SECTION I: ENZYMATIC PHOSPHORYLATION OF N-AcGm
Introduction
The utilization of food materials by living cells proceeds via specific sequences of enzyme reactions - collectively described as metabolic pathway - which fulfil two main functions: they supply the precursors of cell constituents and they supply the energy necessary for biosynthetic and other endergonic processes.

Of the various compounds glucose is one which is oxidized by majority of mammalian tissues and is utilized as a source of energy for biosynthetic processes and other forms of work. The first step in the metabolic route of glucose utilization is a phosphorylation reaction - it being phosphorylated at the 6th carbon atom through the agency of an enzyme. In this type of reaction one phosphoryl group is transferred from a donor molecule to the acceptor molecule by the catalytic action of the enzymes. Enzymes transferring phosphoryl groups from ATP to acceptors have been termed kinases, phosphokinases, phosphotransferases, transphosphorylases etc.

The kinases constitute by far the largest class of known phosphoryl transferring enzymes. The reactions may be thought of as involving nucleophilic attack by the acceptor molecule on the terminal phosphorus atom of the nucleotide (66).

\[
\text{Nucleoside} \quad \overset{\text{O-P-O-P-O}}{\text{O-}} \quad + \quad Y-O-H \\
\downarrow
\]

\[
\text{Nucleoside} \quad \overset{\text{O-P-O-P-O}}{\text{O-}} \quad + \quad Y-O-P-O
\]
The discovery of kinases dates back to 1927 when Meyerhof (67) found that a preparation of yeast autolysate catalyzed the phosphorylation of glucose in presence of ATP according to the following reaction:

\[ \text{Hexose} + \text{ATP} \rightarrow \text{Hexose-6-P} + \text{ADP} \]

This catalyzing factor, designated as hexokinase, was later (68) shown to be an enzyme, and similar or closely related activities were found to be present in most mammalian tissues that can utilize hexoses. The hexokinase activity of many rat tissues, as determined by Long (69) is in the following sequence: (from highest to the lowest) brain, colon, heart, stomach, testis, small intestine, caecum, uterus, spleen, kidney, skeletal muscle, pancreas, lung and liver.

Hexokinase is a common name given to the enzymes catalyzing the transfer of the terminal phosphoryl residue of ATP to any one of the hydroxyl groups of a six carbon monosaccharides. Yeast and animal tissues hexokinases are generally found to be active on more than one hexose. The relative rates of phosphorylation varies from one sugar to another of which glucose has the highest phosphorylation coefficient and is regarded as the physiological substrate. ATP is the phosphoryl group donor for the hexokinase reaction and the enzymes also can utilize ITP only in lieu of ATP at extremely low rates. The hexokinases require \( \text{Mg}^{+2} \) ions for their activity. \( \text{Mg}^{+2} \) may be replaced by other divalent metal ions like \( \text{Mn}^{+2}, \text{Co}^{+2} \) etc. with
lesser degree of effectiveness. Mg$^{+2}$ probably forms complex with ATP and the optimum activity is obtained when the ratio of the two is about unity (70,71).

Several problems concerning the phosphorylation of glucose in liver were resolved upon discovery of a kinase, "glucokinase" (72-75), distinct from the classical hexokinase. The two kinases differ in several ways, most important of which, from a physiological point of view, are their Km for D-glucose ($10^{-2}$ M for glucokinase, $10^{-5}$ M for hexokinase) and the fact that glucokinase is not inhibited by D-glucose-6-phosphate. Although it is not strictly specific for glucose, glucokinase has been given its name to emphasize its role as the kinase responsible for the phosphorylation of D-glucose by the liver in vivo. Since the Km of glucokinase is higher than normal concentrations of blood glucose, the rate of phosphorylation adjusts automatically to charges in the circulating glucose level. Glucokinase activity is depressed by starvation or a carbohydrate-free diet and in alloxanddiabetes; it may be restored by feeding carbohydrates in the first two instances, or by insulin in the last (76-85). Denovo synthesis is necessary for restoration of activity. Hexokinase, on the other hand, is not affected by these hormonal and dietary factors. Mammalian tissues other than liver have only hexokinase. The enzyme is markedly inhibited by D-glucose 6-phosphate in a manner which implies an allosteric inhibitory site (86, 87). But sufficient experimental supports are needed to establish this fact.
The existences of hexokinases which act predominantly or solely on only one hexose in a reaction analogous to the hexokinase reaction has recently been shown. There is evidence now for five such enzymes, namely gluco-6-kinase (88-90), manno-6-kinase (88-90), glucosamine-6-kinase (90,91), N-acetylg glucosamine 6-kinase (25) and fructose-6-kinase (88-90,92). Of these only the fructo-6-kinase (92) from peas has been at all well characterized with regard to substrate specificity. It is of interest to note in this connection that yeast and mammalian tissue hexokinases, although active on various sugars, are very little active on aminosugars except on glucosamine. In mammals aminosugars take an important role in connective tissue metabolism, but fewer informations could be found in literature about the enzymes catalyzing the phosphorylation of aminosugars. Leloir et al. (25) first demonstrated that the extracts of rat kidney and other tissues had the ability of phosphorylating N-AcGm and N-AcGaln with the agency of specific kinases. No purification and properties was studied by them. Later Pattabiraman and Bachhawat (64) purified the N-AcGm kinase about 25 fold from sheep brain extracts and studied some kinetic properties.

Recently we have purified the enzyme, N-AcGm kinase from hog spleen about 3500 fold and studied some of its kinetic properties.
Experimental procedures
Materials

Preparation of N-acetylglucosamine (93)

N-AcGm was prepared by the acetylation of Gm-HCl. D-glucosamine-HCl (10 gms) was added to 80-100 ml. methanol in which 1.1 gm. Na had been dissolved, swirled gently, filtered to remove the precipitated NaCl. Acetic anhydride (6 gm.) was added drop by drop at room temperature with stirring and the mixture was shaken mechanically for about an hour. On standing overnight in the ice-box, a quantitative yield of N-acetyl-D-glucosamine was obtained. This was recrystallised from a water-acetone-alcohol mixture. M.p. -208°.

Preparation of calcium phosphate gel

Calcium phosphate gel was made by the procedure of Keilin and Hartree (94).

Reagents used:

- 0.48M CaCl₂ solution
- 0.32M Na₃PO₄, .12H₂O, solution

150 ml. of the CaCl₂ solution was diluted to a final volume of 1600 ml. and stirred vigorously while 150 ml. of the phosphate solution was added. The pH was adjusted very slowly to 7.4 with dilute acetic acid. The precipitate was washed with distilled water by decantation until chloride free. The precipitate
was finally washed with distilled water in a centrifuge. This should be aged at least for one month before use. The final concentration of the gel was 16.2 mg./ml.

Preparation of Pyruvate Kinase from goat muscle (95)

The muscle (150 gm.) collected from slaughter house, was minced in the cold room and put through a grinder, then weighed. These were then homogenized with 300 ml. of ice-cold 2 x 10⁻³ M EDTA solution in a Waring Blender that had been previously chilled. The resultant thick homogenate was stirred mechanically and vigorously for 30 mins in the cold room, then centrifuged at 10,000 x g for 30 mins. To the supernatant fluid (250 ml.), solid ammonium sulphate was added until a concentration of 1.75M had been attained (total 65 gms would be required). The resulting precipitate was centrifuged at 16,000 x g for 15 mins and discarded. To the measured volume of supernatant, 22 gms solid (NH₄)₂SO₄ was added to bring the molarity to 2.4. The resultant precipitate was collected by centrifugation and dissolved in .02M potassium phosphate buffer, pH 7.6 and used as the source of lactic dehydrogenase and phosphoenol pyruvate kinase.

Preparation of N-AcMm

N-AcMm was prepared by the method of Carroll and Cornforth by epimerization of N-AcGm (96). Satisfactory separation of N-AcMm formed by alkaline epimerization of N-AcGm can be based on differential solubilities. N-acetyl-D-glucosamine was epimerized (pH 11, 2 days) and the product crystallized from water-ethanol. After
separation of N-AcGm, crops of crystalline mixture was obtained. This material was twice stirred (intermittently during 5 min.) in a blender with cold absolute ethanol (25 ml./g.); undissolved N-AcGm was removed, and the filtrate was concentrated (after addition of water) to a small volume. On addition to ethanol, N-acetyl-D-mannosamine monohydrate crystallized. This was chromatographically homogeneous, and its composition, behavior on melting, and mutarotation in aqueous solution agreed with pure N-AcMm.

The following materials were obtained from Sigma Chemical Company, U.S.A., ATP, UTP, CTP, GTP, ADP, AcGm, Gm-HCl, Gl-6-P, Tris, NADH, protamine sulphate, DEAE-cellulose, wheat germ acid phosphatase, and Egg-albumin.

Ammonium sulphate obtained from commercial source was recrystallized from water containing EDTA. Other materials were obtained from commercial source.
Methods

Microdetermination of inorganic phosphate (97)

Reagents used:
Ascorbic acid, 10%
Ammonium molybdate, 2.5%
H₂SO₄, 6N

1 volume of 6N sulfuric acid, 2 volumes of distilled water and 1 volume of 2.5% ammonium molybdate were mixed and 1 volume of 10% ascorbic acid was then added to the mixture (Reagent A).

