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# PHOSPHORYLATION OF ACETYLHEXOSAMINES [1]

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## INTRODUCTION

In a previous paper (1), a liver enzyme system which leads to the formation of free acetylgalactosamine from uridine diphosphate-acetylglucosamine, was described. In experiments designed to discover the further fate of acetylgalactosamine, it was found that liver and kidney enzymes catalyze its phosphorylation by adenosine triphosphate (ATP). Furthermore, a similar process was found to take place with acetylglucosamine as substrate. Phosphorylation of acetylglucosamine with *Escherichia coli* extracts has been reported by Soodak (2) and by Faulkner and Quastel (3). The phosphorylation of monacetylated hexosamines has been studied by several workers. Thus glucosamine has been found to be phosphorylated by ATP and hexokinase to glucosamine 6- phosphate (4, 5). Similarly, galactosamine is transformed into galactosamine 1-phosphate by an enzyme presumably identical with galactokinase (4).

## METHODS

### *Analytical*

The following methods were used: Blix (7) for hexosamines, Morgan and Elson as modified by Reissig *et al.* (8) for acetylhexosamines, Kunitz and McDonald (9) for proteins, Fiske and SubbaRow (10) for phosphate, and a slight modification of the method of Schales and Schales (11) for reducing power.

### *Substrates*

Galactosamine and acetylgalactosamine were purified as described previously (1). Acetylglucosamise 6-phosphate and acetylgalactosamine 1-phosphate were obtained by acetylation of glucosamine 6-phosphate (17) and galactosamine 1-phosphate (1), respectively.

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### *Paper Chromatography*

The procedure described by Cardini and Leloir (1) was used for free acetylhexosamines, and that of Paladini and Leloir (12) for the esters.

### *Preparation of the Enzymes*

The organs were homogenized in 3 vol. of 0.15 *M* KCl containing 0.001 *M* ethylenediamine tetraacetate. The enzymes were prepared from rat liver or kidneys following the procedure described by Roy (13) for the preparation of the sulfate activating system. It consists of a high-speed centrifugation in which the small particles which decompose ATP are removed, followed by ammonium sulfate precipitation between the following molar concentrations: *A*: 0-1.7, *B*: 1.7-2.3, *C*: 2.3-2.9. The precipitates were dissolved in water to a concentration of 80-100 mg. protein/ml.

### *Estimation of the Enzymes*

The disappearance of acetylhexosamine was measured after precipitating the proteins and phosphate esters with $ZnSO_4$ and $Ba(OH)_2$. Usually the reaction mixture contained (in micromoles) 0.1 of acetylglucosamine (or acetylgalactosamine), 0.2 of sodium ATP, 0.3 of $MgCl_2$ and 0.03 ml. of 0.25 *M* tris (hydroxymethyl) aminomethane buffer of pH 7.1 (or 8.2 for acetylgalactosamine) and 0.01 ml. of enzyme solution. Total volume 0.10 ml. Incubation was usually carried out for 2 hr. at 37°, after which 0.1 ml. of 100 % $ZnSO_4$ and 0.1 ml. of 0.3 *M* $Ba(OH)_2$ were added (14). After centrifuging 0.2 ml. of the supernatant was taken for acetylhexosamine analysis. The difference in acetylhexosamine content between a complete sample and one incubated without ATP was taken as the amount esterified. When the esterification of acetylgalactosamine was measured, the precipitation of the phosphate esters with the zinc and barium solutions could be omitted without modifying the results.

