Biochem. J. (1968) **107**, 637
Printed in Great Britain

The Metabolism of D-Galactosamine and *N*-Acetyl-D-galactosamine in Rat Liver

By FRANK MALEY, ANTHONY L. TARENTINO, JOHN F. McGARRAHAN* and RUDOLPH DELGIACCO*
Division of Laboratories and Research, New York State Department of Health, Albany, N.Y. 12201, *U.S.A.*

(*Received* 2 *November* 1967)

D-[1-^{14}C]Galactosamine appears to be utilized mainly by the pathway of galactose metabolism in rat liver, as evidenced by the products isolated from the acid-soluble fraction of perfused rat liver. These products were eluted in the following order from a Dowex 1 (formate form) column and were characterized as galactosamine 1-phosphate, sialic acid, UDP-glucosamine, UDP-galactosamine, *N*-acetylgalactosamine 1-phosphate, *N*-acetylglucosamine 6-phosphate, UDP-*N*-acetylglucosamine, UDP-*N*-acetylgalactosamine and an unidentified galactosamine-containing compound. In addition, [1-^{14}C]glucosamine was found in the glycogen, an incorporation previously shown to result from the substitution of UDP-glucosamine for UDP-glucose in the glycogen synthetase reaction. Analysis of the [1-^{14}C]glucosamine-containing disaccharides released from glycogen by β-amylase provided additional evidence that they consist of a mixture of glucose and glucosamine in a 1:1 ratio, but with glucose predominating on the reducing end. UDP-*N*-acetylgalactosamine was shown to result from the reaction of UTP with *N*-acetylgalactosamine 1-phosphate in the presence of a rat liver extract.

Several years ago (Maley & Lardy, 1956) it was noted that UDP-glucosamine could be synthesized in rat liver extracts by the reaction of UTP with glucosamine 1-phosphate. Subsequently, it was found that a preparation of UDP-galactose 4-epimerase could catalyse the conversion of UDP-glucosamine into UDP-galactosamine (Maley & Maley, 1959). However, attempts to demonstrate the presence of either UDP-glucosamine or UDP-galactosamine in acid-soluble rat liver extracts after intraportal injection (McGarrahan & Maley, 1962) or perfusion (DelGiacco & Maley, 1964) with D-[1-^{14}C]glucosamine were unsuccessful. The main products formed after such treatment were *N*-acetylglucosamine, sialic acid, *N*-acetylglucosamine 1-phosphate, *N*-acetylglucosamine 6-phosphate and UDP-*N*-acetylglucosamine, suggesting that glucosamine is first *N*-acetylated, followed by conversion into the 6-phosphate. Similar results were also obtained by Molnar, Robinson & Winzler (1964) and by Richmond (1965).

In contrast with the results with glucosamine, it has since been noted (Maley, McGarrahan & DelGiacco, 1966*a*) that D-[1-^{14}C]galactosamine is metabolized by rat liver to yield a mixture of radioactive UDP-galactosamine and UDP-glucosamine, as well as a number of other radioactive hexosamines. The incorporation of radioactivity into the glycogen as glucosamine was also observed (Maley, McGarrahan & DelGiacco, 1966*b*), an effect apparently resulting from the substitution of UDP-glucosamine for UDP-glucose in the glycogen synthetase reaction. The present paper is concerned primarily with the isolation and characterization of the UDP-glucosamine–UDP-galactosamine mixture.

* Present address: St Peter's Hospital, Albany, N.Y. 12208, U.S.A.

MATERIALS

D-[1-^{14}C]Galactosamine was purchased from the New England Nuclear Corp., Boston, Mass., U.S.A. Although most of the samples contained small amounts of coloured impurities, the results obtained were similar whether or not the compound was purified on a Gardell (1953) column. Paper chromatography of the purified material in several systems indicated it to be pure galactosamine. *N*-Acetyl-D-[1-^{14}C]galactosamine was prepared by the *N*-acetylation of D-[1-^{14}C]galactosamine. D-Glucosamine 1-phosphate and *N*-acetyl-D-glucosamine 1-phosphate were prepared by a modification of the procedure described by Maley, Maley & Lardy (1956). The former compound was synthesized enzymically or isolated from the acid-soluble fraction by gradient elution chromatography. Both procedures are

described in the Methods section. *N*-Acetyl-D-glucosamine uronic acid was synthesized by the method of Heyns & Paulsen (1955).

METHODS

The paper-chromatographic systems used were described previously (McGarrahan & Maley, 1962), as was the borate electrophoresis procedure for the separation of *N*-acetylhexosamines (Maley & Maley, 1959; McGarrahan & Maley, 1962). For the detection of radioactive areas on the chromatograms, a Nuclear–Chicago 4π strip scanner (Actigraph III) was employed. The relative radioactivity in each region was estimated by the half-height × width procedure.

The radioactivity in the various fractions after column chromatography was determined with a scintillation counter and corrected for quench by the channels-ratio method (Hendler, 1964).

Gas–liquid chromatography of the *O*-trimethylsilyl *N*-acetylhexosamines was performed on an F and M model 402 gas chromatograph equipped with a 4ft. SE-30 Diatoport S column (F and M Division of Hewlitt Packard Corp.). Helium was the carrier gas. The retention time for sorbitol at 170° was 8min. 24sec.

