SECTION II: ENZYMATIC EPIMERIZATION OF N-AcGm
Introduction
The majority of naturally occurring monosaccharides are usually found only as constituents of complex compounds, such as glycosides, oligosaccharides, or polysaccharides. The existence of free sugars mainly results from the catabolism of these complex substances, and is at most transitory. Free monosaccharides are rarely involved in the pathways of biosynthesis. Studies on the metabolism of sugar nucleotides by various enzyme preparation have led to the elucidation of pathways for the biosynthesis of many monosaccharides. One of the main reactions resulting in the transformation of different sugars is "epimerization".

"Epimerization can be defined as the selective inversion of configuration at a single asymmetric center occurring in a compound containing more than one such center". The enzyme responsible for this transformation is termed as epimerase. The enzyme does not act when the two stereoisomers are in a state of equilibrium. The configurational arrangement at the reactive center can be influenced by the stable asymmetric part of the molecule. Stereoisomers differing only in configuration at the carbon atom immediately adjacent to the carbonyl group are called epimers. To extend the definition of epimerisation, Pigman and Goepp (118) suggested to include any pair of stereoisomers that differ solely in configuration about a single asymmetric carbon atom, regardless of its position relative to the carbonyl function. Such an extension of
The definition has been proved useful in the nomenclature of the rapidly increasing list of enzymes that catalyze inversion of configuration at single asymmetric center.

The most common modification is an inversion of the configuration of a carbinol group catalyzed by epimerases. An example is UDP-D-galactose-4-epimerase which catalyzes the reaction:

\[ \text{UDP-D-glucose} \rightleftharpoons \text{UDP-D-galactose} \]

This enzyme and certain other epimerases have been shown to require catalytic amounts of NAD, which suggests that the mechanism of inversion involves an oxidation and reduction at the C-4 position\(^{(119)}\). Several mechanisms for the inversion have been proposed \(^{(119-121)}\) including one in which the pyrimidine ring of the sugar nucleotide actually participates in the reaction \(^{(120,122)}\). The latter mechanism is clearly not a step common to all epimerases as L-ribulose-5-P-4-epimerase \(^{(123,124)}\) interconverts the sugar phosphates, L-ribulose-5-P and D-xylulose-5-P, in a reaction similar to that catalyzed by UDP-D-galactose-4-epimerase.

Epimerization of carbinol groups also occurs during the formation of deoxysugars, but it appears that these epimerizations involve carbon atoms α to a carbonyl function. This would allow the possibility for enediol formation as a mechanism for inversion, analogous to the mechanism for the interconversion of D-ribulose-5-P and D-xylulose-5-P catalyzed by D-ribulose-5-P-3-epimerase \(^{(125,126)}\).
A number of other enzymes that catalyze epimerizations at C-4 of nucleotide-bound sugars have been described. Thus enzymes catalyzing the following pairs are known:

- UDP-glucuronic acid $\rightarrow$ UDP-galacturonic acid (127, 128)
- UDP-D-xylose $\rightarrow$ UDP-L-arabinose (129)
- UDP-glucosamine $\rightarrow$ UDP-galactosamine (130)
- UDP-acetylglucosamine $\rightarrow$ UDP-acetylgalactosamine (131)
- TDP-acetylglucosamine $\rightarrow$ TDP-acetylgalactosamine (132)

Two reports have appeared in which the enzymic epimerization of UDP-N-acetylglucosamine into UDP-N-acetylgalactosamine is described. Maley and Maley (130) found that a partially purified preparation of UDP-galactose-4-epimerase from calf liver acetone powder contains UDP-N-acetylglucosamine-4-epimerase. Whether the two activities are associated with the same enzyme has not yet been reported. UDP-N-acetylglucosamine-4-epimerase has also been found in the extracts of a strain of Bacillus subtilis (131) which is known to synthesize a polysaccharide containing N-acetylgalactosamine. Davidson (121) reported an enzyme from embryonic cartilage which required DPN for catalyzing the epimerization of UDP-N-acetylglucosamine into UDP-N-acetylgalactosamine.

The uridine nucleotides can serve as sources for two other amino sugars, galactosamine and D-mannosamine. Leloir and Cardani (133) in 1957 initially reported that UDP-N-AcGm was converted to
free N-AcGalm, uridine monophosphate and inorganic phosphate by rat liver extracts. This result was not confirmed by Comb and Roseman. Comb and Roseman (134) found that crude extracts of rat liver catalyze the formation of free N-acetyl-D-mannosamine from UDP-N-AcGm. In this epimerization, the acetylamino group at C-2 is apparently the site of enzyme action rather than the hydroxyl group at C-4. The mechanism for this novel biochemical reaction has not yet been determined. This system was not detected in bacteria.