Samples containing inorganic phosphate (upto 8Y of phosphorus) were taken into a 15 ml graduated centrifuge tube and adjusted the volume to 4 ml with distilled water. The reagent blank was consisted of 4 ml of distilled water.

4 ml. of Reagent A was then added to each tube, mixed well and incubated in a 57°C incubator for 1.5 to 2 hours. After incubation, the tubes were cooled to room temperature and readings were taken in Coleman at 610 mp against the blank. The standard curve for phosphate estimation is shown in Fig. 4.

Quantitative estimation of phosphate from sugar phosphates by the treatment of phosphatase (98)

This method is based on the incubation of the substrate, phosphate ester with the enzyme, phosphatase and the determination of the liberated orthophosphate by Chen, Toribara and Warner's method (97).
Reagents used:

Acid phosphatase from wheat germ - a solution containing 0.1 mg./ml. in water was prepared.

Glucose-6-phosphate and N-AcGm-6-P stock solution (0.01M)
0.15M sodium acetate buffer, pH 5.0

Trichloroacetic acid (cold), 10%.

0.1 ml. substrate, 0.3 ml. of acetate buffer and 0.1 ml. of enzyme were taken in a centrifuge tube and incubated for 15 mins. at 37°C. After incubation, 1 ml. of 10% TCA was added in each tube and then the tubes were chilled in ice. After 5 mins, the incubation mixture was diluted to 2.5 ml. and centrifuged. 2 ml. aliquots were taken for determination of inorganic phosphate. 0.1 μmole of inorganic phosphate is formed by enzymic hydrolysis of 0.1 μmole Glucose-6-P.

Preparation of glycolic aldehyde phosphate by periodate oxidation of phosphorylated sugar (99)

This method is based on the fact that when phosphate group is present in the C-6-position of a hexose molecule, the oxidation product of the sugar ester gives rise to glycolic aldehyde phosphate.

Reagents used:

Glucose-6-P and N-acetylglucosamine-6-P (0.01M)
Sodium metaperiodate, 0.2M
Sodium acetate buffer, pH 4.5, 0.4M
Ethylene glycol.
To 0.5 ml. of phosphorylated sugar solution in a calibrated tube, 0.4 ml. periodate solution and 0.25 ml. acetate buffer, pH 4.5 were added. The volume was then made up to 5.5 ml. with H₂O and kept at 5°C for 20 hours. After that, the reaction mixture was treated with 0.3 ml. of ethylene glycol for the destruction of excess periodate in the mixture.

The reaction product i.e. glycolaldehyde phosphate was then isolated by passing the reaction mixture through a column of Dowex-1-acetate (2 ml packed volume) and after washing the column with 25 ml. of water, eluting the phosphate ester with 0.1N H₂SO₄. The phosphate ester of glycolic aldehyde was identified by diphenylamine colour reaction as described by Dische and Borenfreund (100).

Identification of glycolic aldehyde phosphate by the method of Dische et al. (100)

Reagents used:

Diphenylamine reagent - 800 mg. of diphenylamine was dissolved in a mixture of 80 ml. of glacial acetic acid and 0.55 ml. of H₂SO₄.

To 1 ml. of the test sample, 2 ml. of the diphenylamine reagent was added and shaken vigorously. The tubes were then immersed in coiling water bath for 30 mins. Solutions containing glycolic aldehyde in concentrations of as little as 1 mg. percent showed a grass green color whose absorption maxima was at 660 μm.
**Fig. 4.** Standard curve for phosphorus.

**Fig. 5.** Standard curve for N-AcGm
Estimation of N-acylhexosamines by Morgan-Elson color reaction (101)

Reagents used:

Na-tetraborate - A solution of 0.8M borate was prepared and the pH was adjusted to 8.8 with NaOH. Some borate salt crystallised out on standing. The supernatant was used for assay.

p-Dimethylaminobenzaldehyde reagent: 10 gm. of p-dimethyl-aminobenzaldehyde was dissolved in 100 ml. of glacial acetic acid which contained 12.5 percent 10N HCl. This reagent could be stored at 2° for a month without significant deterioration. Shortly before use it was diluted with nine volumes of glacial acetic acid.

To 0.2 ml. samples containing N-acylhexosamine, 0.2 ml. of Na-tetraborate was added. The tubes were heated in a vigorously boiling water bath for exactly 4 mins for N-AcGm (and 10 mins for N-AcMm) and cooled in tap water. 2 ml. of p-dimethylaminobenzaldehyde reagent was then added to each tube and immediately after mixing the tubes were placed in a bath at 36-38°. After 10 mins. the tubes were cooled in tap water and readings were taken at 565 mp. The colour produced in this reaction is proportional to the amino-sugar content of the solution assayed as shown in Fig. 5.

Estimation of proteins

Proteins were generally assayed by the method of Lowry et al. (102). This method depends on the reaction of tyrosine and trypto-
Fig. 6. Standard curve for protein

Fig. 7. Standard curve for glucose
phan residues present in protein with Folin reagent. Since tyro-
sine occurs at regular interval in many proteins, this method is
quite convenient.

Reagents used:

Protein reagent - Reagent A: 2 per cent Na₂CO₃ in 0.1N NaOH.
Reagent B, 0.5 per cent CuSO₄·5H₂O in 1 per cent sodium
or potassium tartarate. 50 ml. of Reagent A was mixed with
1 ml. of Reagent B.

Folin Ciocalteu reagent - A mixture consisting of 100 gm. of
Na-tungstate (Na₂W₀₄·2H₂O), 25 gm. of Na-molybdate (Na₂MoO₄·2H₂O),
700 ml. of water, 50 ml. of 85% phosphoric acid and 100 ml. concen-
trated hydrochloric acid was refluxed gently for 10 hours. 150 gm.
of lithium sulfate, 50 ml. of water and a few drops of bromine water
were added. The mixture was boiled for 15 mins. without condenser
to remove excess bromine. Then it was cooled, diluted to 1 l. and
filtered. The reagent should not have greenish tint. To 0.25 ml.
of a solution containing protein (50 μg. - 200 μg), 2.5 ml. of pro-
tein reagent was added, and after 10 mins a further addition of
0.25 ml. of Folin reagent was made. After another 10 mins, readings
were taken at 660 μ]. The colour produced is proportional to the
concentration of protein in the solution assayed (Fig. 6).

Estimation of glucose

Glucose was estimated according to the method of Nelson (103).
Reagents used:

**Copper Reagent A.** The following reagents (25 gm. Na$_2$CO$_3$ (anhydrous) (25 gm. Rochelle salt (K-Hatartrate) (20 gm. NaHCO$_3$) (200 gm. Na$_2$SO$_4$ (anhydrous) were dissolved in 800 ml. H$_2$O.

**Copper Reagent B**

15% CuSO$_4$, 5H$_2$O containing one or two drops conc. H$_2$SO$_4$ per 100 ml.

**Copper mixture**

25 parts A + 1 part B (prepared before use).

**Arsenomolybdate colour reagent** - 25 gm. of ammonium molybdate was dissolved in 450 ml. H$_2$O and mixed with 21 ml. of conc. H$_2$SO$_4$; 3 gm. of Na$_2$HASO$_4$, 7H$_2$O dissolved in 25 ml. H$_2$O, was added.

**Procedure:** 0.4 ml. of sugar solution and 0.4 ml. of copper mixture were mixed and heated for 20 minutes in boiling water bath. The reaction mixture was then cooled and 0.4 ml. of arsenomolybdate reagent was added in each tube. The volume was then made up to 3 ml. with H$_2$O. The reagent blank contained all reagents except sugar solution. The readings were taken in Bausch & Lomb at 510 μm against blank. The standard curve for glucose estimation is shown in Fig. 7.

**Ionophoresis of sugar and sugar phosphates on paper**

Ionophoresis was carried out under conditions similar to those of Markham and Smith (104).
Reagents: (1) H-AcGm-6-P (.1M)
(2) Borate Buffer, pH 8.4
(3) 5% NaOH in 95% ethanol.

A sheet of filter paper (Whatman No. 3) was dipped into the buffer solution, and the excess moisture was allowed to drain. It is better to have the paper a little too dry rather than too wet when it is placed for equilibration in the moist chamber. 10 μl. of sugar phosphate was applied as a thin line onto the paper by a micropipette. The paper was then fixed in a rectangular glass frame. The frame and paper were placed in a rectangular glass jar. The purpose of the frame was to keep the paper stretched horizontally. The ends of the paper dipped into troughs placed outside the jar and these contained the buffer solution and graphite electrodes. The jar was then filled with carbon tetrachloride until the paper was covered. Carbon tetrachloride is essential as it prevents heating and evaporation and so the rates of movement of the bands are uniform and reproducible. Separation was carried out with a constant current of 50 mA (600 v) for 1 hr. at room temperature. At the end of the ionophoresis, the paper was removed and dried in the oven for 5 mins at 110-120°; ethanolic NaOH was sprayed on the paper. After a few minutes heating at 100-120°, the paper was viewed in ultraviolet light, in which the spots have an intense fluorescence. A clear separation was noticed in between sugar phosphate and free sugar.
Isolation of the product of N-AcGm kinase reaction

In addition to ADP, the product of enzyme action is N-acetyl-D-glucosamine-6-phosphate. In a large scale preparation, a reaction mixture was prepared by adding the following in a final volume of 2 ml: 100 μmoles of N-AcGm, 150 μmoles of ATP, 200 μmoles of MgCl₂, 100 μmoles of 2-mercaptoethanol, 500 μmoles of glycine-NaOH, pH 9.0 and 0.2 ml. of DEAE-cellulose eluate fraction. The reaction mixture was incubated at 37°C until all N-AcGm was converted to its product (about 2 hrs). The pH of the mixture was then adjusted to 4.5 with acetic acid. The mixture was diluted to 50 ml. with water and then treated with 1.0 gm. activated charcoal. The supernatant was collected by centrifugation at 3,000 x g and the residue was rejected. Assay of an aliquot of the supernatant showed that approximately 90% of ADP was removed by charcoal without affecting the concentration of the product very much. The supernatant was further treated with 2.0 gm. activated charcoal and the charcoal adsorbed product was isolated by following treatments. After washing the charcoal with water, the product was eluted with 50 ml. of 96% alcohol. The alcohol was evaporated to dryness and the product was taken up in 1 ml. of water.

