

## Studies on the Golgi Apparatus

### CUMULATIVE INHIBITION OF PROTEIN AND GLYCOPROTEIN SECRETION BY D-GALACTOSAMINE

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1. The administration of D-galactosamine leads to inhibition of protein and glycoprotein secretion by rat liver. To test the secretory function, the secretion times for galactose- and fucose-containing glycoproteins were determined; they were lengthened from 6 to 9 min and from 8 to 13 min respectively. 2. The Golgi apparatus was enriched 100–120-fold relative to the homogenate. A new linked-assay system for the marker enzyme, UDP-galactose-N-acetyl-D-glucosamine galactosyltransferase, is presented. The activity of the enzyme was measured spectrophotometrically by following the formation of UDP coupled to nicotinamide nucleotide reduction. The Michaelis constants were calculated to be 0.11 mM for UDP-galactose with N-acetyl-D-glucosamine as exogenous acceptor and 19 mM for N-acetyl-D-glucosamine itself. 3. The physiological substrate of the galactosyltransferase, UDP-galactose, can be replaced by UDP-galactosamine, which accumulates after D-galactosamine administration. Under conditions *in vitro* the rate of D-galactosamine transfer to an endogenous acceptor protein of the Golgi fraction reaches 9% of that with D-galactose; this finding is noteworthy, because normally a non-acetylated amino sugar does not occur in glycoproteins. 4. The albumin content of the Golgi-rich fraction was diminished to 55% of the reference value 6 h after the injection of 375 mg of D-galactosamine hydrochloride/kg body wt. The transfer of D-[1-<sup>14</sup>C]galactose to an endogenous acceptor protein fell to 60% compared with Golgi-rich fractions from untreated animals. Analysis of the Golgi-rich fraction by polyacrylamide-gel electrophoresis showed a decrease or loss of several protein bands. 5. Protein synthesis can be restored by up to 80% if the UTP pool, decreased after D-galactosamine administration, is filled up by several injections of uridine. 6. From the results presented it can be concluded that the disturbed secretion of proteins and glycoproteins was due to a cumulative effect of galactosamine by: (a) inhibition of protein synthesis leading to a diminution of the endogenous acceptor pool of the galactosyltransferase; (b) inhibition of the galactosyltransferase activity by galactosamine metabolites and (c) replacement of UDP-galactose by UDP-galactosamine.

It is well established that the Golgi apparatus is involved in the synthesis and secretion of lipo- and glyco-proteins (Caro & Palade, 1964; Maley *et al.*, 1969; Rambourg *et al.*, 1969; Morré *et al.*, 1971). Radioautographic studies on the uptake of <sup>3</sup>H-labelled galactose showed the preferential incorporation of radioactivity into the Golgi region of various cells secreting glycoproteins (Neutra & Leblond, 1966). The discovery of specific glycosyltransferases in the Golgi apparatus (Wagner & Cynkin, 1969a; Fleischer *et al.*, 1969) strongly supports the significance of this site in glycosylation of proteins. It is assumed that the first sugar attached, N-acetyl-D-glucosamine (Molnar *et al.*, 1965; Lawford & Schachter, 1966), is transferred directly to the nascent glycoprotein at the rough endoplasmic reticulum, whereas D-mannose (Caccam *et al.*, 1969), additional molecules of N-acetyl-D-glucosamine

(Wagner & Cynkin, 1969b; Tetas *et al.*, 1970), D-galactose (Hagopian *et al.*, 1968; Fleischer *et al.*, 1969), L-fucose (Haddad *et al.*, 1971) and N-acetyl-D-neuraminic acid (Hudgin *et al.*, 1971) are incorporated at the smooth endoplasmic reticulum, including the Golgi apparatus.

During the induction of galactosamine hepatitis (Reutter *et al.*, 1968; Keppler *et al.*, 1968; Lesch *et al.*, 1970), morphological alterations of the endoplasmic reticulum were observed (Medline *et al.*, 1970) and alterations of glycoprotein synthesis found (Reutter *et al.*, 1969). In order to study these effects in more detail, we investigated the influence of D-galactosamine on the function and composition of the Golgi apparatus. An early event after D-galactosamine administration is the accumulation of D-galactosamine metabolites, galactosamine 1-phosphate, UDP-hexosamines, N-acetylhexosamine

phosphates, UDP-*N*-acetylhexosamines (Keppler & Decker, 1969; Bauer *et al.*, 1972). The metabolism of D-galactosamine is accompanied by a marked decrease in UTP+UDP, UDP-glucose and UDP-galactose (Keppler & Decker, 1969; Keppler *et al.*, 1970a). UDP-galactose is the substrate for the galactosyltransferase (Morré *et al.*, 1969; Wagner & Cynkin, 1971), for which a new linked-assay system is described. Data show that the administration of D-galactosamine leads to an inhibition of protein and glycoprotein secretion, which is due to a cumulative effect.

## Materials and Methods

### Animals

Male Wistar rats (Ivanovas, Kisslegg, Germany), weighing about 180 g each, were fed on a commercial diet (Altromin; Altromin G.m.b.H., Lage-Lippe, Germany) and given water *ad libitum*. The diet contained 18–20% (w/w) protein. The animals were kept in a room with natural lighting supplemented by overhead fluorescent lights during daytime.

### Chemicals

D-Galactosamine hydrochloride (puriss.) and *N*-acetyl-D-glucosamine were purchased from C. Roth OHG (Karlsruhe, Germany). The purity was checked by ion-exchange chromatography with an amino acid analyser (Biocal model 200) (Bauer *et al.*, 1972) and paper electrophoresis in 0.1M-sodium borate buffer, pH 8.8.

D-[1-<sup>14</sup>C]Galactosamine (2.7mCi/mmol), UDP-[<sup>14</sup>C]galactose (245mCi/mmol), L-[1-<sup>14</sup>C]leucine (61mCi/mmol) and [<sup>14</sup>C]toluene were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. D-[1-<sup>14</sup>C]Galactosamine 1-phosphate and UDP-D-[1-<sup>14</sup>C]galactosamine were prepared enzymically *in vitro* or *in vivo* as described (Bauer *et al.*, 1972; Bauer & Reutter, 1973).

The enzymes UDP-glucose-NAD<sup>+</sup> oxidoreductase (EC 1.1.1.22), ATP-nucleoside diphosphate phosphotransferase (EC 2.7.4.6), and UTP- $\alpha$ -D-glucose 1-phosphate uridylyltransferase (EC 2.7.7.9), and the coenzymes ATP, NAD<sup>+</sup> and the substrate glucose 1-phosphate were supplied by Boehringer Mannheim G.m.b.H. (Mannheim, Germany). UDP-galactose came from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). 2-(*p*-Iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride was bought from T. Schuchardt (München, Germany), and bovine serum albumin (electrophoretic purity 100%) from Behringwerke A.G. (Marburg-Lahn, Germany). Radiochemically pure rat albumin was a kind gift from Dr. G. Schreiber, Biochemisches Institut der Universität Freiburg, Germany (Schreiber *et al.*,

1969). All other chemicals of analytical grade were obtained from E. Merck A.G. (Darmstadt, Germany).