Perfusion. The rat liver perfusion technique was similar to that described by Miller, Bly, Watson & Bale (1951), except for the enrichment of the media with amino acids. After the perfusion, the liver was extracted twice with 2vol. of 0·6N-$HClO_4$ and the KOH-neutralized extract was chromatographed on a Dowex 1 (X8; formate form; 200–400 mesh) column (15cm. × 1cm.) (McGarrahan & Maley, 1962). Radioactive regions were detected by passage of the column eluate directly through a Nuclear–Chicago scintillation flow-cell system. The glycogen in the column eluate fraction was precipitated, after concentration to 25ml. in a flash evaporator, by the addition of 2vol. of ethanol. The centrifuged precipitate was washed twice with 50% (v/v) ethanol, then dissolved in 5–10ml. of water and dialysed against six 2l. changes of distilled water for 2 days. The glycogen concentration was determined by drying a sample on a planchet and weighing, or by a colorimetric iodine procedure (van der Vies, 1954).

Purification of peak C. The combined fractions constituting this region were freeze-dried, neutralized and rechromatographed on another Dowex 1 (formate form) column (15cm. × 1cm.) by elution with a convex gradient consisting of a 500ml. water mixing chamber and a 4N-formic acid reservoir. The desired compound was freeze-dried, neutralized and placed as a band on Whatman no. 3MM paper, after which it was subjected to descending chromatography in ethanol–M-ammonium acetate, pH7·5 (5:2, v/v). The major radioactive and ultraviolet-light-absorbing region was then excised and eluted with water.

Desalting of peaks E *and* F. Removal of NH_4^+ ion was facilitated by passage of the combined fractions from each peak through a Dowex 50 (H^+ form) column (10cm. × 2cm.) at 4°, followed by freeze-drying.

Nitrous acid. Nitrous acid treatment of free hexosamines glycosidically linked to phosphate results in the production of P_i and the corresponding anhydro sugar (e.g. glucosamine 1-phosphate→anhydromannose + P_i; UDP-glucosamine→UPD + anhydromannose). Cleavage of hexosaminidic bonds by nitrous acid (Foster, Martlew & Stacey, 1953; Yosizawa, 1964) was effected by incubating samples with equivalent volumes of 5% (w/v) $NaNO_2$ and 33% (v/v) acetic acid for at least 10min. To analyse the treated samples for 2,5-anhydromannose and 2,5-anhydrotalose, derived from glucosamine and galactosamine derivatives respectively, the solutions were desalted by passage through a column of Dowex 1 (formate form) over Dowex 50 (H^+ form) (each 3cm. × 1cm.), concentrated to about 1ml. in a flash evaporator and freeze-dried in a conical centrifuge tube. The freeze-dried samples were taken up to about 0·2ml. with water and portions were placed on Whatman no. 3MM paper for borate electrophoresis, a system in which anhydromannose moves to the cathode and anhydrotalose to the anode.

Preparation of UDP-glucosamine and galactosamine 1-phosphate. Rat liver was homogenized with 4vol. of iso-osmotic KCl solution and centrifuged at 35000*g* for 40min. The supernatant was precipitated with $(NH_4)_2SO_4$ (0·35–0·80 saturated). The precipitate was dissolved in as small a volume of 0·05M-potassium phosphate buffer, pH7·5, as possible and dialysed against two 2l. changes of this buffer overnight.

For the preparation of UDP-glucosamine, the following components were used (in μmoles): UTP, 10; glucosamine 1-phosphate, 20; tris, pH8·0, 100; KF, 60; $MgCl_2$, 60; concentrated rat liver extract, 1·0ml. After incubation for 1·5hr. at 37°, the reaction was stopped by heating at 100° for 2min. The precipitate was centrifuged and the extract was passed through a Dowex 1 (formate form) column (15cm. × 1cm.). The UDP-glucosamine was eluted with a formic acid gradient (Maley & Lardy, 1956), and the yield was about 3·5μmoles. A more efficient, but more involved, procedure for the preparation of this compound was to use a purified UDP-glucose pyrophosphorylase from rabbit muscle (Villar-Palasi & Larner, 1960), coupled with inorganic pyrophosphatase, as an enzyme source.

Galactosamine 1-phosphate could be prepared in almost quantitative yields by incubating 0·2ml. of the rat liver extract with the following components (in μmoles): galactosamine, 5·0; ATP, 10; $MgCl_2$, 5·0; KF, 15; tris, pH8·5, 100; the final volume was 0·6ml. The solution was incubated at 37° for 30–60min. and the reaction stopped by heating at 100° for 2min. The supernatant was passed through a column of Dowex 1 (formate form) and eluted as described above. The desired compounds after elution from the columns were concentrated by freeze-drying.

RESULTS AND DISCUSSION

A Dowex 1 (formate form) gradient elution pattern of the acid-soluble fraction after perfusion of a rat liver with [1-^{14}C]galactosamine is presented in Fig. 1(*a*). Fig. 1(*b*) reveals the corresponding elution regions of the indicated known compounds. Tables 1 and 2 present the radioactivity and recovery data from a different experiment in which the galactosamine concentration was four times that employed in the experiment in Fig. 1(*a*). This difference in galactosamine concentration probably accounts for the differences in the relative distribution of radioactivity in the two cases. Regardless of the concentration of galactosamine, the patterns