Purification of N-AcGm kinase

Unless otherwise indicated, all operations were conducted at temperatures between 0° and 4°. All phosphate buffers used in the
enzyme fractionation were prepared in 0.001M EDTA and 0.01M 2-mercaptoethanol. These substances were found necessary to stabilize the enzyme at all stages of purification.

(a) Extraction of tissue

Hog spleen, collected at the abattoir immediately after slaughter, were quickly chilled in ice. The organs can be stored in ice for as long as 5 days or in the deepfreeze at -18°C for several months, if necessary before extraction. The spleen, after thawing, was homogenized with 2 vols of 0.03M potassium phosphate buffer, pH 7.6, in a Waring blender for 20 seconds and after one minute homogenized again for another 20 seconds, both at setting 1 of the blender, then centrifuged at 18,000 x g for 30 mins. The supernatant fluid contained the enzyme and this fraction was referred to as "crude extract".

(b) Protamine step

The first step in the purification involved the precipitation of the enzyme with protamine sulphate solution (2%). To know the actual amount of protamine sulphate in which the enzyme would be precipitated, the following experiment was performed. The crude extract was diluted with equal volumes of water and to each 5 ml. of diluted crude extract taken in a series of test tubes, different volumes of 2% protamine sulphate solution were added, well mixed and then centrifuged at 8,000 x g for 5 mins. The resulting supernatants
were assayed for the kinase activity. It was observed that for 5 ml. diluted crude extract, approximately, 0.4 ml. of protamine sulphate was necessary to precipitate the enzyme quantitatively. The protamine sulphate step was therefore carried out as follows:

The crude extract, 100 ml. was diluted with an equal volume of cold distilled water and treated with 15 ml. of a 2% protamine sulphate solution (dissolved at 37°) with gentle stirring. After 10 mins, the protamine sulphate residue, which contained almost all the enzyme activity, was collected by centrifugation at 8,000 x g for 5 mins and the inactive supernatant fluid was discarded. The protamine precipitate was washed once with 100 ml. and then with 50 ml. of a solution containing 0.1M Tris. The enzyme was then extracted from the residue by stirring with increasing concentrations of potassium phosphate buffer, pH 7.6. It was found that the method of stirring was specially important. For example, vigorous stirring of the precipitate with several types of blenders or homogenizers led to considerable inactivation of the enzyme. The successful procedure involved gentle stirring with a glass rod and slow addition of buffer until a uniform suspension was obtained. The first few extractions each consisting of 50 ml. of 0.02M, 0.05M potassium phosphate buffer, pH 7.6 were rejected. Active enzyme was obtained by four further extractions with 50 ml. each of 0.075M, 0.075M, 0.1M, 0.1M potassium phosphate buffer, pH 7.6; these extracts contained approximately 90% of the enzyme contained in the
crude extract. The four fractions were combined making a total of 200 ml. of "protamine extract".

(c) Ammonium sulphate fraction

The protamine extract (150 ml.) was brought up to 25% saturation with solid (NH₄)₂SO₄ and the inactive residue was discarded by centrifugation at 16,000 x g for 10 mins. The active enzyme was precipitated between 25 to 50 per cent (NH₄)₂SO₄ saturation with solid (NH₄)₂SO₄ and was collected by centrifugation. The precipitate was then extracted with 5 ml. of 25% saturated solution of ammonium sulphate adjusted to pH 7.0. It was then centrifuged at 16,000 x g for 10 mins and inactive residue was then discarded. The supernatant was then treated with solid (NH₄)₂SO₄, until the solution was 50% saturated. The precipitate was then dissolved in 30 ml. of 0.02M potassium phosphate buffer, pH 7.0.

(d) Charcoal fraction

The ammonium sulphate fraction (50 ml.) was then treated with 65 mg. of acid washed charcoal (E. Merck). Protein to charcoal ratio was 1:3. After 10 mins, the supernatant solution was collected by centrifugation at 6,000 x g for 15 mins, and dialysed against 0.005M potassium phosphate buffer, pH 7.6 for 24 hrs. If any precipitate formed during dialysis, it was removed by centrifugation. (Dialysed charcoal fraction).
(e) Calcium phosphate gel adsorption

The next step in the purification involved the adsorption of the enzyme with calcium phosphate gel. In order to determine the minimum amount of gel necessary for adsorption of the enzyme at this stage, a definite volume of dialysed extract was treated with sedimented calcium phosphate gel in different proportions. After centrifugation at 8,000 x g for 5 mins, the supernatants were assayed for enzymic activity. The result is shown in Table 3.

**Table 3**

**Adsorption of N-AcGm kinase by calcium phosphate gel from the dialysed charcoal fraction**

Incubation mixture and other experimental conditions were same as described in the Procedure A. 0.01 ml. supernatant was used in each case for assaying the enzyme activity.

<table>
<thead>
<tr>
<th>Dialysed charcoal fraction (mg./ml)</th>
<th>Vol. of calcium phosphate gel added (ml.)</th>
<th>Total units</th>
<th>Protein concentration mg./ml.</th>
<th>% precipitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 ml</td>
<td>-</td>
<td>3.2</td>
<td>1.2</td>
<td>0</td>
</tr>
<tr>
<td>0.2 ml</td>
<td>0.05 ml</td>
<td>3.0</td>
<td>1.05</td>
<td>10</td>
</tr>
<tr>
<td>0.2 ml</td>
<td>0.1 ml</td>
<td>2.65</td>
<td>0.87</td>
<td>20</td>
</tr>
<tr>
<td>0.2 ml</td>
<td>0.2 ml</td>
<td>1.6</td>
<td>0.51</td>
<td>50</td>
</tr>
<tr>
<td>0.2 ml</td>
<td>0.25 ml</td>
<td>1.0</td>
<td>0.39</td>
<td>70</td>
</tr>
<tr>
<td>0.2 ml</td>
<td>0.4 ml</td>
<td>0.0</td>
<td>0.045</td>
<td>100</td>
</tr>
</tbody>
</table>
The calcium phosphate gel step was therefore carried out as follows:

Calcium phosphate gel (9 ml.; dry wt. 16.2 mg./ml.) was sedimented by centrifugation. The sediment was resuspended with the 30 ml. of dialysed charcoal fraction (protein to gel ratio 1:8) by gentle stirring with a glass rod. After 10 mins. at 0°, the suspension was centrifuged at 8,000 x g for 5 mins and the residue was collected. The next step consisted of extraction of the enzyme from gel residue with phosphate buffer, pH 7.6. After preliminary washing with water (30 ml.), the active enzyme was eluted first with 30 ml. and then with 15 ml. of 0.05M potassium phosphate buffer, pH 7.6 and finally with 15 ml. of 0.07M potassium phosphate buffer, pH 7.6. These extracts (60 ml.) were then combined together and brought to 50% saturation with the addition of solid (NH₄)₂SO₄. After centrifugation at 16,000 x g for 10 mins, the collected proteins were dissolved with 2 ml. of 0.02M potassium phosphate buffer, pH 7.6 and dialysed against 0.005M potassium phosphate buffer, pH 7.6. This fraction represented a purification of 600-fold from the crude extract with an overall yield of 70%.

(Continued)

DEAE-cellulose step

The dialysed calcium phosphate gel fraction was further purified by batchwise DEAE-cellulose chromatography. The calcium phosphate gel fraction (2.9 mg. protein) was applied to a 3 ml. (packed volume) of DEAE-cellulose column, which had been previously
equilibrated with 0.02M KCl -0.01M potassium phosphate buffer, pH 7.6. After adsorption of the enzyme on the column, inert proteins were removed by washing the column with 25 ml. of 0.1M potassium phosphate buffer, pH 7.6. The enzyme was eluted with 15 ml. of 0.2M potassium phosphate buffer pH 7.6. The active fraction was brought to 50% saturation with solid (NH₄)₂SO₄. The precipitate, containing the enzyme, was collected by centrifugation, and dissolved in a small volume of 0.05M potassium phosphate buffer, pH 7.6. (DEAE-cellulose eluate). A summary of the purification procedure was given in Table 5.

Assay of enzymes

Two different assay procedures were used for the estimation of kinases.