In 1964, Ghosh and Roseman (65) reported an enzyme present in animal tissues catalyzing the interconversion of N-AcGm and N-AcMm in presence of a catalytic amount of ATP.

\[
\begin{align*}
\text{N-AcGm} & \overset{(ATP)}{\rightleftharpoons} \text{2-epimerase} \rightarrow \text{N-AcMm}
\end{align*}
\]

They purified the enzyme 2-epimerase about 318 fold from hog kidney extracts and studied some kinetic properties. It was also reported that ATP was neither a direct participant in the reaction nor it was degraded by the enzyme during the course of the reaction. The role of the cofactor at that time was a mystery. They also reported (135) at the same time the presence of a different 2-epimerase in Aerobacter aerogenes. The animal enzyme differs from the bacterial enzyme in two important respects. Firstly, the animal enzyme acts only on the N-acetylhexosamines and not on their 6-phosphate esters.
and secondly, while the bacterial enzyme exhibits no detectable co-factor requirements, the animal epimerase requires a catalytic quantity of ATP. The present investigation was undertaken with a view to determining the actual role of ATP in the epimerization. We have now observed that ATP is not absolutely required for the activity of 2-epimerase, but it exerts a stimulatory effect on the enzyme causing approximately a 20-fold increase of its activity.

Our studies indicate that the 2-epimerase like a regulatory enzyme, possesses two distinct but mutually interacting sites on the enzyme - a catalytic site that binds the substrate and an "allosteric" site that binds the effector molecule, ATP. Kinetic studies with this allosteric enzyme show that ATP not only increases the $V_{\text{max}}$ but it also enhances simultaneously (a) the affinity of the enzyme for its substrate; (b) the cooperativity of substrate binding with the enzyme molecule.
Experimental Procedures
Calcium phosphate gel was made by the procedure of Kellin and Hartree (94). Pyruvate kinase was prepared according to the method of Theodor Bucher and G. Pfleiderer (95). N-AcMm was prepared by the method of Carroll and Cornforth (96).

ATP, PEP, NADH, Gm-HCl, Protamine sulphate, DEAE-cellulose were obtained from Sigma Chemical Company, U.S.A.

Preparation of N-AcGm kinase - N-AcGm kinase, used in the assay of 2-epimerase, was prepared from hog spleen by using the following operations:

Hog spleen (100 g.) was homogenized with 200 ml. of a buffer system (0.03M potassium phosphate buffer, pH 7.6-0.001M EDTA-0.01M 2-mercaptoethanol) in a Waring Blender. The supernatant fluid was collected by centrifugation of homogenized material at 16,000 x g for 30 mins. (crude extract).

The crude extract (150 ml.) was diluted with water (150 ml.) and the enzyme was precipitated by adding 2% protamine sulphate solution (22.5 ml). After a preliminary washing of the residue with 75 ml. of 0.05M potassium phosphate buffer, pH 7.6, the enzyme was extracted with 75 ml. each of 0.075M, 0.075M and 0.1M of the same buffer, pH 7.6. These three extracts were combined (protamine sulphate extract).
The protamine sulphate extract was then brought to 32.5% saturation with solid \((\text{NH}_4)_2\text{SO}_4\) and any precipitate formed at this stage was rejected by centrifugation. The supernatant fluid was brought to a saturation level of 50% with solid \((\text{NH}_4)_2\text{SO}_4\) and the precipitate formed was collected by centrifugation and dissolved in 10 ml. of 0.02M potassium phosphate buffer, pH 7.6 (Ammonium sulphate fraction). This concentrated N-AcGm kinase fraction also contained ATPase. To remove ATPase from the ammonium sulphate fraction, it was equilibrated at 60°C for 2 mins. Coagulated proteins were removed by centrifugation and the supernatant fluid, dispensed in several small test tubes, was kept frozen until used. The heat-treated ammonium sulphate fraction was free of ATPase and other interfering enzymes, and was found quite suitable as a source for specific N-AcGm kinase required for the assay of the 2-epimerase.