### Methods

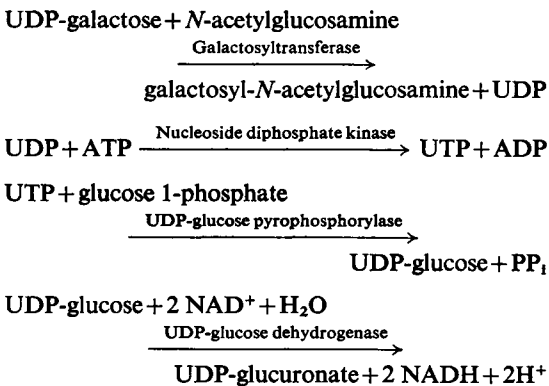
*Measurement of glycoprotein secretion.* Schreiber *et al.* (1969, 1971) have described a method of determining the kinetics of serum protein secretion. This procedure was modified by replacing the radioactive labelled amino acid by D-[1-<sup>14</sup>C]galactose or L-[<sup>3</sup>H]fucose (Reutter, 1971). At 5 or 6 h before the intravenous injection of 100  $\mu$ Ci of D-[1-<sup>14</sup>C]galactose or 100  $\mu$ Ci of L-[<sup>3</sup>H]fucose/kg body wt., 375 mg of D-galactosamine hydrochloride/kg body wt. was administered intraperitoneally. Blood samples (0.2 ml each) were withdrawn from the caval vein at timed intervals while the animals were under pentobarbital anaesthesia. The protein-bound radioactivity was determined as described by Mans & Novelli (1960). The specific radioactivity of glycoproteins was plotted as a function of time. The time between the injection of labelled D-galactose or L-fucose and the appearance of labelled glycoproteins in the serum is called 'galactose-time' or 'fucose-time' respectively.

*Preparation of a Golgi-rich fraction.* Methods for the isolation of the Golgi apparatus have been described in detail by several authors (Morré *et al.*, 1969; Fleischer *et al.*, 1969). This procedure is partly based on techniques developed by Morré *et al.* (1969) and Leelavathi *et al.* (1970). The livers were perfused *in situ* with 25 ml of 0.15M-NaCl solution while the animals were under ether anaesthesia. All subsequent operations were carried out at 4°C. The livers were squeezed through a sieve (sieve pore 0.8 mm diam.) to remove the connective tissue. The minced liver (10 g) was suspended in 5 vol. of 0.5M-sucrose containing 0.1M-Tris-HCl buffer (pH 7.0) and 0.5% (w/v) dextran T-500, followed by homogenization for 30–40 s at slow speed in a Torpedo homogenizer (Emmendinger Maschinenbau G.m.b.H., Emmendingen, Germany), equipped with a thyristor regulator (Janke & Kunkel KG, Staufen, Germany). The mixture was centrifuged for 15 min at 400g, the supernatant collected and centrifuged at 16000g for 30 min. Then the sediments were carefully suspended in 16 ml of 1.6M-sucrose buffer with 10 strokes of a Dounce homogenizer with a type L pestle. A portion (8 ml) of this suspension was placed in the bottom of an SW 27 cellulose nitrate tube and overlaid with the following step gradient: 5 ml of 1.25M-sucrose, 6 ml of 1.0M-sucrose, 8 ml of 0.95M-sucrose, 5 ml of 0.9M-sucrose, and finally 5 ml of 0.5M-sucrose. All gradient solutions were prepared in 0.1M-Tris-HCl buffer (pH 7.0) and 0.5% (w/v) dextran. According to Morré *et al.* (1971) the addition of bivalent cations is not essential for the isolation of the Golgi apparatus and therefore no Ca<sup>2+</sup>, Mg<sup>2+</sup> or Mn<sup>2+</sup> ions were added to the homogenization medium and gradient

solutions. The density of the solutions was controlled with a Zeiss refractometer (C. Zeiss, Oberkochen, Germany). The gradient was centrifuged for 90 min at 25000 rev./min (82000  $g_{av.}$ ) in an SW 27 rotor. The Golgi-rich fraction was localized mainly at the 0.95 and 0.9M-sucrose interface. The membranes were removed from the gradient by using a pipette fitted with a rubber aspirator, washed once in Tris-HCl buffer and were collected after centrifugation at 10000g for 10 min.

To investigate the influence of D-galactosamine on the Golgi apparatus, the rats received a single injection of 375 mg of D-galactosamine hydrochloride (1.74 mmol)/kg body wt. and were killed 3, 6 or 12 h thereafter. For incorporation studies 100 or 300  $\mu$ Ci of L-[1- $^{14}$ C]leucine/kg body wt. was injected into the tail vein 60 min before death.

*Assay of galactosyltransferase activity.* The activity of this marker enzyme of the Golgi apparatus is usually measured by a radiochemical assay originally developed by Babad & Hassid (1966) and later modified by Fleischer *et al.* (1969). Owing to the time-consuming chromatography (50–72 h), this test is not suitable for routine investigations. Therefore a linked-assay system has been developed. Activity is measured spectrophotometrically at 334 nm and 37°C by following the liberation of UDP coupled to nicotinamide nucleotide reduction:



Kalckar & Anderson (1957) described an enzymic assay for the determination of UTP, and Keppler *et al.* (1970b) gave a sequential enzymic assay for the determination of the uracil nucleotides UDP-glucose, UTP, UDP and UMP.

The standard assay mixture in a final vol. of 0.85 ml (pH 7.6) contained glycine (300  $\mu$ mol), sodium cacodylate (120  $\mu$ mol),  $\text{NAD}^+$  (1.5  $\mu$ mol), ATP (2  $\mu$ mol), glucose 1-phosphate (1.5  $\mu$ mol),  $\text{MnCl}_2$  (40  $\mu$ mol), magnesium acetate (10  $\mu$ mol), 2-mercaptoethanol (10  $\mu$ mol), UDP-galactose (0.5  $\mu$ mol), UDP-glucose dehydrogenase (0.1 unit), UDP-glucose pyrophosphorylase (2.5 units), nucleoside diphosphate kinase (2 units), and approx. 100  $\mu$ l of sus-

ended Golgi-rich fraction (0.1–0.2 mg of protein). The reaction was initiated by the addition of 50  $\mu$ mol of *N*-acetyl-D-glucosamine.