Procedure A: This method is based on the specific removal of phosphorylated sugar by the addition of ZnSO₄ and Ba(OH)₂ solutions after enzyme incubation according to the procedure of Somogyi (105), and the estimation of free sugar disappeared from the assay mixture during enzyme reaction. This procedure is quick and can be used for the determination of N-acetylglucosamine kinase activities throughout the purification procedure. Routine assay mixture contained (in μmoles): 1.0 of sugar, 1.0 of ATP, 2.5 of MgCl₂, 12.5 of glycine-NaOH buffer, pH 9.0, and enzyme fraction in a total volume of 0.25 ml. Incubation was usually carried out at 37°C for 10 min.
unless otherwise stated. After incubation, the reaction was stopped by adding 1.0 ml. of 0.4M ZnSO₄, followed by the addition of an equivalent amount of saturated Ba(OH)₂ solution. After centrifugation 0.2 ml of the supernatant was taken for the estimation of free sugar left in the reaction tube by Morgan-Elson colour reaction (101). A control tube was also assayed in each case. This tube contained all the constituents in same amount as described in the text except the enzyme. After incubation, ZnSO₄ was added to the control tube and then the enzyme solution was added followed by the addition of Ba(OH)₂.

It is necessary for any enzyme purification procedure to define an arbitrary unit of enzyme in terms of which the purity and activity of the various fractions may be quantitatively expressed. Here the unit of enzyme is taken as that amount of enzyme required to phosphorylate 1 μM of N-AcGm in 10 mins under the assay conditions of the experiment. The specific activity of any fraction is taken as units of enzyme per mg. protein in that fraction.

Procedure B: In this method N-AcGm kinase activity was measured by estimating the amount of ADP formed during enzyme incubation by NADH-lactic dehydrogenase-pyruvate kinase assay procedure of Kornberg and Pricer (106). Routine assays were performed by incubating the following components (in μmoles) in a total volume
of 0.25 ml: N-AcGm, 1.0; ATP, 1.0; MgCl₂, 2.5; glycine-NaOH, pH 9.0, 12.5 and enzyme fraction. After incubation, the reaction was stopped by heating in a boiling water bath for 1 min. and the reaction mixture was diluted with water to 1.0 ml. An aliquot of this diluted mixture (0.1 ml.) was added to a mixture containing potassium phosphate buffer, pH 7.6 (25 μmoles), MgCl₂ (2 μmoles), EF (20 μmoles), PEP, (0.25 μmole) and NADH (approximately 0.1 μmole) in a final volume of 1 ml. in a Beckman cuvette. Initial absorbance (1 cm light path, 340 μM) was noted and 0.01 ml. lactic dehydrogenase, containing pyruvate kinase (both present in excess) was added. The decrease in absorbance was followed until it reached a constant value (usually in less than 2 min). Assay mixture without sugar or without enzyme served as control.

Procedure A was specially used for determining the specific activities of various fractions during enzyme purification, as the procedure is more suitable for assay of crude preparations containing interfering enzymes. Procedure B was routinely used for kinetic studies with purified enzyme fractions.
Results
A. Distribution of N-acetylglucosamine kinase in different animal tissues:

A survey work for N-acetylglucosamine kinase was undertaken with a view to find out the level of the enzyme in different tissues of animal origin and to select a good source of the enzyme. From the results, shown in Table 4, it can be seen that the spleen has the highest concentration of the N-acetyl D-glucosamine kinase when compared to the other tissues of hog.

Table 4

Distribution of N-AcGm kinase in different animal tissues

Tissue extracts, prepared as described in the text, were assayed for the kinase activity by the procedure A. 0.05 ml of crude extracts was used in each for assaying the enzyme activity.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Sp. activity of* crude extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hog</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>0.25</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.12</td>
</tr>
<tr>
<td>Liver</td>
<td>0.08</td>
</tr>
<tr>
<td>Muscle</td>
<td>-</td>
</tr>
<tr>
<td>Brain</td>
<td>0.12</td>
</tr>
<tr>
<td>Goat</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>0.09</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.08</td>
</tr>
<tr>
<td>Liver</td>
<td>0.036</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.02</td>
</tr>
<tr>
<td>Brain</td>
<td>0.016</td>
</tr>
</tbody>
</table>

* Sp. activity = µmoles of sugar esterified 10 mins/mg protein.
B. Purification of N-acetylglucosamine kinase

When a suitable source for an enzyme has been found, it is necessary to rupture the cell membrane to bring the enzyme into solution. Use of a Waring Blender to homogenize the animal tissues is a common technique used in the extraction of the enzyme, though other methods are available. Hog spleen was homogenized in the presence of a buffer solution (0.03M potassium phosphate buffer, pH 7.6-0.001M EDTA - 0.01M 2-mercaptoethanol) in order to neutralize any acid that might be produced during homogenization and to counteract the inactivating effect of heavy metal ions and oxygen on the enzyme. The extract containing the enzyme will also contain numerous other substances, of both large and small molecular weight. The main part of the purification will consist of a series of fractionations by which the enzyme protein is separated from the other proteins.

Purification of the enzyme by precipitating it from crude extracts with protamine sulphate has been widely used as a technique of protein purification developed in recent times. Protamine sulphate forms a strong complex with nucleoprotein and particulate material. It may also form insoluble complex with some soluble enzymes found in the crude extract of cells. When that happens the enzyme may be solubilized again from this complex by a suitable buffer of appropriately high concentration. In the case of N-AcGm kinase a satisfactory purification (about 27 fold) is achieved by the use of protamine sulphate.
Fractional precipitation of proteins by ammonium sulphate, on account of its large solubility in water and absence of harmful effects on the enzyme is a very widely used method. This step is helpful not only for the purification of the enzyme but also for the concentration of the enzyme.

Charcoal step is necessary for removing particulate material from the enzyme solution to convert a turbid extract into a perfectly clear solution.

Calcium phosphate gel has a long history of use in protein purifications. Adsorption on this gel usually occurs best at low electrolyte concentration. So dialysis of the charcoal fraction is beneficial at this stage; the N-AcGm kinase is quite stable to dialysis.

A most important advance in the subject of protein chromatography was made in 1956 when Peterson and Sober (107) described new types of ion exchanger based on cellulose. These materials, which are made by modifying cellulose chemically, have a very high capacity for proteins and can be endowed with any desired ionexchange characteristic. Of them, Diethylaminoethyl (DEAE)-cellulose is widely used. In the case of N-AcGm kinase purification by stepwise elution from DEAE-cellulose columns was found a more useful procedure than that employing a continuous concentration gradient (gradient elution).
As shown in Table 5, N-AcGm kinase was purified about 3500 fold with 20 percent yield by using the above mentioned steps.

**Table 5**

Purification of N-acetylglucosamine kinase from hog spleen

The composition of incubation mixtures and the other assay conditions were the same as described in Procedure A.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Units</td>
<td>Yield %</td>
<td>*Units/mg Protein</td>
</tr>
<tr>
<td>1. Crude extract</td>
<td>638</td>
<td>100</td>
<td>0.25</td>
</tr>
<tr>
<td>2. Protamine extract</td>
<td>572</td>
<td>90</td>
<td>6.7</td>
</tr>
<tr>
<td>3. Ammonium sulphate</td>
<td>488</td>
<td>76</td>
<td>22.9</td>
</tr>
<tr>
<td>4. Charcoal treatment</td>
<td>480</td>
<td>75</td>
<td>27.3</td>
</tr>
<tr>
<td>5. Calcium phosphate gel</td>
<td>452</td>
<td>71</td>
<td>156.0</td>
</tr>
<tr>
<td>6. DEAE-cellulose eluate</td>
<td>134</td>
<td>21</td>
<td>900.0</td>
</tr>
</tbody>
</table>

* A unit of enzyme catalyzes the conversion of 1 µmole of N-acetylglucosamine to N-acetylglucosamine-6-phosphate in 10 mins. under the conditions described in the text.

**C. Test for homogeneity**

The following experiments were performed to test the homogeneity of the enzyme protein of DEAE-cellulose eluate fraction:
The dialysed DEAE-cellulose eluate fraction (2.0 mg. protein) was applied to a DEAE-cellulose column (5 ml. packed volume) which had been equilibrated with 0.02M KCl-0.01M potassium phosphate buffer, pH 7.6. The enzyme was then eluted with a 200 ml. linear gradient of 0.125M to 0.25M potassium phosphate buffer, pH 7.6 in 0.02M KCl-0.001M EDTA. Fractions were collected, 2 ml. each, and 0.01 ml. from each tube was assayed for N-AcGm kinase by procedure A. Protein concentrations of different fractions were determined by taking the optical density at 280 nm.

-OOO-, enzyme activity; -x-x-x-, Protein
(a) Adsorption on CY-alumina gel

The enzyme fraction (DEAE-cellulose eluate) could not be further purified by the method of CY-alumina gel adsorption. This technique was applied by the following way: the enzyme (DEAE-cellulose eluate) was adsorbed on sedimented CY-alumina gel and eluted with 0.05M potassium phosphate buffer, pH 7.6. The specific activity of the eluate was found to be the same as that of DEAE-cellulose eluate.

(b) DEAE-cellulose column chromatography

DEAE-cellulose eluate fraction was rechromatographed on DEAE-cellulose by gradient elution technique. As can be seen from Fig. 8, a single peak of enzyme activity, which corresponded within limits of the method to a single protein peak, was observed. The shape of the protein peak observed and the specific activities in the fractions across the peak was indicative of no significant heterogeneity.