**Methods**

**Epimerase Assay**

The enzyme was assayed by measuring the ADP formed in the reaction by coupling the 2-epimerase and the specific N-AcGm kinase in the following manner:

\[
\begin{align*}
\text{N-AcMm} + \text{ATP} & \xrightarrow{(ATP)} \text{N-AcGm} + \text{ADP} \\
\text{N-AcGm} + \text{ATP} & \xrightarrow{\text{specific kinase}} \text{N-AcGm-6-P+ADP} \\
\end{align*}
\]
The assay of 2-epimerase activity is based on the following steps:

(1) Incubation of the 2-epimerase with N-AcMm (and ATP, when necessary), (2) treatment of the reaction products with an excess of the specific N-AcGm kinase and ATP for the complete conversion of any N-AcGm formed in the step (1) to N-AcGm-6-P and the formation of an equivalent amount of ADP, and (3) the estimation of ADP enzymatically by the pyruvate kinase-lactic dehydrogenase assay procedure. Since ATP is not degraded in step (1), the amount of ADP estimated in the step (3) equals to the amount of N-AcGm formed by 2-epimerization.

The incubation mixture for measuring the ATP-independent enzyme activity contained the following components in a final volume of 0.25 ml. (in μmole): N-AcMm, 10.0; Tris-HCl, pH 7.3, 12.5; MgCl₂, 2.5 and enzyme fraction. For measuring the ATP-dependent activity each incubation mixture contained the following in a final volume of 0.25 ml. (in μmole): N-AcMm, 2.0; ATP, 1.0, Tris-HCl, pH 7.3, 12.5; MgCl₂, 2.5 and enzyme fraction. After incubation at 37°C for 30 mins, the reaction was stopped by heating 1 min. at 100°C. Each assay mixture was treated with approximately 2 units of specific N-AcGm kinase fraction (see below) and 1.0 μmole of ATP (when ATP was not added in the first incubation mixture). The volume of the mixture was made up to 0.35 ml. and incubated again at 37°C until all N-AcGm was converted to its 6-phosphate ester (usually less than 15 minutes). The mixture was
heated again 1 min. at 100°, cooled and the volume was made up to 1.0 ml. by adding water. For the measurement of ADP, aliquots of the diluted mixture were added to cuvettes containing the following components in a final volume of 1 ml.: potassium phosphate buffer, pH 7.6, 25 μmoles; MgCl₂, 2 μmoles; PEP, 0.5 μmoles; KF, 2.5 μmoles; DPNH, 0.1 μmole (approximately). After noting the initial absorbance at 340 μm, 0.01 ml. solution of pyruvate kinase (containing both lactic dehydrogenase and pyruvate kinase in excess) was added. The absorbance fell immediately and attained a level which did not decrease with time. The fall of absorbance was noted, and the amount of ADP present in the cuvette was calculated from the amount of NADH oxidized. Control tubes used routinely for each set of assay lacked either enzyme fraction or N-AcMm in the first incubation mixture. The missing component was, however, added to the control tube immediately before stopping the reaction by heating.

Specific activity (both ATP-dependent and ATP-independent) has been defined as μmoles of N-AcGm formed per mg. protein in 30 mins. under the assay conditions described above.

One unit of ATP-dependent enzyme activity catalyzes the epimerization of 1 μmole of N-AcGm under the assay conditions stated above.

In the case of N-AcGm kinase, one unit of enzyme activity catalyzes the phosphorylation of 1 μmole of N-AcGm under the assay conditions described above.
Estimation of protein and aminosugars

Protein was determined by the method of Lowry et al. (102). Aminosugars were estimated by a modified procedure of Reissig et al. (101).

Purification of N-AcGm 2-epimerase

Unless otherwise stated all operations were conducted at temperatures between 0°-4°. All buffer systems used in the fractionation procedure were prepared in 0.001M EDTA-0.01M 2-mercapto-ethanol; this was found necessary for enzyme stability.

The first two steps of the purification procedure, namely the preparation of crude extract (step-1) and of protamine extract (step 2) were essentially similar to those as described previously by Ghosh and Roseman (65).

Preparation of crude extract and protamine extract

Kidney Cortex (100 g.) was homogenized in a Waring Blender with potassium phosphate buffer, pH 7.6 (0.03M, 200 ml.) and the soluble supernatant fraction was collected after centrifugation at 16,000 x g for 30 min. (crude extract).