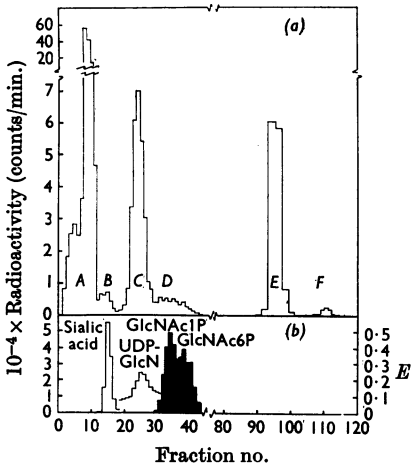

Fig. 1. (*a*) Dowex 1 (formate form) elution pattern of an acid-soluble extract from rat liver perfused for 2hr. with 15·6 μmoles of D-[1-^{14}C]galactosamine (3·4 × 10^6 disintegrations/min./μmole). Each fraction contained 5·4ml. The techniques employed for the perfusion and column elution procedure were described previously (McGarrahan & Maley, 1962; DelGiacco & Maley, 1964). (*b*) Elution pattern obtained with known compounds. The dark areas (GlcNAc1P and GlcNAc6P) represent radioactivity (^{14}C); UDP-glucosamine was determined by measuring E_{260} and sialic acid by the thiobarbituric acid assay (Warren, 1959). UDP-GlcN, UDP-glucosamine; GlcNAc1P, *N*-acetylglucosamine 1-phosphate; GlcNAc6P, *N*-acetylglucosamine 6-phosphate.

Table 1. *Radioactivity distribution after perfusion of rat liver with* [1-^{14}C]*galactosamine*

A 14g. rat liver was perfused with an enriched amino acid solution (Miller *et al.* 1951) containing 56 μmoles of [1-^{14}C]-galactosamine (2·97 × 10^6 disintegrations/min./μmole) for 2·5hr. The quantity of radioactivity in each of the samples isolated from the perfusion was determined as indicated in the Methods section and in Fig. 1. The total initial radioactivity was 166 × 10^6 disintegrations/min. The recovered radioactivity does not take into account conversion into CO_2, loss through extraction of the liver protein and loss in handling.

Sample	10^{-6} × Radioactivity (disintegrations/min.)	Total recovery (%)
Blood	22·8	13·7
Liver protein	6·15	3·7
Liver acid-soluble material	102·0	61·4
Total recovery	131·0	78·8

have always been found to be similar qualitatively. An analysis of the labelled compounds as they were eluted from the column revealed the following information.

Peak A. In addition to the radioactivity eluted in this area, three to four times as much passed through the column with the glycogen fraction. Most of the latter radioactivity (component *A*1) could be retained by a larger column (10cm. × 2·2cm.), suggesting that the column was overloaded with respect to peak *A*. This belief was confirmed through a comparison of radioactive components *A* and *A*1 by the following procedures, which indicate that they are identical. Treatment of components *A* and *A*1 with nitrous acid yielded radioactive material that migrated mainly like anhydrotalose on borate electrophoresis (Maley & Maley, 1959), indicating the compound to be a galactosamine derivative. *N*-Acetylation of components *A* and *A*1 with acetic anhydride (Roseman & Daffner, 1956) provided compounds that were now eluted from Dowex 1 (formate form) in the *N*-acetylhexosamine phosphate region (peak *D* in Fig. 1*a*) and that also chromatographed on paper [ethanol–M-ammonium acetate, pH 7·5 (5:2, v/v)] with the same R_F as *N*-acetylglucosamine 1-phosphate. Before *N*-acetylation, the radioactivity migrated in this system to the same extent as marker glucosamine 1-phosphate. Hydrolysis of purified *N*-acetylated components *A* and *A*1 in 0·1N-hydrochloric acid at 100° for 10min. yielded products that had the same mobility as *N*-acetylgalactosamine when subjected to electrophoresis in 1% borate. The ratio of acid-labile phosphorus (Ames & Dubin, 1960) to *N*-acetylhexosamine (Maley *et al.* 1956), with *N*-acetylgalactosamine as a standard, was 0·99:1·0. Radioautograms showed the presence of small amounts of radioactivity migrating with *N*-acetylmannosamine. The above evidence, as well as the enzymic reaction of *N*-acetylated component *A* with UTP to form UDP-*N*-acetylgalactosamine as

Table 2. *Radioactivity distribution in the liver acid-soluble fraction*

The column eluate is the fraction not retained by the Dowex 1 (formate form) column after passage of the liver acid-soluble material (Table 1) through the column. The total initial radioactivity was 102 × 10^6 disintegrations/min. (Table 1).

Sample	10^{-6} × Radioactivity (disintegrations/min.)
Column eluate	72·0
Glycogen	8·0*
Peak *A*	8·45
Peak *B*	0·74
Peak *C*	4·38
Peak *D*	0·68
Peak *E*	12·5
Peak *F*	1·37
Total	100·1

* Not included in summation since it is part of the column eluate fraction.

described below, strongly suggests the compound to be galactosamine 1-phosphate.

Peak B. This radioactive component was eluted in the sialic acid region (Fig. 1) (peak I in DelGiacco & Maley, 1964), as estimated by the thiobarbituric acid assay (Warren, 1959), and also chromatographed in ethanol–M-ammonium acetate, pH 7·5, butan-1-ol–acetic acid–water (4:1:5, by vol.) and butan-2-ol–acetic acid–water (4:1:5, by vol.) with R_F values corresponding to those of marker sialic acid. To distinguish between *N*-acetylneuraminic acid and *N*-glycollylneuraminic acid, the *n*-butyl acetate–acetic acid–water system was used (Spiro, 1960). Almost all the radioactivity was found to migrate with *N*-acetylneuraminic acid.