### *Purification of the Reaction Products*

Preliminary experiments showed that the reaction products could not be purified using ion-exchange resins, because they were not adsorbed due to the relatively large amounts of inorganic salts. It was observed, that the reaction product could be adsorbed on charcoal from acid solution, and eluted on raising the pH. This observation provided the basis for the method of purification described below. The reaction mixture was made up as follows: 50 μmoles of

acetylhexosamine, 100 μmoles of ATP, 60 μmoles of magnesium chloride, and 5 ml. of enzyme solution. Total volume 20 ml. The pH was adjusted to 7.1 with sodium hydroxide when acetylglucosamine was the substrate and to 8.2 for acetylgalactosamine. The enzyme fraction used corresponded to *A* or *B* or *C*, respectively. After incubating for 2 hr. at 37°, 3 vol. of ethanol was added, the precipitate was filtered off, and the filtrate was freed from ethanol by evaporation under reduced pressure to about one-tenth of the original volume. The solution was then adjusted to pH 4 and poured into a column of 1.8 cm. diameter and 17 cm. long, containing a mixture of 8 g. charcoal (Norit A) and 8 g. filter aid (Hyflo Super-Cel). Before the experiment the column was washed with 0.05 *N* formic acid until the effluent was acid to bromocresol green.

Once the sample has been adsorbed on the charcoal, elution was carried out gradientwise as described by Alm, Williams, and Tiselius (15). A 250-ml. mixing chamber contained three parts of 0.05 *N* formic acid and one part of 0.05 *N* ammonium hydroxide. The inflowing solution was 0.05 *N* ammonium hydroxide. Samples of 6-10 ml. were collected. The sulfate and inorganic phosphate appeared in the first tubes, while the acetylhexosamine esters emerged after 200-300 ml. of solution had passed through the column and when the pH was about 7. In the experiments where acetylglucosamine was the substrate, the samples were analyzed directly for acetylhexosamine. When the substrate was acetylgalactosamine, acid was added to the samples to 0.5 *M*. They were then heated for 5 min at 100°, neutralized with the calculated amount of NaOH solution, and then analyzed for acetylhexosamine. The samples containing acetylhexosamine were pooled, concentrated under reduced pressure, and then stored for several days in a vacuum desiccator over sodium hydroxide and sulfuric acid in order to remove the ammonium formate. The yield was about 60 %. In the experiments with the ester from acetylgalactosamine which is alkali-stable, the evaporation was carried out at pH 7-8, while with the acetylglucosamine ester the pH was maintained around 6.

## Results

### *Conditions for Maximal Activity*

As shown in Fig. 1, the greatest activity was obtained with 0.003 *M* Mg++. The pH optima were 7.1 and 8.2, respectively, for acetylglucosamine and acetylgalactosamine (Fig. 2).

### *Properties of the Enzyme*

As shown in Table I, the activity of the different fractions obtained by precipation with ammonium sulfate is not parallel for the two substrates. The activity on acetylglucosamine is greater in fraction A, and that on acetylgalactosamine in fraction C. No activity on galactosamine was detectable when tested under the same conditions with the rat kidney enzyme. The fraction most active on acetylglucosamine (A) was the least active on glucosamine, so that it seems that the enzymes acting on the acetylated hexosamines are different from those acting on the nonacetylated substances. Under the conditions of the tests, acetylglucosamine 6-phosphate did not disappear when incubated with any of the enzyme fractions. However, after dialysis B catalyzed the disappearance of acetylglcosamine 6-phosphate as has been described previously (17). These reactions are inhibited by ammonium ions, and that is why they cannot be detected with undialyzed preparations. As

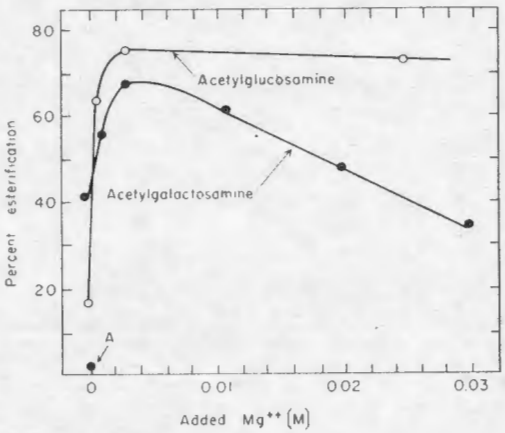


FIG. 1. — Effect of Mg++ concentration. Assays as described in text. Enzyme fraction A was used for acetylglucosamine, and fraction B for acetylgalactosamine. The amounts of ATP were 0.4 and 1.6 μmoles, respectively. Incubation time was 1 hr. Point A corresponds to a reaction mixture containing 0.01 *M* ehtylenediamine tetraacetate.