The crude extract (150 ml.) was diluted with distilled water (150 ml.) and treated with protamine sulphate solution (4.5 ml., 2%) with stirring; the precipitate formed at this stage was discarded after centrifugation. The supernatant fraction was further treated
with protamine sulphate solution (15 ml, 2%), and the precipitate formed was collected by centrifugation. The precipitate was extracted twice with 45 ml. portions of 0.01M potassium phosphate buffer, pH 7.6 and the inactive soluble extracts were centrifuged off. The precipitate was then extracted two more times with 45 ml. portions of 0.025M potassium phosphate buffer pH 7.6; the active soluble fractions obtained after centrifugation were combined. (Protamine extract, 90 ml).

The extraction of the protamine sulphate precipitate as described above was best carried out by suspending the residue, obtained by centrifugation after protamine sulphate treatment, uniformly in buffer solution by slow stirring with a glass rod and then centrifuging at low speed (5,000 x g, 15 min.) to separate the soluble extract.

**Bentonite step**

The enzyme was not adsorbed by bentonite at this stage of purification, but bentonite removed inert proteins. Thus when 90 ml. of protamine extract gently stirred for 10 mins. with 900mg. of bentonite suspension in 0.001M EDTA and centrifuged, almost all the activity was found in the supernatant fluid.

Concentrations of the 2-epimerase in the bentonite supernatant and protamine extract fractions were too low for any satisfactory assay of the ATP-independent enzyme activity. These fractions were
concentrated to small volumes by adsorbing the enzyme on a small DEAE-cellulose column and eluting it with 0.2M potassium phosphate buffer, pH 7.6 (see DEAE-cellulose step). The concentrated enzyme preparations obtained from protamine extract and bentonite supernatant fractions as above were dialyzed against $5 \times 10^{-3}$M potassium phosphate buffer, pH 7.6 and designated as "Protamine concentrate" and "Bentonite sup concentrate".

**DEAE-cellulose step**

Bentonite supernatant fraction (100 ml.) was applied to a DEAE-cellulose column, 1.5 cm. x 10 cm., which had been equilibrated with 0.02M KCl-0.01M potassium phosphate buffer, pH 7.6. After the column had been washed with 200 ml. of 0.05M potassium phosphate buffer, pH 7.6, elution with a 100 ml. linear gradient of 0.05M to 0.15M potassium phosphate buffer, pH 7.6 made in 0.02M KCl-0.001M EDTA-0.01M 2-mercaptoethanol, was carried out at flow rate of 2 ml. per min. Fractions were collected, 5 ml. each, and assayed for ATP-dependent epimerase activity. The enzyme was detected between 0.09M to 0.11M potassium phosphate buffer, pH 7.6, and these fractions were pooled (DEAE-eluate, 45 ml). The pooled fractions (45 ml.) were dialyzed against $5 \times 10^{-3}$M potassium phosphate buffer, pH 7.6 for 5 hr., and the dialyzed preparation was applied again to a DEAE-cellulose column, 1 cm. x 2 cm. equilibrated as before and the enzyme eluted with 3 ml. of 0.2M potassium
phosphate buffer, pH 7.6. The concentrated enzyme fraction was dialyzed against $5 \times 10^{-3}\%$ potassium phosphate buffer, pH 7.6 for 12 hr. and the dialyzed preparation was designated as DEAE-eluate.

**Calcium phosphate gel step**

The DEAE-cellulose concentrate (3 ml.) was treated with 35 mg. of gel (dry weight). After gentle stirring of the mixture for 10 mins. at $0^\circ$, the suspension was centrifuged. The supernatant fluid was designated as calcium phosphate gel eluate.
Results
Fig. 27. Effect of incubation time and protein concentration on the rate of reaction in the absence of ATP: The composition of incubation mixtures and other assay conditions (except the period of incubation) were the same as described for measuring the ATP-independent activity of the epimerase under "Epimerase Assay" (p. 78). The amount of enzyme added per tube was 10 µg (---) and 20 µg protein (—o—) from DEAE-cellulose eluate and 150 µg protein (-X-X--) from bentonite sup concentrate fractions. Incubation of each assay tube was conducted at 37°C for the period shown.
Properties of 2-epimerase

ATP-independent and ATP-dependent activities of various enzyme fractions

Previously, kinetic and other properties of the 2-epimerase were studied in the presence of ATP (65). Similar studies have now been made with the enzyme in the absence of ATP. The enzyme fractions that catalyzed the epimerization of N-AcMm to N-AcGm in the presence of ATP, also catalyzed the same reaction in the absence of ATP, although at a much diminished rate in the latter case. As can be seen from Table 12, the ratio of ATP-dependent and ATP-independent activities of the various enzyme fractions remained nearly constant (18-20) over the 1500-fold purification range.