(c) Ultracentrifugal analysis

Sedimentation measurements of the most purified enzyme preparation (DEAE-cellulose eluate) were made in a Spinco model E ultracentrifuge. The profile presented in Fig. 9 reveals a single principal protein boundary preceded by minor boundaries judged to be contaminant protein. No quantitative measurement of the minor components has been done.
Fig. 9. Sedimentation patterns of N-AcGm kinase. 4.0 mg. protein (from DEAE-cellulose eluate) per ml., in 0.1M phosphate buffer was used. Sedimentation was in the direction of the arrows at 56,100 r.p.m. and was carried out at 20°. The schlieren photographs were taken at a time interval of 8 mins.

Fig. 10. Effect of incubation time on reaction rate; Each incubation mixture contained the following (in micromoles) in final volumes of 0.2 ml: N-AcGm, 1.0; ATP, 1.0; MgCl₂, 2.5; Glycine-NaOH buffer, pH 9.0, 12.5; and 5 µg of calcium phosphate gel fraction. Incubations were conducted at 37°; the reaction was stopped after the indicated period of incubation, the reaction mixture was then diluted to 1 ml. and 0.1 ml. aliquots were assayed for ADP as described in Procedure B.
Fig. 11. Effect of protein concentration on reaction rate; The composition of the incubation mixtures was the same as described in Fig. 10 except for the varying concentrations of protein from calcium phosphate gel fraction. Incubations were conducted at 37° for 20 minutes and the amount of ADP formed in each tube was determined by Procedure B.

Fig. 12. pH-activity curve of N-AeGm kinase: Conditions of standard assay and composition of incubation mixtures as described in Fig. 10, were employed, except for the buffer system. Each incubation tubes contained 12.5 μmoles of either Tris-HCl buffer (7.4-8.9) or Glycine-NaOH buffer (8.6-9.7) and 0.4 μg of DEAE-cellulose eluate in final volumes of 0.2 ml. The tubes were then assayed as described in procedure B.

Tris-HCl buffer; Glycine-NaOH buffer.
Sedimentation velocity was measured at protein concentration of 4.0 mg. per ml. The sedimentation coefficient \( S_{20,w} \) was determined according to the method of Schachman (108) and the value was 5.5.

(d) **Kinetic properties**

**Effect of time and concentration of the enzyme on reaction velocity:** The time course of N-AcGm kinase activity is shown in Fig. 10. It can be seen that the rate of reaction catalyzed by this enzyme is constant for at least a period of 40 mins.

The rate of N-AcGm kinase reaction was found proportional to the amount of enzyme fraction added to the incubation mixture. This is shown in Fig. 11.

**Optimum pH:** N-AcGm kinase activity was determined at various pH values as shown in Fig. 12. Two sets of buffers were used namely, Tris-HCl buffer (pH 7.4-8.9) and Glycine-NaOH buffer (pH 8.6-9.7). The enzyme exhibits a broad pH optima between 8.6 to 9.4.

**Substrate saturation curves:** Substrate concentration is one of the most important factors which determine the velocity of enzyme reaction. When the velocity is plotted against substrate concentration a section of a rectangular hyperbola is generally obtained. When these two variables are plotted in terms of \( 1/[v] \) with respect to \( 1/[S] \) i.e. Lineweaver-Burk plot, a straight line is obtained (109).
Fig. 13. N-AcGm saturation curve of the kinase: The composition of assay mixture and other conditions were the same as described in Procedure B, except for NAcGm which was varied. The amount of enzyme added was 0.4 μg protein of DEAE-cellulose eluate. The inset shows the data plotted in the form of Lineweaver-Burk plot.

Fig. 14. Effect of ATP concentration on N-AcGm kinase activity. The assay conditions were those described under "Procedure B", except for the varying ATP concentration. DEAE-cellulose eluate (0.4 μg protein per assay tube) was used. The inset shows the data plotted in the form of Lineweaver-Burk plot.
In case of N-AcGm kinase, when the N-AcGm concentration \([S]\) (\(\mu\)moles/ml.) is plotted against that of reaction rate \([v]\) (\(\mu\)moles of ADP formation in ten minutes/ml. of incubation mixture under the assay conditions), a hyperbolic form of substrate saturation curve is obtained (Fig. 13). Lineweaver-Burk plot of these data gives a line which is slightly curved instead of a straight line. Apparent \(K_m\) value (concentration of the substrate at half-maximal velocity) for N-AcGm obtained from the above plots is \(1.1 \times 10^{-3}\) M.

The effects of varying concentration of ATP on the reaction velocity catalyzed by the kinase is shown in Fig. 14. In this case, a hyperbolic relationship is obtained when the concentration of ATP \([S]\) (\(\mu\)moles/ml.) is plotted against the reaction velocity \([v]\) (\(\mu\)moles of ADP formation in ten minutes/ml. of incubation mixture under the assay conditions) and Lineweaver-Burk plot yields a straight line. The \(K_m\) value for ATP, determined from these two plots is \(1.8 \times 10^{-3}\) M.

E. Stability

Enzyme stored at 4° in 0.02M potassium phosphate buffer, pH 7.6-0.001M EDTA-0.01M 2-mercaptoethanol, was stable for at least a week. The enzyme was not only unstable to freezing and thawing, but also labile to heat and low pH. Approximately 50% of the total activity would be lost if it was heated at 70° C for 2 mins. The protamine extract and ammonium sulphate fractions were reasonably stable. The loss in activity during storage could
be reversed by adding 2-mercaptoethanol. As indicated in the purification procedure, the enzyme was also stable to dialysis.

F. Substrate specificity

Specificity for sugars: Crane (86) classified hexokinases into single substrate and multisubstrate enzymes. Most of the mammalian kinases come into the second category. It was found that the enzyme, N-acetylglucosamine kinase, was strictly specific for N-acetylglucosamine. No activity was noted by using the following compounds (all D-sugars): N-acetylmannosamine, N-acetyl-galactosamine, Glucosamine, Glucose, Mannose, Galactose and Fructose. The effect of glucose, glucosamine and N-acetylmannosamine on the reaction rate of N-acetylglucosamine phosphorylation was studied; no inhibitory or stimulatory effect of these sugars were found. These results clearly showed the stereo-specificity of the kinase towards N-acetylglucosamine.

Specificity for nucleotide triphosphates: The only good donor of phosphoryl group in the reaction catalysed by the N-AcGm kinase was ATP. Other nucleotides tested for their ability to donate the phosphoryl group were UTP, CTP, GTP and ADP; of these CTP and GTP showed some donating capacity whereas UTP and ADP were completely ineffective. UTP and CTP showed some inhibitory effect on the reaction rate when they were added in the complete incubation mixture (Table 6).
Effect of nucleotides on activity of N-AcGm kinase

Each incubation mixture contained the following components (in μmoles) in a final volume of 0.2 ml: N-AcGm, 1.0; MgCl₂, 2.5; Glycine-NaOH buffer, pH 9.0, 12.5; the indicated nucleotide, 1.25; and 0.4 μg of protein from DEAE-cellulose eluate fraction. The assay conditions were the same as described in Procedure A.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>μmoles of N-AcGm esterified</th>
<th>Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>0.4</td>
<td>100</td>
</tr>
<tr>
<td>UTP</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>GTP</td>
<td>0.07</td>
<td>17.5</td>
</tr>
<tr>
<td>CTP</td>
<td>0.15</td>
<td>37.5</td>
</tr>
<tr>
<td>ATP+UTP</td>
<td>0.3</td>
<td>75</td>
</tr>
<tr>
<td>ATP+CTP</td>
<td>0.3</td>
<td>75</td>
</tr>
<tr>
<td>ATP+GTP</td>
<td>0.4</td>
<td>100</td>
</tr>
</tbody>
</table>

G. Effect of ions

N-AcGm kinase like other kinases required a divalent cation for optimal activity. Though Mg²⁺ satisfied this requirement, other divalent metal ions, Mn²⁺ and Co²⁺ for instance, could replace Mg²⁺ partly while monovalent cations such as Na⁺ and K⁺ and divalent cations e.g. Ca²⁺ and Ba²⁺ were completely ineffective. The effect of these and some other cations are shown in Table 7. It was found that Ca²⁺ played some inhibitory role when it was
Fig. 15. Mg$^{2+}$/ATP ratio for optimum activity. Each incubation mixture contained the following (in micromoles): N-AcGm, 1.0; ATP, 1.0 (or 2.0); Glycine-NaOH, pH 9.0, 12.5; and varying concentration of MgCl$_2$. 0.4 µg protein from DEAE cellulose eluate was added per incubation mixture. Incubations were conducted at 37° for 20 mins. Assays were done according to Procedure B.

Fig. 16. Dependence of reaction rate on Mg$^{2+}$, Mn$^{2+}$ & Co$^{2+}$ ion concentrations: Each incubation mixture contained the following components in final volumes of 0.2 ml. (micromoles); ATP, 1.0; N-AcGm, 1.0; Glycine-NaOH, pH 9.0, 12.5; varying concentrations of cations (Mg$^{2+}$, Mn$^{2+}$ or Co$^{2+}$); and 0.4 µg of protein from the DEAE-cellulose eluate. Incubations were conducted at 37° for 20 mins and assayed as described in Procedure A.
added in the complete incubation mixture. Other cations showed no such activity. The fact that no activity was noted in the absence of cation indicated the absolute requirement of the enzyme for a cation.