The assay does not include a detergent, because it destroys the native membrane structure making it difficult to compare studies *in vitro* and *in vivo*. Probably for this reason Wagner & Cynkin (1971) do not add any detergent when determining the transfer of glucosamine, galactose or *N*-acetylneuraminic acid to an endogenous protein acceptor. The galactosidase does not influence the determination of galactosyltransferase activity, because the extinction remains constant as long as the galactosyl acceptor *N*-acetyl-D-glucosamine has not been added. The possible influence of galactosidase activity on the new linkage formed was not studied. In control experiments the activity of galactosyltransferase was determined in a radiochemical assay with UDP-D-[1- $^{14}$ C]galactose as substrate (Fleischer *et al.*, 1969). In liver cells, free *N*-acetyl-D-glucosamine does not occur and is therefore of minor importance as an acceptor for D-galactose. Therefore, where indicated, activity was determined by using an endogenous trichloroacetic acid-precipitable protein of the Golgi-rich fraction as the galactosyl acceptor as described by Wagner & Cynkin (1969a,b).

*Enzymic assays.* Published procedures were used to measure the enzymes 5'-nucleotidase (EC 3.1.3.5) (Michell & Hawthorne, 1965), glucose 6-phosphatase (EC 3.1.3.9) (de Duve *et al.*, 1955) and succinate-2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride reductase (Pennington, 1961).

*Protein determinations.* The protein content of membrane fractions was determined by the method of Lowry *et al.* (1951). For serum and homogenates the biuret method was preferred (Beisenherz *et al.*, 1953). Before the addition of the biuret reagent protein was precipitated either by 10% (w/v) trichloroacetic acid or by dioxan. Crystalline bovine serum albumin was used as a standard.

*Determination of radioactivity.* Protein-bound radioactivity was determined by the method of Mans & Novelli (1960) with slight modifications. A sample (0.1–0.2 ml) of the homogenate, Golgi-rich fraction or serum was pipetted on to filter-paper discs (Whatman 3MM, 2.3 cm diam.), which had been mounted on pins. The discs were dried and then transferred to chilled 10% (w/v) trichloroacetic acid to precipitate the protein. The discs were left for 60 min at 4°C and then subjected to the following washing procedure to remove free amino acids or non-protein materials: 15 min at 4°C in 5% (w/v) trichloroacetic acid, 30 min at 90°C in 5% (w/v) trichloroacetic acid, 15 min at 4°C in 5% (w/v) trichloroacetic acid, 30 min at 37°C in ether-ethanol (1:1, v/v), and finally 15 min at 22°C in ether. However, when galactosyltransferase was assayed by using an endogenous acceptor protein, heating

was omitted, since under these conditions 10% of the D-galactose incorporated will be liberated by acid hydrolysis.

Since counting efficiency depends on protein concentration (Schreiber *et al.*, 1971), comparable amounts of protein were always applied to the filter paper discs. Radioactivity was determined by counting the air-dried discs in 10 ml of toluene scintillation mixture (Kallmann *et al.*, 1958) in a Packard liquid-scintillation spectrometer. Radiochemically pure rat [ $^{14}\text{C}$ ]albumin was used as a standard. Radioactivity was measured until sufficient counts had accumulated to give a counting error of  $\pm 5\%$  after subtraction of background counts.

**Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis.** A portion (200  $\mu\text{l}$ ) of suspended Golgi membranes (220  $\mu\text{g}$  of protein) was sedimented in an Eppendorf centrifuge (Netheler and Hinz G.m.b.H., Hamburg, Germany) for 10 min at 8000g. Then 200  $\mu\text{l}$  of 0.1M-sodium phosphate buffer (pH 7.1), 1.7  $\mu\text{mol}$  of sodium dodecyl sulphate, and 18  $\mu\text{mol}$  of 2-mercaptoethanol were added to the pellet and the mixture was incubated for 3 min at 100°C. Half of the sample was transferred to a second Eppendorf tube and to both of them 0.2  $\mu\text{mol}$  of Bromophenol Blue and a drop of distilled glycerol were added. The samples were subjected to electrophoresis in a Tris-glycine buffer system, pH 8.3 (Tris, 0.012M; glycine, 0.16M; sodium dodecyl sulphate, 0.004M) for both upper and lower chambers. The preparation of the polyacrylamide gel with 10% (w/v) acrylamide in the running gel was described in detail by Glossmann & Lutz (1970). The current (2mA/tube) was maintained until the dye marker Bromophenol Blue had reached the lower end of the tube. The gels were stained for 90 min with Coomassie Brilliant Blue (0.25%) in ethan-1-ol-acetic acid (9:1, v/v) and were destained for 30 min in methanol-acetic acid-water (2:3:35, by vol.).

**Isolation and determination of albumin.** Albumin was liberated from the Golgi vesicles and purified as described by Glaumann & Ericson (1970). Portions (2–15  $\mu\text{g}$  of protein) of the ethanol extracts were subjected to polyacrylamide-gel electrophoresis with ribonuclease A as the marker protein. Radiochemically pure rat liver albumin was used for comparison or was added to the samples before electrophoresis. Only one major band was seen with the same relative mobility compared with ribonuclease A as rat liver albumin. To determine the amount of albumin present in the sample, density tracings of the gels were recorded with a Gilford spectrometer equipped with the linear transport accessory (Gilford Instruments, Oberlin, Ohio, U.S.A.), with the following operation procedure: wavelength 550 or 630 nm, aperture plate 0.10 mm  $\times$  2.36 mm, filter 410–660 nm, scan rate 1 cm/min, chart speed 1.27 cm (0.5 in)/min. By using various concentrations of standard albumin a calibration curve was established. The maximum extinction value is a more suitable measure for the protein content/band than the area beneath the extinction curve. Up to 10  $\mu\text{g}$  of albumin/band, a linear increase in extinction was observed.

The density tracings were recorded, then the gels were sliced and the bands containing radioactively labelled albumin removed and pressed through a sieve (sieve pores 0.2 mm diam.). The radioactivity was measured in Bray's (1960) scintillation mixture.

## Results

### Secretion of glycoproteins

The normal 'galactose-time' (see the Materials and Methods section) was between 5 and 6 min. Owing to the injection of D-galactosamine this time was prolonged to between 8 and 10 min (Fig. 1). Similar results were obtained with L-[ $^3\text{H}$ ]fucose. The 'fucose-time' was lengthened from 8 to about 13 min (Fig. 1).