Dependence of ATP-independent reaction rate on protein concentration and incubation period

The effect of incubation time and protein concentration on the rate of ATP-independent 2-epimerization of N-AcMm are shown in Fig. 27. The rate showed a linear relationship with time for at least 40 mins. The rate of epimerization was also found proportional to enzyme concentration; when the quantity of enzyme (DEAE-eluate) was doubled in the incubation mixture, a two-fold increase of the rate was observed.
Table 12

Purification of N-acetylg glucosamine 2-epimerase

The composition of incubation mixtures and the other assay conditions were the same as described in the text under "Epimerase assay".

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Protein (mg./ml.)</th>
<th>Total units</th>
<th>Yield %</th>
<th>Sp. activity +ATP (A)</th>
<th>Sp. activity -ATP (B)</th>
<th>Ratio of sp. activities A/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract*</td>
<td>25</td>
<td>600</td>
<td>100</td>
<td>0.12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2. Protamine concentrate</td>
<td>7.76</td>
<td>480</td>
<td>80</td>
<td>3.1</td>
<td>0.16</td>
<td>19.4</td>
</tr>
<tr>
<td>3. Bentonite concentrate</td>
<td>2.0</td>
<td>480</td>
<td>80</td>
<td>30</td>
<td>1.5</td>
<td>20</td>
</tr>
<tr>
<td>4. DEAE-cellulose eluate</td>
<td>0.16</td>
<td>280</td>
<td>46.6</td>
<td>175</td>
<td>8.7</td>
<td>20</td>
</tr>
<tr>
<td>5. Calcium phosphate gel</td>
<td>0.125</td>
<td>226</td>
<td>37.6</td>
<td>180</td>
<td>9</td>
<td>20</td>
</tr>
</tbody>
</table>

* The assay procedure described above is not suitable for measuring the enzyme activity of crude extracts. For the assay of impure preparations the second incubation mixture was treated with ZnSO₄ and Ba(OH)₂ solutions for the quantitative removal of N-AcGm-6-P (105). The amount of N-AcMm disappeared due to epimerization was then estimated by the Morgan-Elson colour reaction (101).
Fig. 28. pH optima of the ATP-independent epimerase activity. Except for the buffer system the composition of incubation mixtures was the same as described for measuring the ATP-independent activity of the epimerase under "Epimerase Assay". Each tube contained 12.5 μmoles of either potassium phosphate buffer (pH 6.2-7.0) or Tris-HCl buffer (pH 7.3-8.5). Bentonite sup concentrate (150 μg protein per assay tube) was used. All assay tubes were incubated at 37° for 30 min.
Fig. 29. Effect of N-acetylmannosamine concentration on the ATP-independent reaction rate. The composition of incubation mixture and the other assay conditions were the same as described in the text for the assay of ATP-independent enzyme activity except for N-AcMm concentration which was varied. Bentonite sup concentrate fraction (540 µg protein per assay mixture) was used.
Double reciprocal plots of ATP-independent reaction rate and N-AcMm concentration: The composition of incubation mixtures was the same as described in the text for the assay of ATP-independent enzyme activity except for the N-AcMm concentration which was varied; 340 μg. protein from the bentonite sup concentrate fraction was used per assay tube. The inset shows the above data plotted according to the empirical Hill equation $V_{\text{max}}$ value was obtained by extrapolation of double reciprocal plots to infinite substrate concentration.

$\vartheta = \mu$ mole N-AcGm formed per ml. of assay mixture in 30 minutes.
Fig. 30. Activation of the 2-epimerase by ATP: Enzyme activity plotted as a function of ATP concentration. The composition of incubation mixtures was the same as described in the assay procedure of ATP-independent activity except for the addition of varying amount of ATP: 150 µg of protein from the bentonite sup concentrate fraction was used. The inset shows the data plotted in the form of Hill equation.

\[ \frac{V_{max}}{V} = \frac{K_m}{K_m + ATP} \]

\( V \) = µmoles N-AcGm formed per ml. of assay mixture in 10 minutes.
The enzyme activity for ATP-independent epimerization showed a pH optimum between 7.4-7.6 (Fig. 28). It may be mentioned that Ghosh and Roseman (65) reported the same pH range for optimum activity of the ATP-dependent epimerization.