Peak C. Indications that this compound is a nucleotide were first revealed by its absorption on charcoal. That it might be related to UDP-glucosamine was suggested by its elution from Dowex 1 (formate form) in the same region as enzymically prepared UDP-glucosamine (Maley & Lardy, 1956) (Fig. 1). Additional evidence was provided by electrophoresis in 0·05M-ammonium formate, pH 3·8, at 17·6v/cm. for 3hr. (migration distances towards anode: UDP-glucosamine, 9·4cm.; UMP, 15·3cm.; UDP-*N*-acetylglucosamine, 20·6cm.) and chromatography in ethanol–M-ammonium acetate, pH 7·5 (R_{UMP} values: UDP-glucosamine, 1·19; UDP-glucose, 1·28; UDP-*N*-acetylglucosamine, 1·54). In both cases, the radioactivity moved identically with the marker UDP-glucosamine. *N*-Acetylation with acetic anhydride converted component *C* into a compound that was eluted from Dowex 1 (formate form) in the same region as carrier UDP-*N*-acetylglucosamine. Hydrolysis (0·1N-hydrochloric acid at 100° for 10min.) of the isolated UDP-*N*-acetylglucosamine, followed by electrophoresis of the desalted *N*-acetylhexosamine in 1% borate, showed the presence of radioactive *N*-acetylglucosamine and *N*-acetylgalactosamine in the ratio 35:65. Colorimetric analysis (Table 3) confirmed this ratio. Nitrous acid treatment of component *C* yielded a mixture of anhydromannose and anhydrotalose with the latter predominating, as expected.

Conversion of component *C* into its hexosamine constituents by hydrolysis in N-hydrochloric acid at 100° for 1hr., followed by chromatography in an amino acid analyser by a slight modification of the procedure of Moore, Spackman & Stein (1958), yielded two ninhydrin-positive peaks that were eluted in the same regions as glucosamine and galactosamine. Analysis for radioactivity, by placing a scintillation flow cell in series with the amino acid analyser, demonstrated that the radioactivity and ninhydrin patterns coincided (Fig. 2). In this case the glucosamine/galactosamine ratio was about 1:1.

Table 3. *Analysis of UDP-hexosamine (peak* C)

The uridine assay was based on ϵ_{mM} 10·0 at 260mμ. Total phosphate was determined by the micro method of Ames & Dubin (1960). The *N*-acetylhexosamine assay was carried out as follows. After *N*-acetylation, peak *C* material was hydrolysed, and the *N*-acetylhexosamines were isolated (see the Methods section), concentrated and assayed by the procedure of Reissig, Strominger & Leloir (1955). The theoretical colour yield of 0·0424μmole of *N*-acetylhexosamine (assuming equivalence to uridine), containing a 35:65 ratio of *N*-acetylglucosamine to *N*-acetylgalactosamine, was determined from the molar extinction coefficients of the latter hexosamines given by Reissig *et al.* (1955). The 35:65 ratio was obtained from the radioactivity in the respective *N*-acetylglucosamine and *N*-acetylgalactosamine regions after borate electrophoresis of the isolated *N*-acetylhexosamine in sample 2.

Sample no.	Assay	Amount (μmole/ml.)	Molar ratio
1	Uridine	0·00678	1
	Total phosphate	0·014	2·06
2	Uridine	0·0424	1
	N-Acetylhexosamine	0·040	0·94

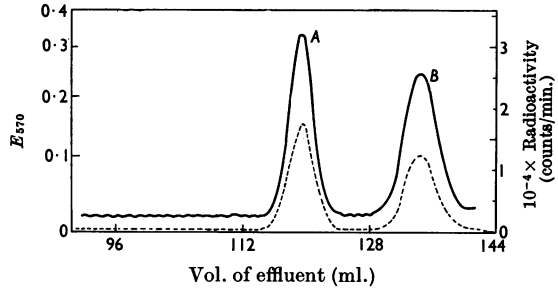

Fig. 2. Chromatography of acid-hydrolysed peak *C* material on an amino acid analyser (Moore *et al.* 1958); *A*, glucosamine; *B*, galactosamine. ——, E_{570}; ------, radioactivity. For details see the text. The eluting buffer was 0·35M-sodium citrate, pH 5·28, at 53°.

To confirm the identity of the nucleotide portion of component *C*, [6-^{14}C]orotic acid was administered intraportally to rats in the presence and absence of unlabelled galactosamine. The elution patterns of the liver acid-soluble fractions (Fig. 3) revealed a new radioactive peak coinciding with peak *C* of Fig. 1(*a*), to be formed only when galactosamine was present. The radioactive elution patterns are otherwise identical after the administration of the [6-^{14}C]orotic acid. As with the hexosamine-labelled peak *C* described above, *N*-acetylation of the nucleotide-labelled component *C* yielded a compound that was eluted from Dowex 1 (formate form) with UDP-*N*-acetylglucosamine, and nitrous acid

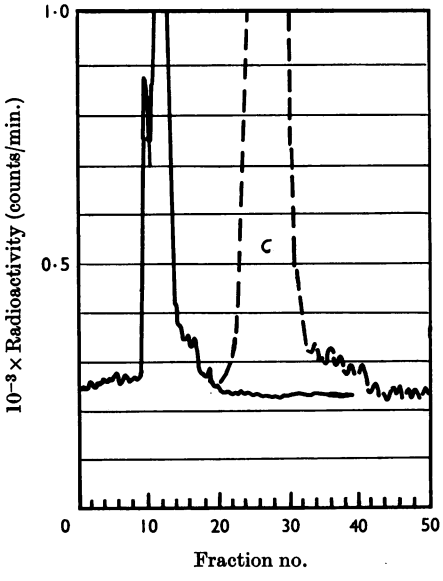

Fig. 3. Dowex 1 (formate form) elution patterns of an acid-soluble extract from rat liver after an intraportal injection with 9·7 μc of [6-^{14}C]orotic acid alone (——) and with 20 μmoles of unlabelled galactosamine hydrochloride (— —). In the latter case, the broken line should be considered an extension of the solid line, as the two patterns were identical except for peak *C*. One hour was allowed from the time of injection to the removal of the liver.