Table 7

Effect of cations on the activity of N-AcGm kinase

Each incubation mixture contained the following components (in μmoles) in a final volume of 0.2 ml: N-AcGm, 1.0, ATP, 1.0; Glycine-NaOH buffer, 12.5; the indicated metal ions, 2.5; and 0.4 μg protein from DEAE-cellulose eluate fraction. Incubated at 37° for 10 min. The assay method was the same as described in Procedure A.

<table>
<thead>
<tr>
<th>Metal ions used</th>
<th>μmoles of the sugar esterified</th>
<th>Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.39</td>
<td>100</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>0.33</td>
<td>56</td>
</tr>
<tr>
<td>Ba²⁺</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>0.33</td>
<td>56</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>K⁺</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Na⁺</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>Mg²⁺+Ca²⁺</td>
<td>0.27</td>
<td>70</td>
</tr>
<tr>
<td>Mg²⁺+K⁺</td>
<td>0.39</td>
<td>100</td>
</tr>
<tr>
<td>Mg²⁺+Na⁺</td>
<td>0.39</td>
<td>100</td>
</tr>
</tbody>
</table>

As shown in Fig. 15 for optimal activity of the kinase, the Mg²⁺/ATP ratio was approximately 2:1 which is unusual, since most hexokinases have a ratio of about 1:2 (110).
Fig. 17. Effect of anions (PP$\scriptsize{\text{I}}$ & P$\scriptsize{\text{I}}$) on reaction rate. The incubation mixtures and experimental conditions were the same as described in Fig. 10 except for the addition of different amounts of Na-Pyrophosphate, pH 9.0 and Na-phosphate, pH 9.0. All tubes were incubated for 10 mins. Amount of enzyme added per tube was 0.4 µg of protein from DEAE-cellulose eluate. The amount of the sugar phosphorylated was determined by procedure A. — — — — Na-pyrophosphate; — — — — Na-phosphate.
The competitive activities of Mg$^{2+}$, Mn$^{2+}$ and Co$^{2+}$ with respect to the stimulation of the reaction rate is presented in Fig. 16.

It is interesting to note that the enzyme is strongly inhibited by anions like pyrophosphate and inorganic phosphate (Fig. 17). These inhibitions could be reversed by the addition of excess Mg$^{2+}$ in the incubation mixture (Table 8).

**Table 8**

**Reversal of pyrophosphate inhibition by Mg$^{2+}$**

Except for the varying concentration of Mg$^{2+}$, the composition of incubation mixture, experimental conditions and method of assay were the same as described in Procedure A. 0.4 μg protein from DEAE-cellulose was added in each incubation time.

<table>
<thead>
<tr>
<th>Concentration of pyrophosphate</th>
<th>μmoles of sugar phosphorylated</th>
<th>+4x10^{-3}M Mg$^{2+}$</th>
<th>+8x10^{-3}M Mg$^{2+}$</th>
<th>+16x10^{-3}M Mg$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.912</td>
<td>1.2</td>
<td>1.32</td>
<td></td>
</tr>
<tr>
<td>4x10^{-3}M</td>
<td>0.36</td>
<td>1.04</td>
<td>1.26</td>
<td></td>
</tr>
<tr>
<td>8x10^{-3}M</td>
<td>0.24</td>
<td>0.44</td>
<td>1.08</td>
<td></td>
</tr>
<tr>
<td>16x10^{-3}M</td>
<td>0.06</td>
<td>0.4</td>
<td>0.428</td>
<td></td>
</tr>
<tr>
<td>32x10^{-3}M</td>
<td>0</td>
<td>0</td>
<td>0.36</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 18. Effect of PCMB on N-AcGm kinase and reversal of the effect by cysteine. Incubation mixtures were prepared as described in Fig. 10 except that 0.4 µg of protein of from dialysed DEAE-cellulose eluate was used. Incubations were conducted at 37° for 20 mins in the presence of indicated concentrations of PCMB. In the experiments in which cysteine was used, 10 µmoles of freshly neutralized cysteine hydrochloride were added along with PCMB to incubation mixtures as described above. The amount of N-AcGm phosphorylated was determined by Procedure A.
Fig. 19. Lineweaver-Burk plot of the response of reaction rate to increasing concentration of ATP: Each incubation mixture contained the following (in micromoles) in final volumes of 0.2 ml: N-AcGm, 1.0; MgCl₂, 2.5; Glycine-NaOH buffer pH 9.0, 12.5 and the varying concentration of ATP. The reaction rate of N-AcGm kinase was measured in the presence and in the absence of ADP following the assay Procedure A. ADP when added to the incubation mixture had the concentration of $8 \times 10^{-3}$M. DEAE-cellulose eluate (0.4 µg/assay tube) was used for this purpose.

$\psi =$ µmoles of N-AcGm phosphorylated/assay tube.

$[\text{ATP}] =$ µmoles of ATP present/assay tube.
H. Effect of p-chloromercuribenzoate

Sulphydryl reagent like p-chloromercuribenzoate markedly inhibited the enzyme activity. For a protein concentration of 20 μgms/ml, PCMB at the concentration of 4x10^{-4} M inactivated the N-acetylglucosamine kinase completely. This inactivation was reversed almost 80 percent by the addition of cysteine at a concentration of 5x10^{-2} M (Fig. 18). The activity of highly purified N-acetylglucosamine kinase was found to be stimulated in the presence of the thiol compound, 2-mercaptoethanol.

Increase in activity of the enzyme was observed when the thiol compound was added either directly to the standard assay system or when it was preincubated with the enzyme.

I. Inhibitory effect of ADP

Inhibition of the forward reaction by either or both of the products has been reported with several of the hexokinases. In this case, ADP was a strong competitive inhibitor of the forward reaction. As shown in Fig. 19, inhibition due to ADP was competitive with ATP with the K_i being 0.9x10^{-3} M. It was found that the sensitivity of the enzyme to ADP lost at the same rate as the catalytic activity of the enzyme during heat treatment.

J. Effect of N-AcGm 6-P and UDP-N-AcGm

N-AcGm 6-P and UDP-N-AcGm were the potent inhibitors of the kinase reaction. These inhibitory effect could not be reversed
Fig. 20. N-AcGm saturation curves of the kinase with or without added N-AcGm-6-P. The composition of the assay mixture was the same as described in the Procedure B except for N-AcGm which was varied. The amount of enzyme added per incubation tube was 0.4 μg protein from DEAE-cellulose eluate. N-AcGm-6-P when added was 1.6 μmole per assay mixture of 0.25 ml. --- control; —+—— + N-AcGm 6-P.
by excess of the substrate, N-AcGm. So this was a non-competitive type of inhibition. Substrate saturation curve in the presence of N-AcGm (Fig. 20) showed that the inhibitor affected the $V_{\text{max}}$ of the kinase whereas apparent $K_m$ remained approximately the same. A similar type of effect of UDP-N-AcGm on $V_{\text{max}}$ has been observed. However, UDP-N-AcGm is a much more effective inhibitor of the kinase than N-AcGm 6-P as one can see from Table 9; this is so because of the stronger depressing effect of the former on the $V_{\text{max}}$ of the enzyme. As shown in Table 9, 60% inhibition was achieved in presence of $3.0 \times 10^{-3}\text{M} \text{N-AcGm-6-P}$.

**pH effect on the action of inhibitors**

To study the effect of pH on the inhibitory action of UDP-N-AcGm and N-AcGm 6-P, the kinase activity was determined in the presence and absence of these inhibitors at various pH's. It was found that the inhibitory function was more pronounced at the region of pH 7.5 (Table 9) whereas the kinase showed a broad catalytic pH optima between 8.6 to 9.4 (Fig. 12).
### Table 9

Effect of pH on the inhibitory activity of UDP-N-AcGm and N-AcGm-6-P on the kinase

<table>
<thead>
<tr>
<th>pH</th>
<th>N-AcGm phosphorylated (µmole)</th>
<th>Inhibition of activity (Percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control +N-AcGm-6-P +UDP-N-AcGm</td>
<td>+N-AcGm-6-P +UDP-N-AcGm</td>
</tr>
<tr>
<td>7.0</td>
<td>0.17</td>
<td>0.13</td>
</tr>
<tr>
<td>7.5</td>
<td>0.17</td>
<td>0.07</td>
</tr>
<tr>
<td>8.1</td>
<td>0.20</td>
<td>0.13</td>
</tr>
<tr>
<td>8.6</td>
<td>0.22</td>
<td>0.21</td>
</tr>
</tbody>
</table>

