ENZYMATIC FORMATION OF ACETYLGALACTOSAMINE*

By C. E. CARDINI and LUIS F. LELOIR

(From the Instituto de Investigaciones Bioquímicas, Fundación Campomar, Buenos Aires, Argentina)

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Several papers have been published on the biosynthesis of glucosamine in different organisms (1–3), but the reactions which lead to the synthesis of galactosamine have hardly been explored. The first indication of its mode of synthesis came from the isolation of uridine diphosphate[1] acetylgalactosamine from liver by Pontis (4). This finding showed that the transformation of acetylhexosamines is similar to that of the pair glucose-galactose which has been studied in detail (5, 6). Pontis mentioned that, upon incubation of UDP-acetylglucosamine with a crude liver extract, some acetylgalactosamine was formed, but he did not investigate whether the substance appeared in the free or combined form. Further work on the point is reported in this paper. The products arising by the action of rat liver homogenate on UDP-acetylglucosamine were found to be free acetylgalactosamine and uridine monophosphate.

Methods

Analytical—The methods of Reissig *et al.* (7) for acetylhexosamine and of Fiske and Subbarow (8) for phosphate were used.

Substrates—UDP-acetylglucosamine was obtained from yeast by chromatography on an anion exchange column with the use of chloride solutions as displacing agents. The procedure, which was similar to that used previously (9), was developed by Dr. H. Pontis, to whom we are indebted for generous gifts of UDP-acetylglucosamine.

Acetylglucosamine-1-phosphate was prepared as described previously (2). Galactosamine (10) and acetylgalactosamine (11) were found to be contaminated with a small amount of a substance which migrated slowly in paper chromatograms. This substance, probably a disaccharide, could be removed by chromatography on charcoal (Norit A). After an aqueous solution of acetylgalactosamine was run into the column, elution was carried out gradient-wise with water and ethanol up to 2 per cent concentra-

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[1] The following abbreviations are used: UDP for uridine diphosphate and UMP for uridine 5′-monophosphate.

tion. The fractions containing the substance were pooled and evaporated under reduced pressure. From the residue, acetylgalactosamine was crystallized with methanol and ethyl ether.

The same procedure was used for the purification of galactosamine, except that the solutions which were run into the column contained in addition 0.01 N HCl.

Preparation of α-Galactosamine-1-phosphate—The procedure was similar to that described previously (12), but the purification was carried out by anion exchange chromatography. A solution containing 20 mg. of galactosamine hydrochloride in 20 ml. of water, 0.5 ml. of 0.1 M $MgCl_2$, 0.5 ml. of 2 M tris(hydroxymethyl)aminomethane buffer, pH 7.4, 3 ml. of 0.04 M adenosine triphosphate, and 3 ml. of *Saccharomyces fragilis* extract (prepared by extracting the cells dried by air with 3 volumes of 0.1 M $HNaCO_3$ during 24 hours at 5°) was incubated at 37°. After 2 to 3 hours, the proteins were coagulated by heat and filtered off, and the clear liquid was poured into a Dowex 1 acetate column (100 cm. × 2.5 sq. cm.). Elution was then carried out gradient-wise with acetic acid, and the galactosamine phosphate was separated as the barium salt as described previously for glucosamine-6-phosphate (2).

Preparation of N-Acetyl-α-galactosamine-1-phosphate—50 μmoles of barium galactosamine-1-phosphate in 2 ml. of water were acetylated at 0° by adding 500 μmoles of acetic anhydride in several portions. The pH was maintained near neutrality by adding dilute ammonium hydroxide. The mixture was treated with a slight excess of basic lead acetate solution and made alkaline to phenolphthalein with ammonium hydroxide. The mixture was centrifuged and the precipitate washed with water and decomposed with hydrogen sulfide. After aeration, the liquid was taken to pH 8 with $Ba(OH)_2$, and 3 volumes of ethanol were added. The yield was about 35 μmoles. Analysis showed that equal amounts of inorganic phosphate and acetylgalactosamine were liberated by acid hydrolysis (7 minutes at 100° in N acid), the identity of which was checked by paper chromatography.

After deacetylation (3 hours at 100° in 2 N acid), galactosamine was identified by paper chromatography. Determination of the rotatory power of *N*-acetylgalactosamine-1-phosphate gave $[\alpha]_D$ +178° calculated for the free acid from the labile phosphate content. Calculation of the molar rotatory power ($[\alpha]_D$ × molecular weight) from this value gives 54,000. For methyl-*N*-acetyl-α-galactosaminide, the values are +187.3 and 44,000, respectively, with the use of the data of Masamune *et al.* (13). The similarity of these two values indicates that the acetylgalactosamine-1-phosphate is the α anomer.