Substrate (N-AcMm) saturation curve of the epimerase without its activation by ATP

The effect of N-AcMm concentration on the rate of reaction is shown in Fig. 29. Apparent Km value of N-AcMm (conc. of N-AcMm required for half-maximal velocity) calculated from Lineweaver-Burk plot was $9 \times 10^{-3} \text{M}$ (Fig. 29a) whereas Km value for N-AcMm in presence of ATP was $1.7 \times 10^{-3} \text{M}$ (Fig. 34). As reported earlier (65), Km value for N-AcMm in presence of ATP was $3.0 \times 10^{-3} \text{M}$.

Effect of ATP on the reaction rate

When the rate of epimerization was plotted against ATP concentration in the presence of excess N-AcMm, a typical sigmoid shaped curve was obtained. N-AcMm concentration used in this study was high enough ($4 \times 10^{-2} \text{M}$) for the enzyme to maintain maximal ATP-independent activity. As can be seen from Fig. 30, ATP increased the $V_{\text{max}}$ of the enzyme-catalyzed reaction by about 18-fold. Sigmoid shape of the ATP-saturation curve is indicative of interactions between ATP-binding sites.
For an enzyme possessing \( n \) number of mutually interacting substrate binding sites it has been shown by Atkinson et al. (111) that Michaelis equation may be put in the form

\[
\log \frac{\frac{V}{V_{\text{max}}}}{\frac{V_0}{V_{\text{max}}}} = n \log (s) - \log K \quad \cdots \cdots \cdots \quad (1)
\]

where \( V \) is velocity of the enzyme catalyzed reaction, \( (s) \) is substrate concentration, \( V_{\text{max}} \) is the maximum velocity of the reaction, and \( K \) is a constant. Equation (1) which is also otherwise known as Hill equation, has been used widely in the study of enzyme-substrate (or enzyme-effector) interactions in the case of regulatory enzymes showing sigmoidal response of activity to increasing substrate (or effector) concentration.

For an enzyme where an effector enhances the rate of reaction as has been observed with \( N^1\beta\text{Gm}^2\)-epimerase, one can show that the Hill equation can be transformed to

\[
\log \frac{\frac{V}{V_{\text{max}}}}{\frac{V_0}{V_{\text{max}}}} = n \log (A) - \log K \quad \cdots \cdots \cdots \quad (2)
\]

where \( V_0 \) = velocity of reaction in the absence of effector, and \( (A) \) = concentration of effector. As can be seen from Fig.30. Plotting \( \frac{\frac{V}{V_{\text{max}}}}{\frac{V_0}{V_{\text{max}}}} \) against \( \log (\text{ATP}) \) gave a straight line whose slope \( n \) (Hill coefficient or interaction coefficient) was equal to 2.6. It has been shown by Atkinson et al. (111) that the numerical value of the slope \( n \) will depend both on the number of interacting sites and the strength of interactions between them.
Fig. 31. Effect of temperature on sensitivity to ATP activation of N-AcMm-2-epimerase: Bentonite supernatant, concentrated to 1.5 mg. protein/ml. and dialysed, was kept at 50°C and at 60°C for the indicated time. The heat treated enzyme fractions were assayed immediately for the ATP-dependent and ATP-independent epimerase activities using 15 μg and 150 μg. protein respectively by the procedure described in the text under "Epimerase Assay".
In the case where interaction is strong, the value of $n$ will approach a limiting integral value to the number of sites. The numerical value of the slope $n = 2.6$ suggests that there are at least three ATP binding sites per molecule of the 2-epimerase.

**Desensitization of the enzyme to ATP**

Since the site for an effector molecule on a regulatory enzyme is not identical with the site for its substrate, it has been possible in many instances to desensitize a regulatory enzyme to allosteric effector by structural modification of the protein without loss of its catalytic activity (111,60,136,137). Among the methods used to desensitize an enzyme to allosteric control, treatment with p-chloromercuribenzoate and heating are quite common. Both these methods could be successfully used to desensitize the 2-epimerase to ATP.