*a* N-AcGm kinase activities were determined in the presence and in the absence of N-AcGm-6-P and UDP-N-AcGm at various pH's as follows: In the control tubes, the reaction mixture contained the following components in a final volume of 0.25 ml (in µmole): N-AcGm, 0.25; ATP, 1.25; MgCl₂, 2.5; buffer (potassium phosphate, pH 7.0 and Tris-HCl pH's 7.5, 8.1 and 8.6) 12.5 and 174 µg protein from ammonium sulphate fraction. Experimental tubes contained, in addition to the above components, either UDP-N-AcGm (0.125 µmole) or N-AcGm-6-P (0.6 µmole). After incubation at 37° for 30 min. the reaction mixtures were treated with ZnSO₄ and Ba(OH)₂ solutions for the quantitative removal of N-AcGm-6-P (and also UDP-N-AcGm when present). The amount of N-AcGm left in each tube after ZnSO₄-Ba(OH)₂ treatment was estimated by the Morgan-Elson colour reaction. The value of N-AcGm left when subtracted from 1.0 µmole gives the amount of N-AcGm-6-P formed by the catalytic reaction.
Fig. 21. Effect of heat treatment on the sensitivity of the kinase to N-AcGm 6-P inhibition. DEAE-cellulose eluate fraction (80 μg/ml) was quickly heated to 55°C and then kept at that temperature for the indicated time period. The enzyme fraction (0.01 ml.) was cooled after the heat treatment and its activity was assayed as described in Procedure B in the presence and absence of N-AcGm-6-P. The amount of N-AcGm-6-P when added was 1.0 μmole per 0.25 ml of assay mixture, —o-o— control; —△-△— + N-AcGm-6-P.
Fig. 22. Double reciprocal plots of reaction rate of N-AcGm kinase and N-AcGm concentration. The composition of the assay mixture and the assay procedure were the same as described under procedure B except for the varying concentration of N-AcGm. DEAE-cellulose eluate (0.4 µg) was used per incubation mixture. The inset shows the data plotted in the form of Hill equation. $V = \mu$moles ADP formed/ml assay mix.

Fig. 25. Double reciprocal plots of reaction rate and substrate (N-AcGm) concentration in the presence of HAcGm 6-P. The experimental procedure was similar as described in Fig. 22 except that 3 µmole of AcGm-6-P was added in each incubation tube. The inset shows the Hill plot of the above data. $V = \mu$moles of ADP formed/ml of assay mixture.
Fig. 24. Double reciprocal plots of reaction rate and substrate (N-AcGm) concentration in the presence of UDP-N-AcGm. The experimental procedure was similar as described in Fig. 22 except that 1 jimole of UDP-N-AcGm was added in each incubation tube. The inset shows the Hill plot of the above data.

\( \nu = \) moles of ADP formed/ml of assay mixture.

Fig. 25. Lineweaver-Burk plot of reaction rate and substrate concentration of desensitized (N-AcGm) of the desensitized enzyme. The experimental conditions were similar to those as described under Fig. 22, except that 0.4 \( \mu \)g of "desensitized enzyme" (heated at 55°C for 15 mins, as described in Fig. 21) was used. The inset shows the data plotted in the form of Hill equation.
K. Desensitization of the kinase to N-AcGm 6-P and UDP-N-AcGm

Allosteric enzymes possess at least one regulatory site (inhibitory or activatory) other than the catalytic site. Controlled heat treatment has been a well known method of destroying selectively the regulatory site. The effect of heat treatment on the kinase in relation to its catalytic activity and sensitivity to N-AcGm-6-P was shown in Fig. 21. The enzyme completely lost its sensitivity to N-AcGm 6-P after 10 mins at 55° but it still retained about 50% of its initial catalytic activity.

L. Characteristics of substrate saturation curves of the native and desensitized N-AcGm kinase

In Fig. 20, is portrayed a graphical representation of the results of plotting substrate concentration with respect to activity of the native N-AcGm kinase in the absence as well as in the presence of the inhibitor. A hyperbolic relationship of these variables is noted in absence of the inhibitor. When these data are plotted interms of 1/v with respect to 1/[s], the line obtained was slightly curved (Fig. 22). But the curve was more pronounced in the presence of the inhibitor when the data plotted in the double reciprocal form (Fig. 23 & 24). From the double reciprocal plot, the $V_{\text{max}}$ was obtained by extrapolation to infinite substrate concentration.

The substrate saturation curves in the presence or in the absence of inhibitor are also fitted to the empirical Hill equation.
\[
\log \frac{v}{v_{\text{max}}} = n \log [s] + \log k
\]

where \(v_{\text{max}}, v, [s], n\) and \(k\) are maximal velocity, initial reaction velocity, N-AcGm concentration, a complex function of an interaction coefficient and the number of binding sites (111) and a complex equilibrium constant, respectively.

It can be seen that the presence of inhibitor caused a change of the interaction coefficient (11) from 1.66 to 2.0. On the other hand, the substrate saturation curves of the desensitized enzyme obtained by heating followed the normal Michaelis-Menten kinetics (Fig. 25). Lineweaver-Burk plot yielded a straight line and the slope of the Hill plot (11) reduced from 1.66 to 1.0.

M. Stoichiometry of reaction

Stoichiometry of a reaction is done for characterizing the reaction unequivocally. From Table 10, it can be seen that the amount of N-AcGm-6-P formed was equal to the amount of ADP in the reaction. The ratio was approximately 1:1. Further the amount of N-AcGm disappeared from the incubation system during the reaction could be fully accounted for by the appearance of an equivalent amount of N-AcGm-6-P. These results indicate that the reaction proceeds according to the equation:

\[
\text{N-AcGm} + \text{ATP} \rightleftharpoons \text{N-AcGm-6-P} + \text{ADP}
\]
Table 10

Stoichiometry of N-AcGm kinase reaction

A typical incubation mixture which contained the following components (in µmoles) was used in a final volume of 1 ml: N-AcGm, 10; ATP, 10; MgCl₂, 10; Glycine-NaOH, pH 9.0, 30; and 0.07 mg of the calcium phosphate gel fraction. The reaction was conducted at 37°C for 30 mins. After incubation, the reaction was stopped by the addition of 1 ml of 0.1N acetic acid. The reaction mixture was passed through a column of Dowex-1-acetate (2 ml packed volume) and washed with water (10-15 ml). Free sugar (N-AcGm), eluted in this fraction, was collected and estimated according to the method of Morgan-Elson colour reaction (101). The adsorbed sugar ester was then eluted quantitatively with 0.1N H₂SO₄ (15-20 ml). The amount of N-AcGm 6-phosphate eluted out was estimated by the Morgan-Elson colour reaction (101).

ADP formed in the reaction mixture was determined by Procedure B. The value obtained was corrected for any ADP formed in the absence of N-AcGm during incubation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Increase or decrease µmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. N-acetyl D-glucosamine</td>
<td>-5.6</td>
</tr>
<tr>
<td>2. N-acetyl D-glucosamine-6-phosphate</td>
<td>+5.8</td>
</tr>
<tr>
<td>3. ADP</td>
<td>+5.8</td>
</tr>
</tbody>
</table>
Fig. 26. Absorption maxima of glycoaldehyde phosphate colour produced by diphenylamine reaction. 5μmoles (approx.) of the product was treated with periodate according to the method of Jeanloz and Forchielli (99). The reaction product was then purified by chromatography on Dowex-1-acetate column and then the phosphate ester was treated with diphenylamine according to the procedure of Dische and Borenfreund (100). Glucose-6-P was identically treated with periodate; the product purified and treated with diphenylamine for the colour reaction. The absorption of the grass green colour in different wavelength was determined in automatic Beckman DK2 spectrophotometer.
N. Identification of the product

The product isolated from the reaction mixture of N-AcGm kinase was identified as N-AcGm-6-P by the following experiments:

(a) The product gave characteristic Morgan-Elson colour reaction of aminosugar. On electrophoresis in paper, the product migrated at the same rate as an authentic sample of N-AcGm-6-P.

(b) The product was incubated with acid phosphatase as described in Method (98), and the amount of inorganic phosphate and free N-AcGm formed in the incubation medium were estimated according to the method of Chen et al. (97) and Reissig et al. (101) respectively. Inorganic phosphate to N-AcGm ratio was found in the proportion of 1.00:0.99.

(c) The position of the phosphate group of the product was established by periodate oxidation in acetate buffer (pH 4.5) by the method of Jeanloz and Porchielli (87). Glucose-6-phosphate was taken as a standard. The periodate treated products were purified by chromatography on a Dowex-1-acetate column (Method) and the purified phosphate ester derivative gave characteristic diphenylamine colour reaction of glycolaldehyde (100). Both derivatives gave a grass green colour whose absorption maxima was at 660 m\(\mu\) (Fig. 26). The identification of glycolaldehyde phosphate as a periodate oxidation product confirmed the position of phosphate ester at C-6 of N-AcGm.
These results suggested that in addition to ADP, N-acetyl-D-glucosamine-6-phosphate is the product of the reaction catalyzed by N-AcGm kinase.
Discussion
In mammalian tissues specific kinase for N-AcGm has been detected, although no extensive purification of the enzyme has been done so far. But purification of the enzyme is necessary to study the properties of the enzyme, specially the allosteric phenomenon which regulates enzyme activity.

N-AcGm kinase has now been purified from hog spleen about 3500 fold with a slight contamination of impurities, which catalyzes the reaction:

\[ \text{N-AcGm} + \text{ATP} \xrightarrow{\text{Mg}^{+2}} \text{N-AcGm 6-P} + \text{ADP} \]

\( \text{Mg}^{+2} \) and ATP are essential for this enzymatic reaction.