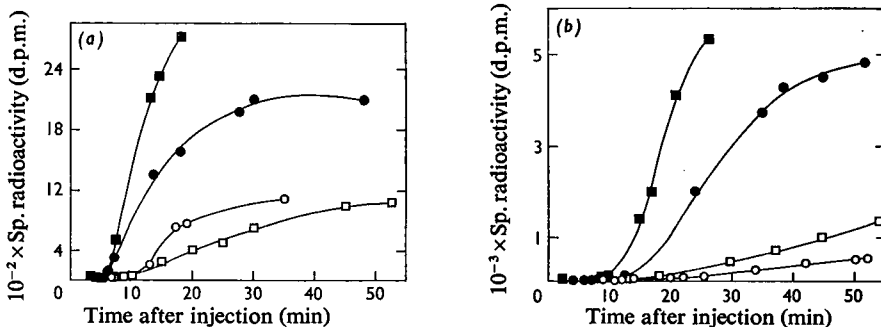


Fig. 1. Specific radioactivity of glycoproteins in the serum of rats

D-Galactosamine was injected at 08.30 h; at 13.30 h the rats received an intravenous injection of 100  $\mu\text{Ci}$  of D-[1- $^{14}\text{C}$ ]galactose (a) or L-[ $^3\text{H}$ ]fucose (b) /kg body wt. ●, ■, Sp. radioactivity of control animals; ○, □, Sp. radioactivity after D-galactosamine administration.

Table 1. Activities of marker enzymes of the homogenate and the Golgi-rich fraction

D-Galactosamine hydrochloride (375 mg/kg body wt.) was injected intraperitoneally. The injections were given at 08:30h, and the animals were killed either at 14:30h or 20:30h. Units of specific activity are given as  $\mu\text{mol}$  of P<sub>i</sub> released/h per mg of protein, except for succinate-2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride reductase which is given as  $\mu\text{mol}$  of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride reduced/h per mg of protein. All enzymes were assayed at 37°C. Values are means from ten determinations  $\pm$ s.d.

Enzyme	Cell fraction	Specific activity ( $\mu\text{mol}/\text{h}$ per mg of protein)		
		Control	At 6h after D-galac- tosamine	At 12h after D-galac- tosamine
5'-Nucleotidase	Homogenate	3.9 $\pm$ 0.6	3.5 $\pm$ 0.5	3.1 $\pm$ 0.6
	Golgi-rich	3.6 $\pm$ 0.4	3.6 $\pm$ 0.3	3.4 $\pm$ 0.6
Glucose 6-phosphatase	Homogenate	4.3 $\pm$ 0.9	5.2 $\pm$ 0.6	5.0 $\pm$ 0.6
	Golgi-rich	4.5 $\pm$ 1.1	6.2 $\pm$ 1.0	6.3 $\pm$ 0.6
Succinate-2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride reductase	Homogenate	0.74 $\pm$ 0.11	0.85 $\pm$ 0.10	0.80 $\pm$ 0.10
	Golgi-rich	0.04 $\pm$ 0.01	0.05 $\pm$ 0.01	0.07 $\pm$ 0.02

Table 2. Specific activity of UDP-galactose-N-acetyl-D-glucosamine galactosyltransferase

For comparison, the activity of the transferase was determined in a radiochemical assay. The incubation mixture of galactosyltransferase contained 6  $\mu\text{mol}$  of sodium cacodylate buffer (pH 6.75), 4  $\mu\text{mol}$  of MnCl<sub>2</sub>, 1  $\mu\text{mol}$  of MgCl<sub>2</sub>, 3  $\mu\text{mol}$  of 2-mercaptoethanol, 0.15  $\mu\text{mol}$  of UDP-[<sup>14</sup>C]galactose and 50  $\mu\text{l}$  of Golgi-rich fraction (50-150  $\mu\text{g}$  of protein), in a total volume of 0.1 ml. The reaction was started by the addition of 4  $\mu\text{mol}$  of N-acetyl-D-glucosamine. Incubation was carried out for 30 or 60 min at 37°C. Blank incubation mixtures contained no N-acetyl-D-glucosamine. The incubation was terminated by addition of 12  $\mu\text{mol}$  of EDTA (pH 7.4) and heating for 60s at 90°C, then the entire reaction mixtures were applied to Whatman DE-81 paper and chromatographed for 48-72h with butan-1-ol-propan-1-ol-water (3:1:1, by vol.). The amount of radioactivity was determined by counting successively 0.5 or 1.0cm-wide strips in 10ml of toluene scintillation mixture (Kallmann *et al.*, 1958). The unit of specific activity is expressed in nmol of D-galactose transferred/h per mg of protein at 37°C. Values are means  $\pm$  s.d. from five determinations.

Cell fraction	Specific activity (nmol/h per mg of protein)
Homogenate	2.6 $\pm$ 0.3
Supernatant after the first centrifugation	8.0 $\pm$ 1.5
Membrane fraction after the second centrifugation	52.0 $\pm$ 4.3
Golgi-rich fraction after density-gradient centrifugation	310.0 $\pm$ 20

Moreover, the amount of secreted glycoprotein was markedly decreased, as shown by measurements of the specific radioactivity of serum glycoproteins.

*Purity of the Golgi-rich fraction*

The purity of the Golgi-rich fraction was checked by both morphological (Plate 1) and biochemical criteria. In comparison with data from the literature (Morré *et al.*, 1969; Leelavathi *et al.*, 1970; Fleischer & Fleischer, 1970), the preparations were contaminated to a lesser extent by plasma membranes but showed a slightly higher content of fragments from the endoplasmic reticulum. The specific activity of 5'-nucleotidase, as a marker enzyme for plasma membranes, was only approx. 5% of that of enriched plasma-membrane fractions (Reutter & Bachmann, 1971). The contamination by mitochondria was negligible (Table 1), as shown by measuring succinate-2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride reductase activity as a sensitive marker enzyme for mitochondria (Pennington, 1961). The best-known marker enzyme of the Golgi apparatus is UDP-galactose-N-acetyl-D-glucosamine galactosyltransferase (Morré *et al.*, 1969; Fleischer *et al.*, 1969). This enzyme was enriched 100-120-fold relative to the homogenate (Table 2). Injection of D-galactosamine hydrochloride had no significant influence on the purity of the Golgi-rich fraction, as shown by the specific enzyme activities (Table 1).

*Characteristics of liver galactosyltransferase*

**pH optimum.** Fig. 2 shows the effect of pH on the transfer of D-galactose from UDP-galactose to the exogenous acceptor N-acetyl-D-glucosamine. A broad pH optimum was found between pH 7.4 and 8.2. In glycine-cacodylate buffer maximum activity occurred at about pH 7.5.