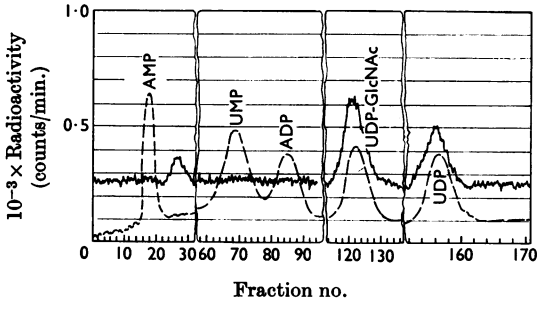

Fig. 4. Dowex 1 (formate form) chromatography of combined *N*-acetylated peak *C* material (126000 disintegrations/min.) and nitrous acid-treated peak *C* material (63000 disintegrations/min.). Peak *C* material was isolated after the intraportal injection of [6-^{14}C]orotic acid. A constant-volume reservoir (250 ml. of water) was employed with the following changes of reservoir solutions: 4 N-formic acid to tube 72; 4 N-formic acid + 0·2 M-ammonium formate to tube 135; 4 N-formic acid + 0·4 M-ammonium formate to termination. Each tube contained about 4·7 ml. and was collected at a rate of slightly less than 1 ml./min. The column size was 15 cm. × 1 cm. and contained the indicated marker nucleotides (——). UDP-GlcNAc, UDP-*N*-acetylglucosamine.

treatment of component *C* converted it into a radioactive compound that was eluted with carrier UDP from Dowex 1 (formate form) (Fig. 4). The latter result was also confirmed by chromatography in ethanol–M-ammonium acetate, as well as by electrophoresis in 0·05 M-ammonium formate. Acid hydrolysis of component *C* (N-hydrochloric acid for 30 min. at 100°) yielded a radioactive compound that chromatographed in the above systems with UMP. Also, as indicated in Table 3, the phosphorus/uridine ratio of purified peak *C* was 2:1. From the above evidence, it would appear that peak *C* consists mainly of a mixture of UDP-galactosamine and UDP-glucosamine.

Peak D. As indicated in Fig. 1, this is eluted similarly to *N*-acetylglucosamine 1-phosphate and *N*-acetylglucosamine 6-phosphate from Dowex 1 (formate form) (peaks II and III in DelGiacco & Maley, 1964). Chromatography in ethanol–M-ammonium acetate gave similar results. Because of the limited amount of material, a more complete characterization was not possible, but preliminary studies based on borate electrophoresis of the phosphate-free sugars released on mild acid hydrolysis (0·1 N-hydrochloric acid at 100° for 10 min.) and those sugars released on acid phosphatase treatment of the acid-stable component indicate the *N*-acetylgalactosamine 1-phosphate:*N*-acetylglucosamine 1-phosphate:*N*-acetylglucosamine 6-phosphate proportions to be 30:6:64.

Peak E. The radioactivity in this area coincides exactly with UDP-*N*-acetylglucosamine (peak IV in DelGiacco & Maley, 1964) and was characterized as described by McGarrahan & Maley (1962). The ratio of *N*-acetylglucosamine to *N*-acetylgalactosamine was 70:30.

Peak F. Because of limitations in the amount of material, this region was not analysed completely, but hydrolysis in 4 N-hydrochloric acid at 100° for 6 hr. followed by *N*-acetylation and borate electrophoresis revealed the radioactivity to migrate like *N*-acetylgalactosamine. On chromatography in ethanol–M-ammonium acetate, pH 7·5, component *F* migrated with R_{UMP} 0·68. However, hydrolysis of component *F* in 0·1 N-hydrochloric acid at 100° for 10 min. yielded a product that was retained by Dowex 1 (formate form) and that was eluted on convex-gradient elution chromatography (Fig. 1) just before *N*-acetylglucosamine 1-phosphate (peak *D*). It does not appear to be a uronic acid, as chemically prepared *N*-acetylglucosamine uronic acid (Heyns & Paulsen, 1955) was eluted much faster than hydrolysed component *F*.