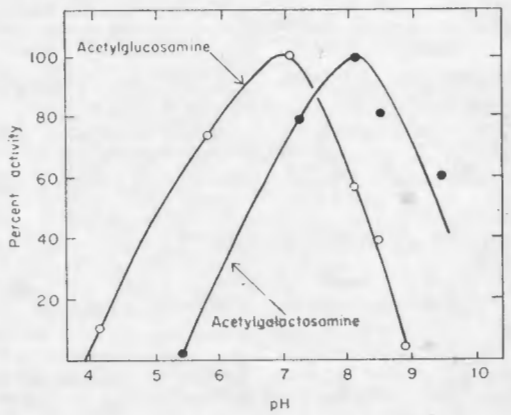


FIG. 2. — pH optimum: The pH was measured on aliquots with a glass electrode. The buffers were tris (hydroxymethyl) aminomethane with maleate (pH 5-7) or chloride (pH 7-9) (16).

TABLE I

*Activity on Different Substrates*

Methods as described in text. The rate of esterification was calculated from the linear part of a time, curve.

| Kidney fractions | Micromoles esterified/hr. Acetylglucosamine | Acetylgalactosamine | Glucosamine |
|---|---|---|---|
| A | 0.06 | 0 | 0 |
| B | 0.05 | 0.03 | 0.007 |
| C | 0.024 | 0.06 | 0.014 |

to the stability of the enzymes, they could be stored for a month or more at −10°C. without great loss in activity. Dialysis overnight led to complete inactivation of the acetylgalactosamine enzyme. No reactivation could be obtained by adding heated extracts. The enzyme acting on acetylglucosamine could be dialyzed overnight at 0° without apreciable loss of activity.

### *Activity in Different Organs*

Table II shows the results obtained with extracts of different organs. Kidney extracts were most active on acetylgalactosamine, followed by heart, spleen, and liver. The activity on acetylglucosamine was slightly lower than with acetylgalactosamine as substrate. The negative finding of Faulkner and Quastel (3) with brain extracts and acetylglucosamine as substrate may have been due to the high ATP-descomposing activity of their extracts.

TABLE II

*Activity in Different Organs*

Assays as in Table I except thar the amount of ATP was 1.0 μmoles and the final volume was 0.2 ml. The enzyme solutions were obtained as described in the text, except that only one precipitation was carried out with 2.9 *M* ammonium sulfate.

| | Rate of esterification in micromoles/hr./mg. protein Acetylgalactosamine | Acetylglucosamine |
|---|---|---|
| Kidney | 0.10 | 0.06 |
| Heart | 0.07 | 0.05 |
| Spleen | 0.06 | 0.02 |
| Liver | 0.05 | 0.04 |
| Brain | 0.03 | 0.03 |
| Lung | 0.01 | 0.01 |

### *Properties of the Reaction Products*

*Acetylglucosamine.* When the tests for enzyme activity were carried out without precipitation of the phosphate esters, no change was detectable in the amount of acetylhexosamine. This shows that the reaction products gives this reaction similar to free acetylglucosamine.

Analysis of the product obtained after purification with charcoal gave the following results: (total phosphate taken as 1.0): inorganic phosphate, 0; Morgan and Elson reaction (with acetylglucosamine as standard) 1.1; reducing power (with acetylglucosamine as standard) 0.9.

After hydrolysis of the phosphate group with phosphate, acetylglucosamine was identified by paper chromatography on borate-treated paper.