**Protein- and time-dependency.** Fig. 3 shows that the enzyme activity directly depends on protein content, i.e. the transfer of D-galactose increases with

the amount of Golgi-rich fraction added. A similar correlation was measured in investigations of time-dependency. After initiation of the reaction by addition of *N*-acetyl-D-glucosamine the progress curve showed a short lag period for about 4 min and was then linear with time up to 30 min.

*Effects of substrate and acceptor concentrations.* The effect of UDP-galactose and *N*-acetyl-D-glucosamine on transferase activity was determined.

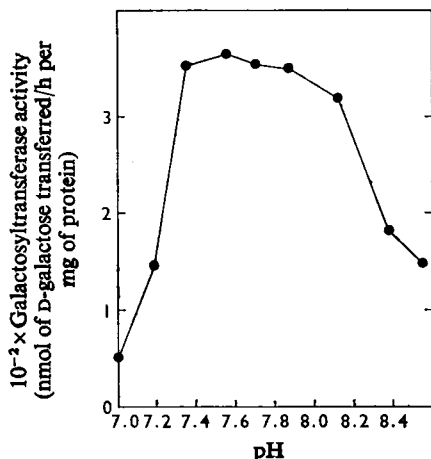
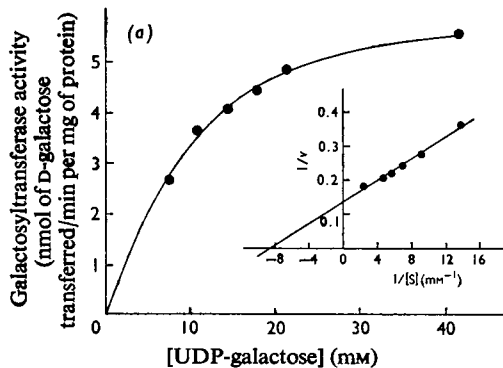


Fig. 2. Effect of pH on UDP-galactose-*N*-acetyl-D-glucosamine galactosyltransferase

Activity was measured spectrophotometrically at 334 nm and 37°C by following the liberation of UDP coupled to nicotinamide nucleotide reduction. Assays were carried out as described in the Materials and Methods section.



When the data were plotted by the method of Lineweaver & Burk (1934), straight lines were observed (Fig. 4). The Michaelis constants ( $K_m$ ) are calculated to be 0.11 mM for UDP-galactose with *N*-acetyl-D-glucosamine as exogenous acceptor, and 19 mM for *N*-acetyl-D-glucosamine itself. These values agree well with the data from radiochemical assays (Spiro & Spiro, 1968; Wagner & Cynkin, 1971). For comparison the  $K_m$  value was determined by using

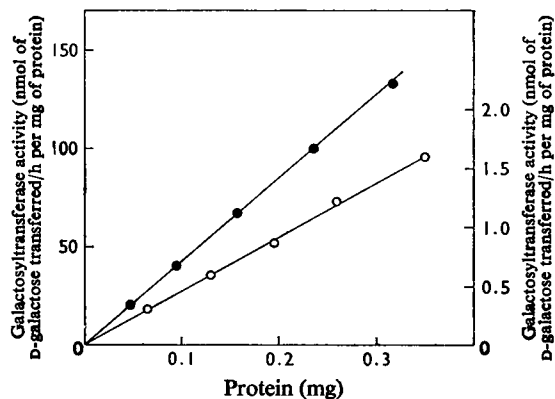


Fig. 3. Protein-dependency of the transfer of D-galactose to an exogenous and endogenous acceptor

The transfer of D-galactose to the exogenous acceptor *N*-acetyl-D-glucosamine was measured spectrophotometrically (left ordinate, ●), whereas the transfer of D-[<sup>14</sup>C]galactose to the endogenous protein acceptor of the Golgi-rich fraction was determined in a radiochemical assay (right ordinate, ○). For other details, see the legend to Table 2 and the Materials and Methods section.

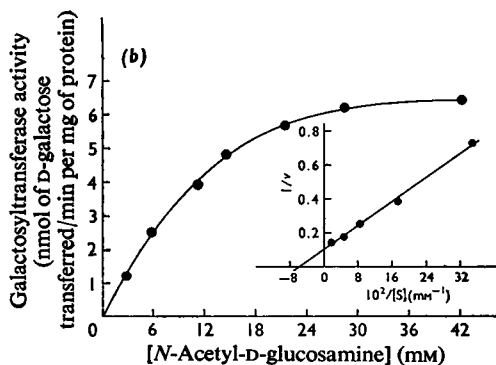
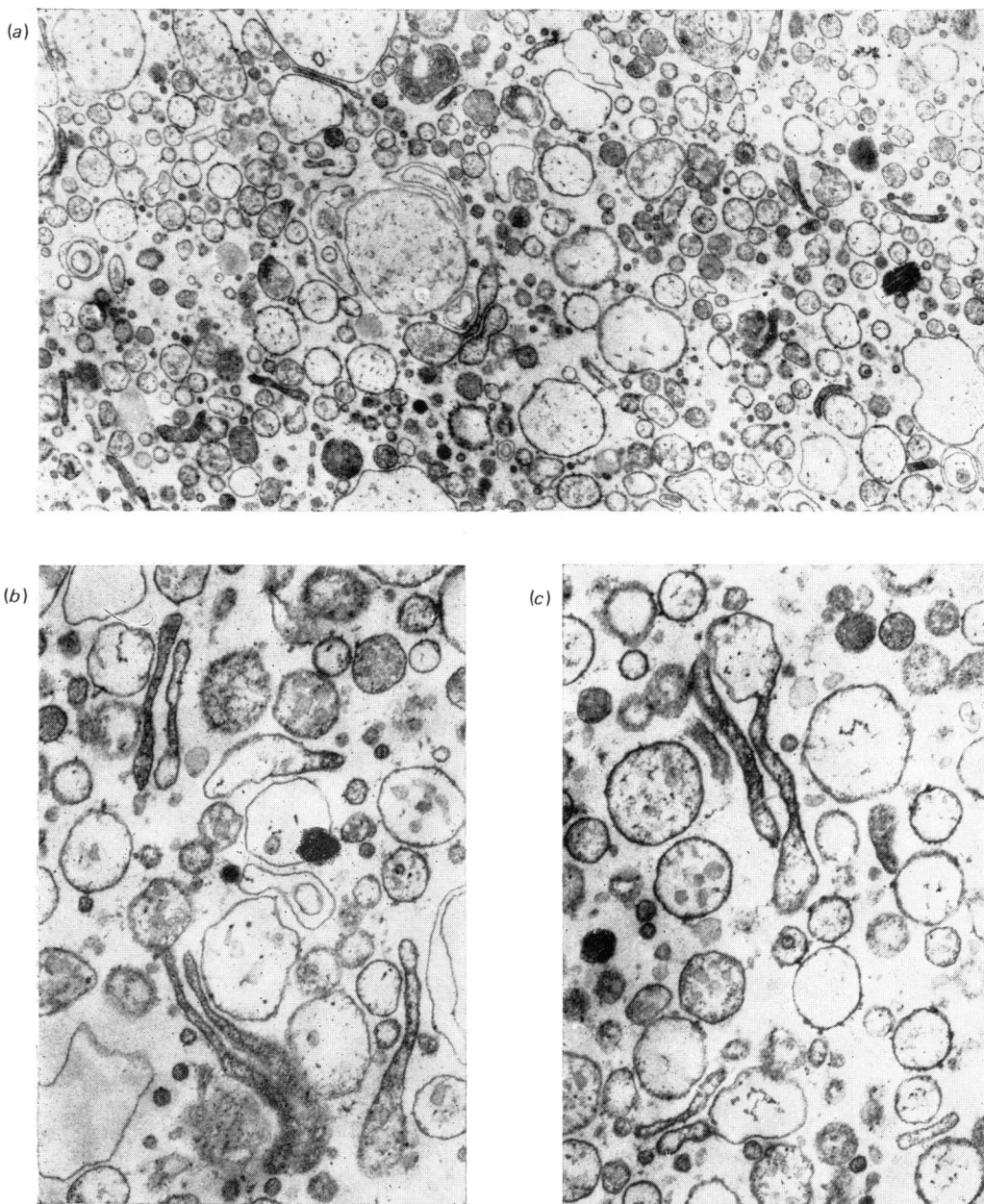