Incorporation of radioactivity into glycogen. As much as 5–10% of the radioactivity in the acid-soluble fraction was found to be associated with the glycogen in a form non-diffusible through a dialysis membrane. As indicated by Maley *et al.* (1966*b*), the

radioactivity is glycosidically linked to the glycogen and could be released by nitrous acid as well as by treatment with α-amylase or β-amylase. Analysis of the disaccharide released on β-amylase treatment of the above labelled glycogen and its *N*-acetylated derivative provided unequivocal evidence that the radioactivity was associated with neither glucose nor galactosamine, but with glucosamine (Maley *et al.* 1966*b*). The chemical, enzymic and chromatographic evidence presented by Maley *et al.* (1966*b*) suggests that the product released by the β-amylase treatment of glycogen consists of glucose and glucosamine in an alternating sequence and the data in Table 4 support this contention. The nitrous acid treatment should release free glucose when it is on the reducing end of disaccharide I [*O*-2-amino-2-deoxy-α-D-glucopyranosyl-(1→4)-D-glucopyranose], whereas disaccharide II [*O*-α-D-glucopyranosyl-(1→4)-2-amino-2-deoxy-D-glucopyranose] should be converted into a disaccharide with anhydromannose on the reducing end. It is assumed in Table 4 (Expt. A) that the colour yield of free anhydromannose is equivalent to anhydromannose on the reducing end of a disaccharide. As indicated in Table 4 (Expt. A), approx. 60% of the disaccharide mixture consists of disaccharide I. The data in Table 4 (Expt. B) confirm the 1:1 ratio of glucose to glucosamine in the disaccharide mixture. In the four cases examined to date disaccharide I varied from 60 to 75%. The finding that a specific β-*N*-acetylglucosaminidase could not hydrolyse disaccharide I, whereas a mixture of α-*N*-acetylglucosaminidase and β-*N*-acetylglucosaminidase from rat epididymis (Findlay, Levvy & Marsh, 1958) could, supports the α-anomeric nature of the glycosidic bond in disaccharide I. The latter studies are not definitive, however, because of the admitted crudeness of the epididymal extract. Since glycogen synthetase is most likely responsible for the incorporation of glucosamine into glycogen (Maley *et al.* 1966*b*), the glucosamine–glucose glycosidic bond could be expected to be α. By analogy with maltose the β-amylase product would appear to consist of a mixture of *O*-2-amino-2-deoxy-α-D-glucopyranosyl-(1→4)-D-glucopyranose (I) and *O*-α-D-glucopyranosyl-(1→4)-2-amino-2-deoxy-D-glucopyranose (II). However, the limited amounts of material thus far available have prevented more desirable chemical methods of characterization from being employed.

It remains to be seen whether other enzymes acting on glycogen can mobilize glucosamine from its peripheral position in glycogen to an interior location, and whether such a transfer affects the structure and utilization of glycogen. The glucosamine is believed to be peripheral since almost all of the radioactivity can be released by β-amylase, without affecting the precipitation of the residual glycogen by ethanol. Preliminary studies suggest that phosphorylase may not be able to release the incorporated glucosamine. If this is the case and sufficient glucosamine can be incorporated into glycogen, a glycogen-storage defect could result.

Synthesis of UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine. Though an explanation can be provided for the synthesis of UDP-galactosamine from galactosamine by invoking the reactions entailed in the Leloir–Kalckar pathway (Leloir, 1951; Kalckar, Braganca & Munch-Petersen, 1953; Kalckar, 1965), the reactions responsible for the synthesis of UDP-*N*-acetylgalactosamine are not as obvious. Attempts to demonstrate the synthesis of this nucleotide by the direct *N*-acetylation of

Table 4. *Analysis of product of β-amylase hydrolysis*

The product of β-amylase hydrolysis was isolated as described by Maley *et al.* (1966*a*). Anhydromannose was determined by the method of Dische & Borenfreund (1950). Glucose was determined by a micro modification of the coupled hexokinase–glucose 6-phosphate dehydrogenase assay (Slein, Cori & Cori, 1950; see below). The total was estimated from the radioactivity of the sample assuming no dilution of the [1-^{14}C]galactosamine precursor ($1{\cdot}80 \times 10^6$ counts/min./μmole). The *N*-acetylglucosamine assay was carried out as follows. *N*-Acetylated product of β-amylase hydrolysis was hydrolysed for 2 hr. in 2 N-HCl at 100°. Excess of HCl was removed by evaporation *in vacuo* followed by *N*-acetylation of the hexosamine and removal of salt by passage of the mixture through a column of Dowex 1 (formate form) over Dowex 50 (H^+ form) (each 3 cm. × 1 cm.). The eluate was concentrated to dryness and the residue was taken up in about 0·5 ml. for glucose (Slein *et al.* 1950; see above) and *N*-acetylglucosamine (Reissig *et al.* 1955) assays.

	Treatment	Products formed (μmole)	
Expt. A	Nitrous acid	Anhydromannose	0·0282
		Glucose	0·0150
		(Total)	(0·0252)
Expt. B	HCl hydrolysis + *N*-acetylation	*N*-Acetylglucosamine	0·0198
		Glucose	0·0200
		(Total)	(0·0205)

peak *C* material with acetyl-CoA were not successful, nor could fully supplemented rat liver extracts effect this synthesis from [1-^{14}C]galactosamine or UDP-[1-^{14}C]hexosamine (peak *C*). Since all of the reactions presented below, except (3), were described previously (Maley & Maley, 1959; Ballard, 1966; Chou & Soodak, 1952; Leloir, Cardini & Olavarría, 1958; Glaser, 1959), the following route of synthesis was therefore considered:

Galactosamine $\xrightarrow{1}$ *N*-acetylgalactosamine $\xrightarrow{2}$ *N*-acetylgalactosamine 1-phosphate $\xrightarrow{3}$ UDP-*N*-acetylgalactosamine $\xrightarrow{4}$ UDP-*N*-acetylglucosamine