Leiour and Cardini (25) first reported that rat kidney contains the maximal activity of N-AcGm kinase followed by heart, spleen and liver. But the specific activity of N-AcGm kinase from kidney extracts was reported to be 0.1 (sp. activity = \( \mu \)moles of N-AcGm esterified/hr/mg. protein), which was very low, whereas in our experiments extracts from hog spleen showed a specific activity (defined as above) of 1.5. The presence of high concentration of the enzyme in hog spleen is probably because of the blood cell degradative activities of the spleen which makes free aminosugars available for rephosphorylation in that organ.

Hog spleen was chosen as the source of the kinase throughout this study on the ground that it contains not only large amount of the enzyme but is also free from other interfering enzymes which disturb the kinase catalyzed reaction.
The kinetic properties of \(N\)-AcGm kinase from hog spleen differ widely from other kinases isolated from different sources (Table 11). The kinase is strictly specific for \(N\)-AcGm and shows a broad pH optima between 8.6 and 9.4. But the kinase isolated from sheep brain showed a distinct pH optimum at 7.4. As can be seen in Table 11, the affinities of the sheep brain enzyme for the substrates, \(N\)-AcGm and ATP are significantly higher than that of the spleen enzyme.

Table 11

<table>
<thead>
<tr>
<th>Source of (N)-AcGm kinase</th>
<th>(K_m) (N)-AcGm</th>
<th>(K_m) ATP</th>
<th>pH optima</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hog spleen</td>
<td>(8 \times 10^{-4}) M</td>
<td>(1.2 \times 10^{-3}) M</td>
<td>Plateau from pH 8.6-9.4</td>
</tr>
<tr>
<td>Sheep brain (64)</td>
<td>(3.34 \times 10^{-4}) M</td>
<td>(6.11 \times 10^{-4}) M</td>
<td>Optimum at pH 7.4</td>
</tr>
<tr>
<td>\textit{S. aureus} (112)</td>
<td>(4 \times 10^{-4}) M</td>
<td>(1.2 \times 10^{-3}) M</td>
<td>Plateau from pH 7.3-8.8</td>
</tr>
<tr>
<td>\textit{E. coli} (113)</td>
<td>(5 \times 10^{-5}) M</td>
<td>(1.4 \times 10^{-4}) M</td>
<td>Plateau from pH 7.5-9.5</td>
</tr>
<tr>
<td>\textit{S. pyogenes} (114)</td>
<td>(0.77 \times 10^{-3}) M</td>
<td>-</td>
<td>Plateau from pH 7.0-8.0</td>
</tr>
</tbody>
</table>

During the purification procedure, it became apparent that unlike the brain enzyme, the purified spleen enzyme is not inactivated by dialysis. The enzyme is strongly inhibited by p-chloro-
mercuribenzoate and this inhibition can be reversed almost totally by cysteine. This result suggests that a -SH group is involved in the activity of the enzyme. N-AcGm kinase has been found to undergo activation in the presence of 2-mercaptoethanol. Highly purified N-AcGm kinase loses its activity slowly on storage at 4°C. It may lose its activity completely after a few weeks. However, the inactivated enzyme could be fully reactivated by preincubation with 2-mercaptoethanol.

It has been observed that the kinase activity is also inhibited by the presence of ADP which is a product of the enzyme catalyzed reaction. However, the inhibitory effect of ADP could be competitively prevented by ATP. The competitive antagonism between ATP and ADP in the reaction catalyzed by the kinase is shown in Fig. 19. The Lineweaver-Burk plots (1/v against 1/[s]) of ATP saturation curves, one in the presence and the other in the absence of ADP, yield straight lines showing different slopes that meet at a point on the ordinate at infinite substrate concentration. This means that the presence of ADP cannot alter $V_{\text{max}}$ but it increases the apparent $K_m$ for ATP of the enzyme. This effect of ADP could be due to a competitive displacement of ATP by the former from a common receptor site (catalytic site) on the enzyme. The above possibility is also suggested by our observation that, during heat treatment of the enzyme, the catalytic activity and the inhibitory activity of ADP were abolished at
the same rate even when the heating period was extended to a point of complete inactivation of the enzyme.

Our investigation has revealed that the catalytic activity of N-AcGm kinase is very sensitive to UDP-N-AcGm and N-AcGm-6-P. Within a certain pH range, UDP-N-AcGm and N-AcGm-6-P both can strongly inhibit the enzyme activity. One can see from Table 9 that the maximum inhibitory effect is shown by these metabolites around pH 7.6, whereas the catalytic activity is only slightly altered due to a change of pH between 7.0 and 8.6, the activity being a little higher at the higher pH's within this range. The pronounced effect of pH on the inhibitory activity of these metabolites on the kinase is probably due to a Bohr effect (i.e., the enzyme affinity for the inhibitors is a function of pH).

N-AcGm failed to reverse the inhibitory activity of UDP-N-AcGm and of N-AcGm-6-P. Therefore, the inhibitory action of these two metabolic inhibitors is of non-competitive type. The presence of the inhibitor, decreased the $V_{\text{max}}$ of the kinase quite appreciably. So, according to Monod et al. (53), N-AcGm kinase may be regarded as one belonging to negative V-system.

The fact that the catalytic activity of the kinase and the inhibitory activity of its two effectors vary in a non-identical fashion with the change of pH indicates that the effector molecules may have receptor site(s) separate from the catalytic site.
on the protein molecule. A better indication of the separate receptor site(s) for UDP-N-AcGm and N-AcGm-6-P from the catalytic site is given by the fact that the kinase, by careful heating, can be so modified that it becomes completely insensitive to these inhibitors but still capable of catalysing the phosphorylation reaction in the presence of the substrates. This result supports the possibility of a specific site for inhibitor on the enzyme molecule other than the active site.

It has been shown by a number of workers that the presence on an enzyme of more than one kinetically important binding site may be recognized by a nonlinear double reciprocal plots (115-117). From the observation of such nonlinear plots with N-AcGm as the variable substrate and the value of $n = 1.66$ of the slope of the Hill plot suggest that at least two molecules of N-AcGm are involved in the reaction and that these may bind at different sites and exhibit a homotropic cooperative effect.

It was found that in the presence of both inhibitors (UDP-N-AcGm and N-AcGm-6-P), the value of $n$ becomes 2.0. This increment of the value suggests the stronger interaction between the substrate ligands in the presence of inhibitors.

It was observed that the substrate saturation curve for desensitized enzyme followed the normal Michaelis-Menten kinetics. In this case, Lineweaver-Burk plot yielded a straight line and...
the value of $n$ of the slope of the Hill plot was close to 1.0. This reduction of interaction coefficient suggests the loss of the cooperativity of substrate binding due to heat treatment.

All these results clearly indicate that the inhibition of the kinase activity by UDP-$\text{N-AcGm}$ and $\text{N-AcGm-6P}$ is most likely due to an allosteric effect.
Summary
A highly specific kinase which phosphorylates \( \text{N-AcGm} \) has been purified approximately 500-fold from the extracts of hog spleen. The enzyme contains slight contamination of impurities which has been revealed from the ultracentrifugal studies. An \( S_{20,w} \) of 5.5 is obtained for the native enzyme. The enzyme phosphorylates \( \text{N-AcGm} \) in the presence of ATP to form \( \text{N-AcGm-6-P} \) and ADP. The products of the kinase reaction (\( \text{N-AcGm-6-P} \) and ADP) have been unequivocally identified.

The enzyme is maximally active within a pH range of 8.6 and 9.4. The enzyme is strictly specific for \( \text{N-AcGm} \) as phosphate acceptor. \( K_m \) values for \( \text{N-AcGm} \) and ATP are \( 1.1 \times 10^{-3} \) M and \( 1.8 \times 10^{-3} \) M respectively. ATP is the phosphoryl group donor for the \( \text{N-AcGm} \) kinase reaction and the enzyme also can utilize CTP and GTP in lieu of ATP at low rates. The kinase requires \( \text{Mg}^{+2} \) ions for its activity. \( \text{Mg}^{+2} \) may be replaced by other divalent metal ions like \( \text{Mn}^{+2} \) and \( \text{Co}^{+2} \) with lesser degree of effectiveness.

The enzyme is inhibited by inorganic phosphate and pyrophosphate. This inhibition can be overcome by stoichiometric amounts of \( \text{Mg}^{+2} \). The enzyme is also inactivated by p-chloromercuribenzoate and this inactivation can be reversed by the addition of -SH compounds e.g. cysteine and 2-mercaptoethanol.

The enzyme is inhibited by the reaction products i.e. ADP and \( \text{N-AcGm-6-P} \). The inhibition by ADP is competitive with ATP whereas the inhibition by \( \text{N-AcGm-6-P} \) is of non-competitive type. UDP- \( \text{N-AcGm} \), the end product of the pathway of \( \text{N-AcGm} \) meta-
holism, is found to be a strong non-competitive inhibitor of the kinase reaction.

Hill plot of N-AcGm saturation kinetics of the enzyme in the presence of its non-competitive inhibitors (UDP-N-AcGm or N-AcGm-6-P) showing \( n=2 \) suggest that there may be more than one site for binding the substrate on the enzyme molecule. The enzyme protein could be desensitized to UDP-N-AcGm and N-AcGm-6-P by controlled heat treatment without much loss of its catalytic activity. All these results strongly suggest that the hog spleen N-AcGm kinase, is a regulatory enzyme, and the end-product UDP-N-AcGm and the reaction product N-AcGm-6-P, both act on the enzyme as feed-back inhibitor.