When run on paper with the ethanol-ammonium acetate solvent of pH 3.8, the product gave the same *Rf* as a sample of acetylglucosamine 6-phosphate obtained by acetylation of glucosamine 6-phosphate with acetic anhydride. Moreover, both substances gave approximately the same results when tested as activators of the deamination of glucosamine 6-phosphate with kidney enzymes (17). Another test which was carried out consisted in adding the deaminase together with glucose 6-phosphate dehydrogenase, isomerase, and triphosphopyridine nucleotide. Thus the formation of fructose 6-phosphate could be followed by reduction of triphosphopyridine nucleotide in a spectrophotometer. In this test the two above-mentioned preparations were indistinguishable. The phosphorus liberated in 0.2 *N* alkali at 100° in 3 min. was 60 %. A sample of glucose 6-phosphate which was run at the same time gave 64 %. All the properties of the reaction product which have been investigated are those of acetylglucosamine 6-phosphate. This substance has been prepared previously by Brown (18) by enzyme acetylation of glucosamine 6-phosphate, and by Roseman and Ludowieg (19) and by Leloir and Cardini (17) using acetic anhydride. Maley and Lardy (20) have published a purely chemical method of synthesis.

*Acetylgalactosamine.* In this case the result of activity tests was the same with or without precipitation of the phosphate esters, thus showing that some change occurred in the group involved in the reaction with *p*-dimethylaminobenzaldehyde. Analysis of the product obtained by purification with charcoal gave the following results (total phosphate taken as 1.0):

| | Direct | After acid hydrolysis |
|---|---|---|
| Inorganic phosphate | 0 | 1.00 |
| Morgan and Elson reaction (acetylgalactosamine as standard) | 0 | 1.1 |
| Reducing power (acetylgalactosamine as standard) | 0 | 1.1 |

The substance was compared with a sample of acetylgalactosamine 1-phosphate obtained by acetylation of galactosamine 1-phosphate with acetic anhydride (1). When run on paper with ethanol-ammonium acetate solvent of pH 3.8, the two substances were indistinguishable. After acid hydrolysis, acetylgalactosamine was detected by paper chromatography. The rate of acid hydrolysis in 1 *M* acid at 37° was measured and, as shown in Fig. 3, the substance is hydrolyzed about three times more rapidly than α glucose 1-phosphate. The lack of reducing power before hydrolysis shows that the phosphate is in position one. Moreover, the molar rotatory power ($\alpha M = \alpha_D \times$ mol. wt) measured in water was 52,000, which is the same within the experimental errors as the value of + 54,000 found (1) for a preparation obtained by acetylation of α galactosamine 1-phosphate. Thus all the properties of the substance obtained by enzymic phosphorylation of acetylagalactosamine indicate that it is α-acetylgalactosamine 1-phosphate.

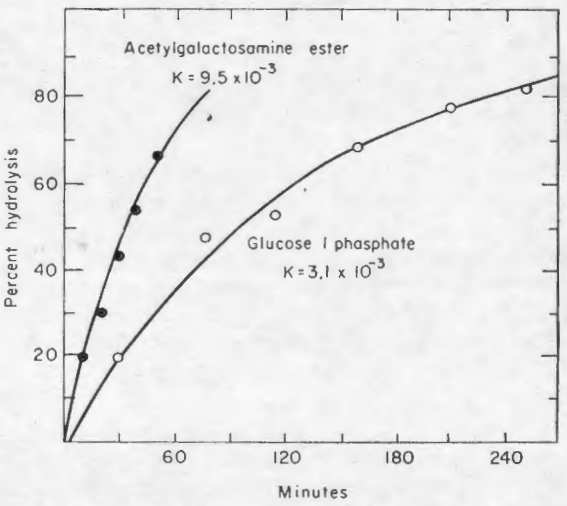


FIG. 3. — Acid hydrolysis of the ester obtained from acetylgalactosamine. After incubation in 1 *N* sulfuric acid at 37° the samples were analyzed for inorganic phosphate. The value of the hydrolysis constant *K* was calculated using $\log_{10}$ and minutes.

## SUMMARY

The phosphorylation of acetylglucosamine and acetylgalactosamine by adenosine triphosphate catalyzed by extracts of rat organs has been studied. The reaction products had the properties of acetylglucosamine 6-phosphate and acetylgalactosamine 1-phosphate, respectively. The enzymes appear to be different from other kinases.

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