(a) **Effect of heat on ATP-dependent and ATP-independent activities of the 2-epimerase**:

In an effort to obtain the enzyme in a state insensitive to ATP but retaining its ATP-independent catalytic function, Bentonite supernatant fraction was subjected to various degrees of thermal denaturation treatment. As can be seen from Fig. 31, the enzyme fraction could be fully desensitized to ATP by heating at $60^\circ$ for 1 min, whereas the same treatment was accompanied by only a slight loss of ATP-independent activity. At $50^\circ$, the desensitization was
Fig. 32. Protection of ATP against the thermal inactivation of the ATP-binding site: The dialysed bentonite sup concentrate, 1.5 mg. protein/ml. was kept at 45°C and at 50°C with 5 x 10^{-3} M ATP and without ATP for the indicated time. The heat treated enzyme fractions (aliquots containing 15 μg protein) were assayed immediately for the ATP-dependent epimerase activities following the procedure described in the text under "Epimerase Assay".
Fig. 35. Effect of PCMB on sensitivity to ATP activation of N-AcMm-2-epimerase: Bentonite supernatant (concentration to 1.5 mg. protein/ml. and dialysed) was preincubated at 27°C for 3 mins. with PCMB at the given concentrations. The treated fraction was immediately diluted with the assay medium. ATP-dependent and ATP-independent activities by using 0.5 µg and 150 µg protein respectively were assayed as described in the text under "Epimerase Assay".
complete in 5 mins, but the desensitized enzyme retained about 80% of its initial ATP-independent activity even after 20 minutes of heating. The most probable explanation of this differential effect is that the catalytic site and the site that binds ATP are not identical in nature.

Allosteric enzymes are frequently found to become more resistant to heat denaturation by the presence of effector molecules (138). When ATP was present during the time of heating of the enzyme a protective effect against heat inactivation was observed. As can be seen from Fig. 32, there is a distinct protective effect of ATP on the ATP-dependent activity during heating on the enzyme at 45° and at 50°.

(b) **Effect of PCMB on ATP-dependent and ATP-independent activities:**

When the 2-epimerase was treated with PCMB under controlled conditions, one can obtain the enzyme catalytically active but completely insensitive to ATP. This is shown in Fig. 33. The enzyme was found to be inactivated by PCMB in two distinct steps. The first inactivation was accomplished by preincubating the enzyme with PCMB at a concentration below $5 \times 10^{-5}$M. PCMB below $5\times10^{-5}$M concentration is particularly effective in destroying the sensitivity of the enzyme to ATP. The second step which corresponded to the inactivation of the catalytic site, become operative
Fig. 34. Double reciprocal plots of reaction rate of native enzyme and substrate (N-AcMm) concentration in the presence of ATP: The composition of incubation mixture and other assay conditions were the same as described in the text for the assay of ATP-dependent activity except for the varying concentration of N-AcMm; Bentonite supconcentrate (34 μg protein per assay tube) was used. The inset shows the above data plotted according to the empirical Hill equation: $V = \mu$moles of N-AcGm formed per ml. of assay mixture in 30 mins.
Fig. 35. Lineweaver-Burk plot of reaction rate of heated enzyme to increasing concentrations of N-AcMm: The composition of incubation mixtures was the same as described in the text for the assay of ATP-independent enzyme activity except for the N-AcMm concentration which was varied; 340 μg of protein from the desensitized enzyme preparation (heated 50°C for 5 mins, as described in Fig. 31) was used per assay tube.

The inset shows the above data plotted according to the empirical Hill equation.

$$\vartheta = \mu \text{moles N-AcGm formed per ml. of assay mixture in 30 mins.}$$
Fig. 36. Lineweaver-Burk plot of reaction rate of PCMB-treated enzyme to increasing concentrations of N-AcGm. Bentonite supconcentrate was preincubated at 27°C for 3 mins with 5 x 10^{-5} M PCMB; dialysed against 0.025M potassium phosphate buffer, pH 7.6-0.001M EDTA. The treated fraction (340 µg protein) was immediately diluted with the assay medium. The composition of incubation mixture and the assay conditions for the assay of ATP-independent enzyme activity were the same as described in the text under "Epimerase Assay". The inset shows the above data plotted in the form of Hill equation. \( V^q = \mu \text{moles N-AcGm formed per ml. of assay mixture in 30 min.} \)
Whether the binding of subunits of the epimerase and their disso-
ciation are analogous to aspartate transcarbamylase system studied
by Gerhart and Pardee (60) is not known. Further work is necessary
to elucidate the mechanism of allosteric action in the epimerase.

