation of L-leucine-C^{14} into TCA-insoluble material. — — — — Incorporation of D-glucosamine-C^{14} into TCA-insoluble material. Identical patterns were obtained when 20-min or 120-min incubation times were used, except that the leucine-C^{14} content of the polysomes was much reduced. The peak in optical density near the midpoint in the gradient corresponds to the 80*S* ribosomes. Fractions (0.8 ml) were collected from the bottom of the gradient for optical density and radioactivity determinations. For the radioactivity determinations, 2 mg of ovalbumin was added to each fraction as carrier.

methanol were made. Sinohara and Sky-Peck,[22] using glucosamine-C^{14}, found that not more than 5 per cent of the total radioactivity of a microsomal fraction from mouse liver could be ascribed to glycolipid.

In order to locate the cellular site of glycoprotein biosynthesis, the sucrose density gradient analysis of desoxycholate-dissociated rough-surfaced membranes was performed following 4 min incubation with isotope. Appreciable quantities of leucine were associated with polysomal and ribosomal RNA (Fig. 2), as expected of normal protein biosynthesis.[23] However, with glucosamine, no activity was found in either polysomes or ribosomes. After incubation for 20 min, and 2 hr with leucine or glucosamine, no appreciable activity was found in polysomes or ribosomes. A mechanism for glycoprotein biosynthesis at the ribosomal level involving an sRNA-amino acid-hexosamine intermediate, or a synchronized attachment of carbohydrate to polysomal polypeptide, would therefore seem untenable. From our data, a nonsynchronous mechanism involving the synthesis of polypeptide at the polysome, followed by carbohydrate attachment within the membraneous complex of the rough endoplasmic reticulum, would seem more appropriate. Such a mechanism has been suggested by Sarcione[24] for liver glycoprotein biosynthesis.

It has been suggested[1] that the smooth endoplasmic reticulum is very diverse in origin, and possibly consists of the golgi apparatus, other regional differentiations of the endoplasmic reticulum, and plasma membranes. In view of the extensive evidence[2, 4, 25] based on neuraminidase treatment and electrophoresis that most of the sialic acid of Ehrlich ascites tumor cells, like erythrocytes,[26] resides on the surface membrane, this acid might therefore be considered a surface marker. Furthermore, Gasic and Berwick[27] showed by electron microscopy that sialic acid is largely associated with the surface regions of mouse ascites cells. Thus, large amounts of sialic acid of the smooth endoplasmic reticulum fraction, about 75 per

cent of the total,[25] indicate that surface membranes are present in this fraction. It is highly probable, therefore, that the data presented here describes the biosynthesis of surface membrane glycoprotein.

Summary.—The separation of smooth and rough endoplasmic reticulum and the incorporation of labeled glucosamine and amino acids into these fractions have been described. The inhibition of the protein synthesis by puromycin and tenuazonic acid greatly exceeded inhibition of carbohydrate synthesis up to 2 hr, whereas both protein and carbohydrate syntheses were inhibited by azauridine. Labeled hexosamine of the microsomes was found to be associated with the membranes only, and not with the ribosomes. It was hypothesized from these experiments that the biosynthesis of the glycoprotein of the surface membrane, which is present in the smooth endoplasmic reticulum fraction, takes place at the microsome in two steps. First, the polypeptide is synthesized at the polysome, and then the polypeptide is linked to the carbohydrate within the membraneous complex.

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