Though reaction (1) was quite slow (about 30 $\mu\mu$moles/min./mg. of protein), sufficient amounts of *N*-acetylgalactosamine might be formed during a 2hr. perfusion to contribute significantly to the UDP-*N*-acetylhexosamine pool. To determine if the latter effect could be observed, the utilization of *N*-acetylgalactosamine was investigated after an intraportal injection of the 1-^{14}C-labelled compound (Fig. 5). Three major peaks are apparent in Fig. 5 and were characterized as described in the galactosamine incorporation studies: peak *B*, sialic acid; peak *D*, about 80% *N*-acetylgalactosamine 1-phosphate; peak *E*, 70:30 mixture of UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine. In contrast with the galactosamine studies, no radioactivity was found in the glycogen, a result in agreement with the absence of peak *C* material, a compound shown previously to be a glycogen donor (Maley *et al.* 1966*b*). Since no apparent precursor of peak *D* material, other than *N*-acetylgalactosamine 1-phosphate, was evident in the elution patterns, it seemed possible that the latter compound might be directly incorporated into component *D*, i.e. without first undergoing a conversion into *N*-acetylglucosamine 1-phosphate. That this is the case is indicated in Fig. 6, where a comparison of the rates of synthesis of UDP-*N*-acetylglucosamine, galactosamine 1-phosphate, UDP-*N*-acetylgalactosamine and *N*-acetylgalactosamine 1-phosphate is presented. As noted in Fig. 6, the most rapid reaction is the

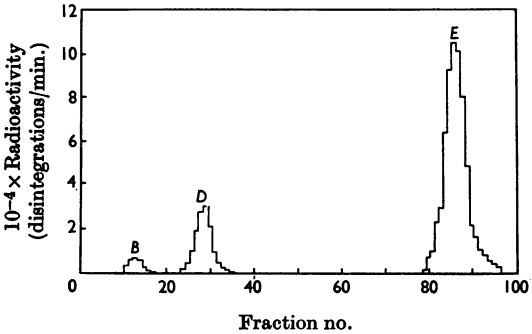

Fig. 5. Dowex 1 (formate form) elution pattern of an acid-soluble extract from a rat liver after intraportal injection of *N*-acetyl[1-^{14}C]galactosamine (3·5 μmoles; 32·8 × 10^6 disintegrations/min.). The liver (9·8 g.) was removed from the rat 1 hr. after the injection and extracted with $HClO_4$ as described in the Methods section. The neutralized extract was chromatographed as described previously (McGarrahan & Maley, 1962; DelGiacco & Maley, 1964).

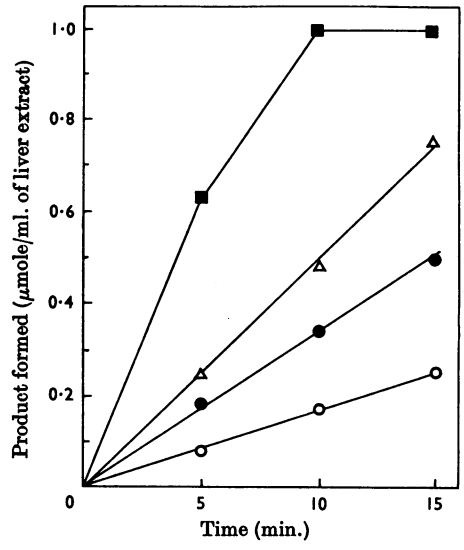

Fig. 6. Formation of UDP-*N*-acetylgalactosamine (●) and UDP-*N*-acetylglucosamine (■). The reaction mixtures contained (in μmoles): tris–HCl buffer, pH 8·5, 20; $MgCl_2$, 5; mercaptoethanol, 2; UTP, 4·9; *N*-acetyl[1-^{14}C]galactosamine 1-phosphate (2·88 × 10^6 disintegrations/min./μmole), 0·12, or *N*-acetyl[1-^{14}C]glucosamine 1-phosphate (2·88 × 10^6 disintegrations/min./μmole, 0·12; 0·1 ml. of the supernatant fraction from a 30% iso-osmotic-KCl homogenate of rat liver centrifuged for 30 min. at 144000*g*. The final volume was 0·4 ml. with the reactions being terminated by heating in a boiling-water bath for 2 min. at the indicated times. The supernatant fractions were placed on Dowex 1 (formate form) columns (4 cm. × 1 cm.) and excess of radioactive material was eluted with 4 N-formic acid until a background count was obtained. The UDP-*N*-acetylhexosamine was then eluted with 15 ml. of 4 N-formic acid + 0·2 M-ammonium formate. Formation of galactosamine 1-phosphate (△) and *N*-acetylgalactosamine 1-phosphate (○). The reaction mixtures contained (in μmoles): tris–HCl buffer, pH 8·5, 20; $MgCl_2$, 3; NaF, 6; mercaptoethanol, 3; ATP, 10; [1-^{14}C]galactosamine (7·08 × 10^6 disintegrations/min./μmole), 0·33, or *N*-acetyl[1-^{14}C]galactosamine (1·04 × 10^6 disintegrations/min./μmole), 0·9; 0·1 ml. of a rat supernatant fraction as described above; the final volume was 0·6 ml. The reactions were terminated by heat and assayed by means of Dowex 1 (formate form) columns as described by McGarrahan & Maley (1962).