Fig. 4. Determination of the  $K_m$  values of the UDP-galactose-*N*-acetyl-D-glucosamine galactosyltransferase

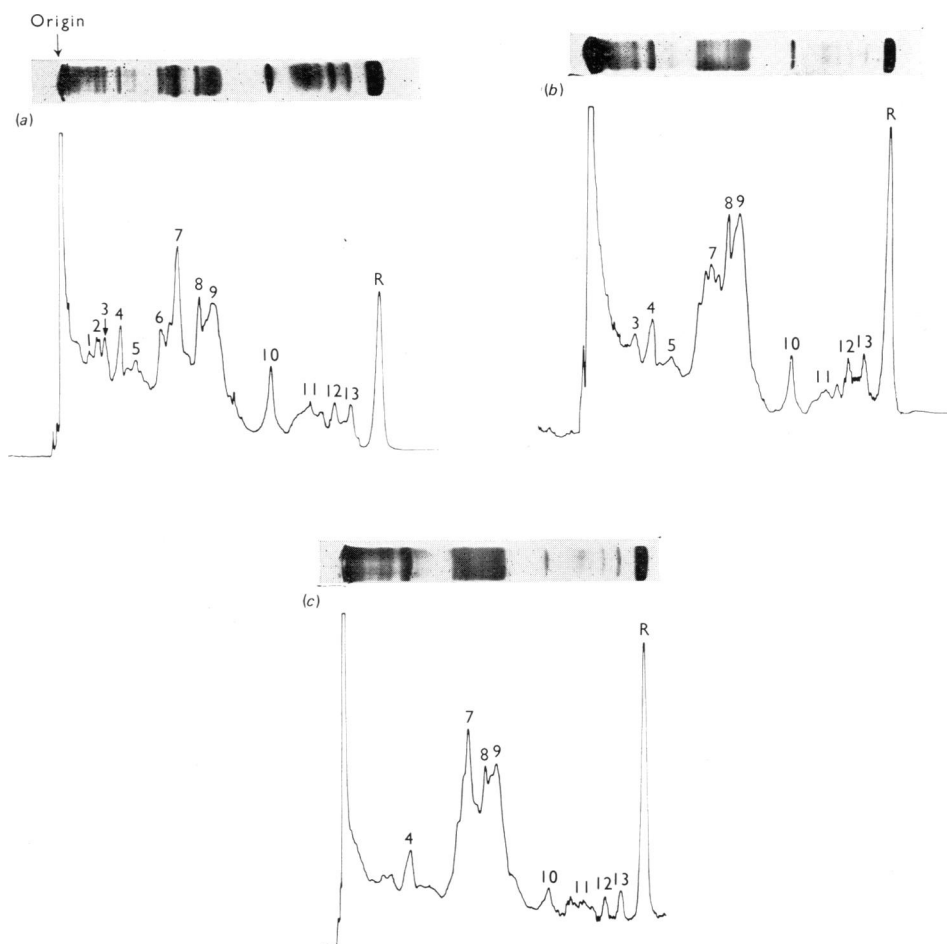
The standard incubation mixture as described in the Materials and Methods section was used except that either the substrate UDP-galactose (a) or the acceptor *N*-acetyl-D-glucosamine (b) was added at different concentrations to the reaction mixture. The regression equations of the Lineweaver-Burk plots were calculated and are shown as insets.



EXPLANATION OF PLATE I

*Electron micrographs of Golgi-rich fractions isolated by discontinuous gradient centrifugation*

(a) Micrograph shows that the fraction consists mainly of very-low-density-lipoprotein-loaded Golgi elements (magnification  $\times 25\,000$ ). (b) and (c) Characteristic features of Golgi cisternae with associated vesicles (magnification  $\times 50\,000$ ).



EXPLANATION OF PLATE 2

*Electrophoretic comparison of Golgi-rich fractions at different times after D-galactosamine administration*

The Golgi apparatus was enriched 100–120-fold relative to the homogenate. Experimental details are given in the Materials and Methods section. (a) Control animals, (b) 6 h after D-galactosamine injection, (c) 12 h after D-galactosamine injection. The running gel contained 10% (w/v) acrylamide. All gels were stained with Coomassie Brilliant Blue, destained and analysed on a Gilford spectrophotometer equipped with a gel-scanning device. The numbers 1–13 indicate the main protein bands. The band marked with 'R' represents ribonuclease A, which was added to the samples before electrophoresis.

Table 3. *Transfer of D-[1-<sup>14</sup>C]galactosamine from UDP-[1-<sup>14</sup>C]galactosamine to an endogenous protein acceptor*

The incubation mixture was similar to the assay system described for Table 2. UDP-[<sup>14</sup>C]galactose was replaced by UDP-[1-<sup>14</sup>C]galactosamine and the exogenous acceptor *N*-acetyl-D-glucosamine was replaced by the endogenous protein acceptor present in the Golgi-rich fraction as described by Wagner & Cynkin (1969*a,b*). The reaction was initiated by addition of the substrate UDP-[1-<sup>14</sup>C]galactosamine. Incubation was carried out for 5 min at 37°C. Protein-bound radioactivity was determined as described by Mans & Novelli (1960). For further experimental details, see the legend to Table 2 and the Materials and Methods section.