Paper Chromatography—The method for separating acetylglucosamine

from acetylgalactosamine with borate-treated paper described by Cabib *et al.* (9) was used. In order to avoid tailing and an irregular shape of the spots, it was found that, after immersion in the borate solution, the papers had to be pressed firmly between two sheets of blotting paper and then allowed to dry at room temperature. The borate solution was prepared by adding 8 ml. of 5 N HCl to a solution containing 15.3 gm. of sodium metaborate and adding water to complete 200 ml. The pH was about 8. The solvent for chromatography was *n*-butanol-pyridine-water, 6:4:3.

The spots were revealed by dipping the papers in 0.5 N NaOH in ethanol, heating for 10 minutes at 100°, and then dipping into a *p*-dimethylaminobenzaldehyde solution prepared as described by Reissig *et al.* (7) for the stock solution. The pink color appeared rapidly at room temperature. The rate of migration of acetylgalactosamine was about 40 per cent that of acetylglucosamine.

Application of Method to Non-Acetylated Hexosamines—Since hexosamines can be easily converted to the *N*-acetyl derivatives, the method mentioned above could be used for the detection of glucosamine and galactosamine. The procedure was as follows. The solutions containing 0.05 to 0.2 μmole were evaporated to dryness in a test plate, and the following reagents were added: 0.1 ml. of water, about 0.001 ml. of acetic anhydride, a small drop of bromothymol blue, and sufficient dilute ammonia to keep the indicator blue. The solutions were evaporated to dryness in a vacuum desiccator and chromatographed as described above.

Since the separation of the acetyl derivatives is clear cut, this method may be useful whenever glucosamine or galactosamine has to be identified.

Preparation of Enzyme System—Rat liver (about 7 gm.) was homogenized in 2 volumes of water and centrifuged for 10 minutes at 3000 r.p.m. The supernatant solution (crude extract) was treated with 0.4 volume of 50 per cent (w/v) ammonium sulfate solution neutralized to pH 7.0 with ammonium hydroxide. The precipitate was separated by centrifugation at 0° and discarded. To the supernatant solution 0.2 volume of ammonium sulfate solution was added. This precipitate was dissolved in 0.4 ml. of water and stored at −10°.

Estimation of Enzymes—The test system was made up as follows: 0.02 ml. of 0.01 M UDP-acetylglucosamine, 0.01 ml. of 0.5 M magnesium sulfate, 0.01 ml. of 2 M tris(hydroxymethyl)aminomethane buffer, pH 7.5, 0.04 ml. of 0.1 M cysteine, and enzyme solution; total volume, 0.12 ml. The amount of enzyme was 0.04 ml. of the crude or 0.01 ml. of the purified extract containing about 1 mg. of protein. The incubation time was usually 15 minutes at 37°.

When the liberation of free acetylgalactosamine was measured, the mix-

ture was deproteinized by adding 0.1 ml. each of zinc sulfate and barium hydroxide and 0.4 ml. of water (14). After centrifuging, 0.5 ml. of the supernatant fluid was analyzed for acetylhexosamine.

For the chromatographic tests for free acetylhexosamines, the zinc sulfate-barium hydroxide supernatant fluids were concentrated and spotted on paper. When it was necessary to chromatograph the free and bound acetylhexosamines, deproteinization was carried out by adding 0.5 ml. of 5 per cent trichloroacetic acid. The supernatant fluid was heated for 10 minutes at 100° in order to hydrolyze the UDP-hexosamine compounds, extracted twice with 3 ml. of ethyl ether, neutralized with concentrated ammonium hydroxide, and spotted on paper.

TABLE I

Paper Chromatography of Nucleotides

The enzyme system is as described in the text. Protein was precipitated with 5 per cent trichloroacetic acid. The extract was washed with ether, neutralized with ammonium hydroxide, and spotted on Whatman No. 1 paper. The solvent was ethanol ammonium acetate, pH 7.5 (16), containing ethylenediaminetetraacetate. The substances were localized under ultraviolet light.

	Distance from origin
	cm.
UDP-acetylglucosamine + enzyme system, $t = 0$	29
" + " " $t = 15$ min	28, 20.2
UMP	20.1
UDP	10.5

Results

Detection of Enzyme—The incubation of UDP-acetylglucosamine with crude liver extracts was found to lead to the formation of a substance which behaved like acetylgalactosamine when chromatographed on borate-treated paper or upon deacetylation and degradation with ninhydrin (15). The substance reacts directly with the modified Morgan and Elson reagent, whereas the acetylhexosamines bound to UDP give a negative reaction. Therefore the activity of the enzyme system could be detected qualitatively by paper chromatography with or without acid hydrolysis and quantitatively by measuring the formation of free acetylhexosamine.