synthesis of UDP-*N*-acetylglucosamine. The product resulting from the reaction of UTP and *N*-acetylgalactosamine 1-phosphate was isolated by anion-exchange chromatography followed by paper chromatography in ethanol–M-ammonium acetate, pH 7·5 (5:2, v/v). The purified nucleotide was found to contain UMP, acid-labile phosphorus (0·1 N-hydrochloric acid at 100° for 10 min.) and *N*-acetylgalactosamine in the proportions 1:1:1. The *N*-acetylhexosamine portion of the molecule was identified by colour yield in the modified Morgan–Elson reaction (Reissig *et al.* 1955), borate electrophoresis and gas–liquid chromatography. In the last case, the unknown compound was eluted with a retention time relative to sorbitol of 1·5; known samples of *N*-acetylglucosamine and *N*-acetylgalactosamine had relative retention times of 1·75 and 1·50 respectively (see the Methods section). Whether the formation of UDP-*N*-acetylgalactosamine is effected by a specific UDP-*N*-acetylgalactosamine pyrophosphorylase or is due to a lack of specificity on the part of UDP-*N*-acetylglucosamine pyrophosphorylase is still to be determined. The next reaction in the sequence, (4), the epimerization of UDP-*N*-acetylgalactosamine to UDP-*N*-acetylglucosamine, was described previously with a partially purified calf acetone-dried powder extract as an enzyme source (Maley & Maley, 1959). The absence of *N*-acetylglucosamine from the isolated UDP-*N*-acetylgalactosamine is in support of the direct synthesis of the latter compound.

The isolation of UDP-*N*-acetylgalactosamine after the reaction of UTP and *N*-acetylgalactosamine 1-phosphate represents the first clear-cut demonstration of this synthesis. Strominger & Smith (1959) indicated that a pyrophosphorolysis of UDP-*N*-acetylgalactosamine to the extent of 3% that of UDP-*N*-acetylglucosamine occurred in a partially purified enzyme preparation from *Staphylococcus aureus*; however, the products of the reaction were not characterized, nor was the possibility of epimerization of the UDP-*N*-acetylgalactosamine to UDP-*N*-acetylglucosamine followed by pyrophosphorolysis of the latter eliminated.

We are grateful to Dr Thomas H. Plummer, jun., for his aid with the amino acid analyser. This work was supported in part by Grant CA-6406 from the U.S. Public Health Service and Grant GB-4589 from the National Science Foundation.

REFERENCES

Ames, B. N. & Dubin, D. T. (1960). *J. biol. Chem.* **235**, 769.
Ballard, F. J. (1966). *Biochem. J.* **98**, 347.
Chou, T. C. & Soodak, M. (1952). *J. biol. Chem.* **196**, 105.
DelGiacco, R. & Maley, F. (1964). *J. biol. Chem.* **239**, PC 2400.
Dische, Z. & Borenfreund, E. (1950). *J. biol. Chem.* **184**, 517.
Findlay, J., Levvy, G. A. & Marsh, C. A. (1958). *Biochem. J.* **69**, 467.
Foster, A. B., Martlew, E. F. & Stacey, M. (1953). *Chem. & Ind.* p. 825.
Gardell, S. (1953). *Acta chem. scand.* **7**, 207.
Glaser, L. (1959). *J. biol. Chem.* **234**, 2801.
Hendler, R. W. (1964). *Analyt. Biochem.* **7**, 110.
Heyns, K. & Paulsen, H. (1955). *Chem. Ber.* **88**, 188.
Kalckar, H. M. (1965). *Science*, **150**, 305.
Kalckar, H. M., Braganca, B. & Munch-Petersen, A. (1953). *Nature, Lond.*, **172**, 1038.
Leloir, L. F. (1951). *Arch. Biochem. Biophys.* **33**, 186.
Leloir, L. F., Cardini, C. E. & Olavarría, J. M. (1958). *Arch. Biochem. Biophys.* **74**, 84.
McGarrahan, J. F. & Maley, F. (1962). *J. biol. Chem.* **237**, 2458.
Maley, F. & Lardy, H. A. (1956). *Science*, **124**, 1207.
Maley, F., McGarrahan, J. F. & DelGiacco, R. (1966*a*). *Fed. Proc.* **25**, 526.
Maley, F., McGarrahan, J. F. & DelGiacco, R. (1966*b*). *Biochem. biophys. Res. Commun.* **23**, 85.
Maley, F. & Maley, G. F. (1959). *Biochim. biophys. Acta*, **31**, 577.
Maley, F., Maley, G. F. & Lardy, H. A. (1956). *J. Amer. chem. Soc.* **78**, 5303.
Miller, L. L., Bly, C. G., Watson, M. L. & Bale, W. F. (1951). *J. exp. Med.* **94**, 431.
Molnar, J., Robinson, G. B. & Winzler, R. J. (1964). *J. biol. Chem.* **239**, 3157.
Moore, S., Spackman, D. H. & Stein, W. H. (1958). *Analyt. Chem.* **30**, 1185.
Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955). *J. biol. Chem.* **217**, 959.
Richmond, J. E. (1965). *Biochemistry*, **4**, 1834.
Roseman, S. & Daffner, I. (1956). *Analyt. Chem.* **28**, 1743.
Slein, M. W., Cori, G. T. & Cori, C. F. (1950). *J. biol. Chem.* **186**, 763.
Spiro, R. G. (1960). *J. biol. Chem.* **235**, 2860.
Strominger, J. L. & Smith, M. S. (1959). *J. biol. Chem.* **234**, 1822.
van der Vies, J. (1954). *Biochem. J.* **57**, 410.
Villar-Palasi, C. & Larner, J. (1960). *Arch. Biochem. Biophys.* **86**, 61.
Warren, L. (1959). *J. biol. Chem.* **234**, 1971.
Yosizawa, Z. (1964). *Nature, Lond.*, **201**, 926.