Concentration of UDP-[1- <sup>14</sup> C]galactosamine in the assay system (nmol/ml)	Transfer of D-[1- <sup>14</sup> C]galactosamine to an endogenous protein acceptor (nmol/h per mg of protein)
13.4	0.13
26.8	0.20
40.2	0.24
53.6	0.28
134.0	0.36

the endogenous acceptor protein of the Golgi-rich fraction. Under these conditions the Michaelis constant was calculated to be 11 μM for UDP-galactose.

#### Substrate specificity

Since UDP-galactosamine only occurs in significant amounts after administration of D-galactosamine hydrochloride (Keppler *et al.*, 1970*c*; Bauer & Reutter, 1973), it was decided to study whether this compound can be used by the galactosyltransferase instead of UDP-galactose. In order to simulate the situation found in the living cell the enzyme activity was determined by using an endogenous trichloroacetic acid-precipitable protein of the Golgi-rich fraction as glycosyl acceptor as described by Wagner & Cynkin (1969*a,b*). Incorporation of radioactivity from UDP-[1-<sup>14</sup>C]galactosamine into the acceptor did indeed occur (Table 3). Under optimum conditions the amount incorporated reached 9% of that with D-galactose (see Table 4).

#### Inhibition of galactosyltransferase by UDP-galactosamine and galactosamine 1-phosphate

The transfer of [<sup>14</sup>C]galactose from UDP-[<sup>14</sup>C]galactose to an endogenous acceptor protein of the Golgi-rich fraction is inhibited by galactosamine metabolites. The *K<sub>i</sub>* value is calculated to be 1.4 mM for UDP-galactosamine. Galactosamine 1-phosphate is only effective at higher concentrations. Half-maximal inhibition is reached at 10 mM-galactosamine 1-phosphate.

Table 4. *Transfer of D-[1-<sup>14</sup>C]galactose from UDP-D-[1-<sup>14</sup>C]galactose to an endogenous and exogenous acceptor*

D-Galactosamine hydrochloride (375 mg/kg body wt.) was injected intraperitoneally. Units of specific activity are expressed in nmol of D-galactose transferred/h per mg of protein at 37°C, ±s.d. from eight measurements. For experimental details, see the legend to Table 2 and the Materials and Methods section.

Type of acceptor	Sp. activity (nmol/h per mg of protein)		
	Control	At 3 h after D-galactosamine	At 6 h after D-galactosamine
Endogenous protein acceptor	4.9 ± 0.1	4.1 ± 0.05	2.9 ± 0.2
Exogenous acceptor <i>N</i> -acetyl-D-glucosamine	300 ± 15	292 ± 10	295 ± 12

#### Diminution of endogenous protein acceptor concentration

At 3 and 6 h after the administration of D-galactosamine, the activity of the galactosyltransferase was determined with both endogenous and exogenous acceptors. From the data (Table 4) it can be concluded that the activity of the transferase was not altered. Nevertheless the incorporation of D-galactose into the endogenous protein acceptor was decreased to 60% of the control value. We assume that this decrease is due to a fall in the acceptor protein concentration in the Golgi apparatus after D-galactosamine administration.

#### Inhibition of incorporation of L-[1-<sup>14</sup>C]leucine

At different times after D-galactosamine injection the rats received a single dose of 100 μCi of L-[1-<sup>14</sup>C]leucine/kg body wt. The animals were killed 1 h later and the protein-bound radioactivity was determined in serum, homogenate and Golgi-rich fraction (Table 5). Already 3 h after the administration of the amino sugar an appreciable decrease in L-[1-<sup>14</sup>C]leucine incorporation was observed. The pool of free L-leucine has not been altered at this time (Reutter, 1971). At the same time a diminution of serum protein content was found.

Minimum incorporation was measured 6 h after the administration of D-galactosamine. The Golgi-rich fraction contained only 40% of the radioactivity compared with controls. Determinations of radioactivity in homogenate and serum show a similar time-course of inhibition of L-[1-<sup>14</sup>C]leucine incorporation. To study the influence of a diminished uridylyte concentration on protein synthesis, D-galactosamine together with uridine was given to

Table 5. *Effect of D-galactosamine on the incorporation of L-[1-<sup>14</sup>C]leucine*

The specific radioactivity was determined as a function of time after a single injection of 375 mg of D-galactosamine hydrochloride/kg body wt. At 1 h before death 100  $\mu$ Ci of L-[1-<sup>14</sup>C]leucine/kg body wt. was given via the tail vein. Protein-bound radioactivity was determined as described in the Materials and Methods section. Values are means  $\pm$ s.d. from five determinations.

Cell fraction	Sp. radioactivity (d.p.m./mg of protein)			
	Control	At 3 h after D-galactosamine	At 6 h after D-galactosamine	At 12 h after D-galactosamine
Serum	2240 $\pm$ 150	1400 $\pm$ 240	790 $\pm$ 150	940 $\pm$ 80
Homogenate	2240 $\pm$ 320	1650 $\pm$ 160	1280 $\pm$ 130	1760 $\pm$ 80
Golgi-rich fraction	15020 $\pm$ 1050	9450 $\pm$ 510	7250 $\pm$ 570	11460 $\pm$ 230

Table 6. *Relationship between the incorporation of L-[1-<sup>14</sup>C]leucine and the concentration of uracil nucleotides after the administration of D-galactosamine*

The dose of uridine used was 1.2 g/kg body wt. All injections were given intravenously. The first dose was given together with 375 mg of D-galactosamine hydrochloride/kg body wt. at 08.30h. In cases where several doses of uridine were administered, they were injected at time-intervals of 2 h. At 13:30h the rats received a single injection of 100  $\mu$ Ci of L-[1-<sup>14</sup>C]leucine/kg body wt. via the tail vein; the rats were killed at 14:30h. Values are means  $\pm$ s.d. from five measurements.

No. of doses of uridine (1.2 g/kg) injected	Incorporation of L-[1- <sup>14</sup> C]leucine (d.p.m./mg of protein)			Concentration of uracil nucleotides (nmol/g wet wt. of liver)		
	Serum	Homogenate	Golgi-rich fraction	UTP+UDP	UDP- glucose	UDP- galactose
One	1580 $\pm$ 100	1360 $\pm$ 150	11440 $\pm$ 400	255 $\pm$ 41	149 $\pm$ 11	74 $\pm$ 3
Two	1870 $\pm$ 270	1650 $\pm$ 160	13550 $\pm$ 820	689 $\pm$ 140	282 $\pm$ 17	86 $\pm$ 25
Three	1960 $\pm$ 180	2180 $\pm$ 200	14760 $\pm$ 640	820 $\pm$ 132	329 $\pm$ 29	85 $\pm$ 7
Controls:						
(a) Uridine alone	2650 $\pm$ 190	2600 $\pm$ 220	16700 $\pm$ 750			
(b) D-Galactosamine alone				78 $\pm$ 12	40 $\pm$ 3	27 $\pm$ 5
(c) Untreated animals	2300 $\pm$ 140	2350 $\pm$ 170	15800 $\pm$ 620	310 $\pm$ 14	355 $\pm$ 31	88 $\pm$ 5

fill up this nucleotide pool (Keppler, 1973; Decker *et al.*, 1973).