The enzyme system was found to be very labile so that the only purification step which could be carried out was a precipitation with neutralized ammonium sulfate. When stored frozen, the concentrated enzyme solution or the ammonium sulfate precipitate was still active after 4 to 5 days, but after a short dialysis (3 hours) the enyzme system became so labile that the activity disappeared overnight even when stored at −10°. No

reactivation could be obtained by the addition of heated liver extracts or diphosphopyridine nucleotide.

Distribution of Enzyme System—The activity of several tissues was investigated both by chromatography and by measuring the liberation of acetylgalactosamine. Only rat and guinea pig liver were found to be active. Rat kidney, lung, muscle, brain, and spleen were inactive under the conditions of the test. *Saccharomyces fragilis* extracts were also inactive, although they contain a very active galacto-waldenase.

TABLE II

Action of Enzyme System on Different Substrates

The complete system is as described in the text. The amount of substrate added was about 0.2 μmole.

	Δ acetylgalactosamine	Δ inorganic P
	μmole	*μmole*
UDP acetylglucosamine	0.22	0.23
Acetylgalactosamine-1-phosphate	0	0
Acetylglucosamine-1-phosphate	0	0
UDP		0.42
UMP		0.15
β-Glycerophosphate		0.04
Phenyl phosphate		0.05

TABLE III

Free and Combined Acetylhexosamines in Reaction Products

	Charcoal filtrate		Charcoal adsorbate	
	Acetylglucosamine	Acetylgalactosamine	Acetylglucosamine	Acetylgalactosamine
Complete system, $t = 0$	0	0	+++	0
Same plus 0.3 μmole acetylgalactosamine	0	++	+++	0
Complete system, $t = 15$ min.	0	++	+++	0

The reaction mixture was as in the text, but the amounts were increased 5-fold, and the crude enzyme was used. After 15 minutes at 37° the proteins were precipitated with 0.5 ml. of 5 per cent trichloroacetic acid. 50 mg. of charcoal (Norit A) were added to the supernatant solution. The mixture was filtered after 15 minutes. The filtrate (charcoal filtrate) was saved for chromatography. The charcoal was washed three times with 10 ml. of water containing 2 per cent ethanol, suspended in 0.5 ml. of 5 per cent trichloroacetic acid, and heated for 10 minutes at 100°. After filtration this fraction (charcoal adsorbate) and the filtrate were freed from acid by extraction with ethyl ether, neutralized with ammonium hydroxide, and dried for chromatography on borate-treated paper as described in the text.

Reaction Products—The liberation of free acetylgalactosamine was accompanied by a liberation of inorganic phosphate which varied somewhat with different lots of enzyme. The product arising from the uridine moiety of UDP-acetylglucosamine was investigated by paper chromatography, and, as shown in Table I, after incubation with the enzyme system a substance was formed which migrated like uridine monophosphate. No UDP could be detected chromatographically or by analysis (17).

The liberation of inorganic phosphate and acetylgalactosamine from different substrates is shown in Table II.

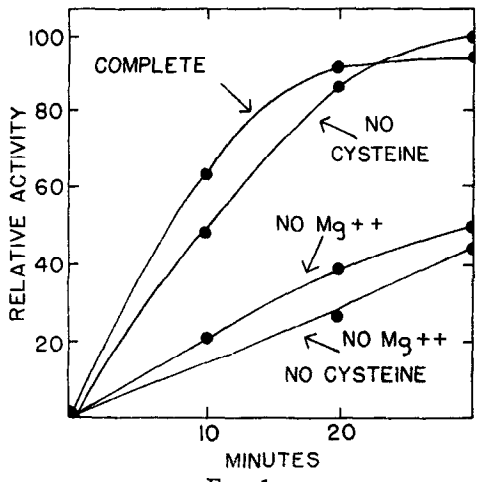

FIG. 1

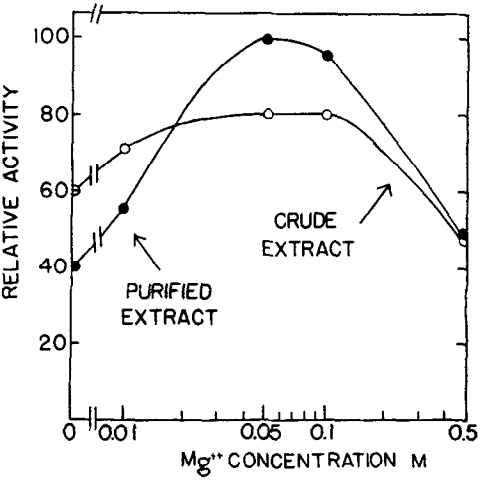

FIG. 2

FIG. 1. The action of magnesium ions and cysteine. The substrate was UDP-acetylglucosamine. The activity was measured in terms of acetylgalactosamine formation by the purified enzyme preparation from liver.

FIG. 2. The influence of Mg^{++} concentration. Determination of the optimal concentration with the complete system was described in the text.

Several experiments were carried out in order to detect the accumulation of UDP-acetylgalactosamine, by separating the nucleotides from the free acetylhexosamines by adsorption on charcoal. As shown in Table III, no nucleotide-bound acetylgalactosamine could be detected after incubation of UDP-acetylglucosamine with a crude rat liver extract. Similar tests with the enzyme precipitated with ammonium sulfate or guinea pig liver were also negative.

Conditions for Optimal Activity—The system was found to be activated by cysteine and by Mg^{++} ions (Fig. 1). The maximal rate was obtained with a concentration of cysteine from 0.01 to 0.1 M and with 0.05 Mg^{++} (Fig. 2). The activation obtained with Mn^{++} was smaller than with

Mg^{++}. The optimal pH was found to be between pH 7.5 and 8.0 (Fig. 3).

Specificity—No free acetylgalactosamine was formed with acetylglucosamine-1-phosphate, acetylgalactosamine-1-phosphate, or free acetylglucosamine.

Inhibitors—The influence of different substances on the liberation of acetylgalactosamine was as follows: Ethylenediaminetetraacetate 0.01 M 50 per cent inhibition. No effect was obtained with borate (0.01 to 0.1 M), penicillin, cortisone, hydrocortisone, fluoride, or acetyl salicylate. Complete inhibition was produced by adding 0.001 M Ni^{++} or Co^{++} instead of Mg^{++}.

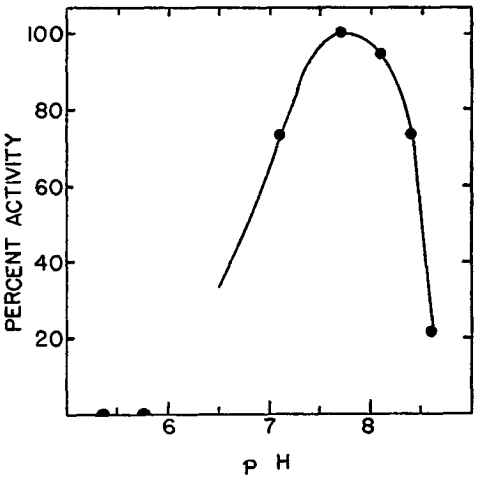

FIG. 3. pH optimum. Complete system as described in the text with 0.2 ml. of tris(hydroxymethyl)aminomethane buffer. pH determined on aliquots with a glass electrode.

DISCUSSION

On the basis of the knowledge on the glucose-1-phosphate-galactose-1-phosphate transformation and in consideration of the fact that UDP-acetylgalactosamine has been found in liver, the results reported in this paper can be interpreted as being due to the action of three enzymes as follows:

(1) UDP-acetylglucosamine $\rightleftarrows$ UDP-acetylgalactosamine

(2) UDP-acetylgalactosamine $\rightarrow$ UDP + acetylgalactosamine

(3) UDP $\rightarrow$ UMP + P

Reaction 1 would be an inversion at C_4 similar to the galacto-waldenase

reaction in which diphosphopyridine nucleotide is involved (6). Reaction 2 would be catalyzed by another enzyme of rather high specificity, since, under the same conditions, neither UDP-acetylglucosamine nor acetyl-α-glucosamine-1-phosphate is appreciably hydrolyzed.

Reaction 3 in which UDP is hydrolyzed has been detected by several workers (18, 19) and has been found to be catalyzed by the extracts used in this work.

The fact that no UDP-acetylgalactosamine could be detected would be a consequence of a faster rate of Reaction 2 as compared with Reaction 1, and may be also attributed to the lack of sensitivity of the analytical methods.

If it is taken for granted that the enzyme system is equally active in intact liver and that the acetylgalactosamine formed appears free in the cells, we are confronted with the problem of what is the fate of free acetylgalactosamine. Preliminary experiments have shown that under certain conditions acetylgalactosamine may be transformed by liver enzymes. This point is now under investigation.

SUMMARY

The action of liver enzymes on uridine diphosphate acetylglucosamine has been studied. The products obtained were free acetylgalactosamine, uridine monophosphate, and inorganic phosphate.

The preparation of *N*-acetyl-α-galactosamine-1-phosphate is described.

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