After three injections of 1.2 g of uridine/kg body wt. at 2 h intervals all uridylates were at least in the normal range at the time when L-[1-<sup>14</sup>C]leucine was given (Table 6). A single injection of uridine together with D-galactosamine led to a decrease in the specific radioactivity to only 70% of normal values; without uridine administration the specific radioactivity fell to 40%. Even after three injections of uridine the incorporation of L-[1-<sup>14</sup>C]leucine does not reach normal values again.

The administration of uridine alone to control rats induced a slight stimulation (10-15%) of the incorporation of L-[1-<sup>14</sup>C]leucine.

#### Polyacrylamide-gel electrophoresis

At 6 h after D-galactosamine administration the disc electrophoresis patterns and the densitometer tracings of Golgi-rich fractions show a decrease or even a disappearance of various protein bands (Plate 2). This is especially characteristic of band (7) (relative mobility 0.63 compared with ribonuclease A).

As judged from the migration behaviour and solubility in trichloroacetic acid and ethanol this band represents albumin. Therefore the albumin content of the Golgi-rich fraction was determined (see the Materials and Methods section). The results confirm the findings of the electrophoretic analysis. At 6 h after D-galactosamine administration the albumin content was diminished by 45% compared with untreated rats. Further, the incorporation of L-[1-<sup>14</sup>C]leucine into albumin was decreased by 30% compared with control animals. From 6 h after D-galactosamine administration onwards the protein content increased again, although at 12 h normal values had not yet been reached. Protein patterns of the gels revealed that minor bands were still missing.

#### Discussion

The methods described allow studies on the secretion of glycoproteins and on the function and composition of the Golgi apparatus of rat liver. The purity of the Golgi-rich fraction is indicated by electron-microscopic and biochemical data.

According to Eylar (1965) glycosylation facilitates the secretion of proteins. With regard to this concept the enzyme galactosyltransferase, which attaches the galactose molecule to the nascent glycoprotein, may play an essential role in the secretion of glycoproteins. Therefore it has been studied more thoroughly during galactosamine hepatitis, which leads to an altered secretion of plasma proteins.

With *N*-acetyl-D-glucosamine as an exogenous acceptor, the transferase activity itself does not change after D-galactosamine injection, whereas the transfer of D-galactose to an endogenous acceptor protein of the Golgi-rich fraction falls to 60% of the normal value (Table 4). From these results it is concluded that it is the endogenous acceptor protein concentration of D-galactosamine-treated livers that has been diminished.

Owing to the administration of D-galactosamine the substrate of the galactosyltransferase, UDP-galactose, decreases from  $88 \pm 12$  to  $25 \pm 5$  nmol/g wet wt. of liver (Table 6). *In vitro*, with an endogenous acceptor protein, the Michaelis constant ( $K_m$ ) is calculated to be  $11 \mu\text{M}$  for UDP-galactose. Consequently the decrease in the substrate concentration caused by D-galactosamine injection should only be of limited influence on transferase activity.

It is noteworthy that the physiological substrate UDP-galactose can partially be replaced by the normally non-occurring UDP-galactosamine, which accumulates in the liver after D-galactosamine administration. Under conditions *in vitro* the rate of D-galactosamine transfer reaches 9% of that with D-galactose. It should be recognized that *in vivo*, under the conditions of galactosamine hepatitis, the concentration of UDP-galactosamine is 20 to 25 times higher than that of UDP-galactose. In glycoproteins, however, even a small amount of D-galactosamine instead of D-galactose may cause substantial alterations in their specificity and their behaviour, because a sugar with a non-acetylated amino group has been introduced.

According to the endomembrane concept (Morré *et al.*, 1971), the Golgi apparatus is the source of vesicles capable of fusing with the plasma membrane. By this route a non-acetylated D-galactosamine can become part of this subcellular structure. Reutter & Bachmann (1971) showed that the plasma membrane alters during induction of galactosamine hepatitis.

The measurements presented have shown that D-galactosamine caused a decrease of protein and glycoprotein synthesis as well as a lengthening of the time of secretion for galactose- and fucose-containing glycoproteins. Two reasons may be considered responsible, first, an inhibition of protein synthesis, and secondly, an inhibition of the secretion process itself. The decrease of the albumin content in the Golgi-rich fraction and the measurements of the total protein content in serum and homogenate

show a marked decrease in protein synthesis. Moreover, the diminished rate of L-[1- $^{14}\text{C}$ ]leucine incorporation and the lowered content of endogenous protein acceptor for the galactosyltransferase further support the finding of disturbed protein synthesis. The decreased rate of protein synthesis is mostly due to a trapping of uracil nucleotides after the injection of D-galactosamine. The pool of UTP+UDP has been lowered below a value that is no longer sufficient to provide enough substrate for a rapidly-turning-over mRNA. Studies on rat liver tyrosine aminotransferase show that a defined concentration of UTP+UDP is needed for the corticosteroid-mediated induction of this enzyme (Reynolds & Reutter, 1973).

The present paper shows that protein synthesis can be restored to 80% of normal values, if uridine is injected repeatedly. Similar results have been published by Shinozuka *et al.* (1973). Therefore, apart from the trapping of uracil nucleotides, a different mechanism must be responsible for the inhibition of the synthesis of several proteins. Previous investigations have shown that galactosamine 1-phosphate and UDP-galactosamine inhibit UDP-glucose pyrophosphorylase (Keppler & Decker, 1969) and UDP-glucose dehydrogenase (Bauer & Reutter, 1973), and therefore it seems conceivable that some galactosamine metabolites may influence an enzymic step involved in protein synthesis. Shinozuka *et al.* (1973) speculate that the UDP-hexosamines may be responsible for the formation of ribosomal aggregates.

From the present data, it can be concluded that the disturbed secretion of proteins and glycoproteins of rat liver is due to a cumulative effect: (a) inhibition of protein synthesis leading to a diminution of the endogenous acceptor pool of the galactosyltransferase, (b) inhibition of the galactosyltransferase activity by galactosamine metabolites and (c) replacement of UDP-galactose by UDP-galactosamine.

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