Substrate saturation curve of the enzyme desensitized to ATP:

N-AcMm saturation curve of the native enzyme in the absence
of ATP showed some deviation from Michaelis-Menten relationship
as one can see from its Lineweaver-Burk plot in Fig. 29a. The
Lineweaver-Burk plot was slightly curved instead of being linear.
When the effect of increasing N-AcMm concentration on the enzyme
activity was measured in the presence of ATP, a more pronounced
deviation from Michaelis-Menten relationship was observed (Fig.
34). The substrate saturation curves, in absence and in presence
of ATP have been plotted in the Hill system of coordinates and
are shown in Fig. 29a and 34 respectively. It can be seen that
the presence of ATP induced a change in the slope of the Hill plot
from 1.66 to 2.0. On the otherhand, the substrate saturation
curves of the desensitized enzyme obtained by PCMB treatment or by
heating conformed to Michaelis-Menten Kinetics as can be seen from
the linear form of their Lineweaver-Burk plots (Fig. 35,36). As
expected Hill plots of these curves yielded straight lines having
their slope close to 1.
The values of apparent Km and Hill coefficients for the native and desitized enzyme are given in a tabular form (Table 14).

Table 14

Hill coefficients and apparent Km values with respect to N-AcMm of the 2-epimerase

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>Addition</th>
<th>Apparent Km conc. of N-AcMm at $1/(2V_{max})$ x $10^{-3}$ M</th>
<th>Hill coefficient n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bentonite sup concentrate</td>
<td>ATP</td>
<td>1.7</td>
<td>1.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.0</td>
<td>1.66</td>
</tr>
<tr>
<td>Bentonite sup concentrate</td>
<td></td>
<td>29.0</td>
<td>1.2</td>
</tr>
<tr>
<td>desensitized by heat</td>
<td></td>
<td>38.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Bentonite sup concentrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>desensitized by PCMB</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Discussion
The non essentiality of ATP in the reaction catalyzed by the 2-epimerase has been observed when the assay was performed using a high substrate and a high enzyme concentration. Previously (65) the enzyme was purified about 318 fold from crude extracts of hog kidney; the 2-epimerase has now been purified by about 1500-fold from the crude extracts of hog kidney. The ATP-dependent and ATP-independent epimerase activities could not be separated and the ratio of the specific activities remained nearly constant throughout the range of 1500 fold purification of the enzyme (Table 1). This result suggests the same enzyme is responsible for both the activities. Allosteric enzymes whose activity depended absolutely on the presence of their effector molecules, though rare, have been reported in the literature. The requirement for citrate and acetyl CoA for the catalytic activities of acetyl CoA carboxylase (139) and pyruvate carboxylase (140) respectively are well known. Glucose-6-P has also been shown to be an essential effector molecule required for the activation of frog muscle glycogen synthetase D (141). N-AcGm-2-epimerase at first appeared to be an enzyme depending absolutely on ATP for its catalytic activity. However, our present study demonstrates conclusively the non-essentiality of ATP for the activity of 2-epimerase. ATP appeared to be essential because its absence diminished the $V_{\text{max}}$ of the 2-epimerase by a factor of 20. When the activator of an enzyme enhances the $V_{\text{max}}$ by this order of magni-
In view of the fact that the 2-epimerase activity is powerfully stimulated by ATP, it was this particular response that was studied more extensively. The 2-epimerase apparently belongs to the allosteric enzyme of the type known as positive 'V system', as defined by Monod et al. (41), since the effector ATP produces a remarkable effect on the $V_{\text{max}}$ in the positive direction. However, the activation of the enzyme by ATP is also accompanied by an increase of the enzyme affinity for its substrate, N-AcGm.

Allosteric enzymes whose effector molecules are capable of influencing both $K_m$ and $V_{\text{max}}$, as observed with N-AcGm 2-epimerase, are known. Glucose-6-P has been found to decrease the apparent $K_m$ for UDP-glucose and increase the $V_{\text{max}}$ by 50 fold in the case of rabbit muscle glycogen synthetase D (142). Trehalose has been shown to increase the apparent $K_m$ for glucose-6-P and to decrease the $V_{\text{max}}$ of the enzyme trehalose synthetase (143). One usually refers these group of allosteric enzymes as belonging to mixed 'V and K system'.

In Fig. 29 & 30 are portrayed graphical representations of the effect of substrate concentrations on the enzyme activity in the presence and absence of ATP. A hyperbolic relationship of
these two variables is noted in the absence of the activator, while in its presence a sigmoidal relationship is seen. When these data are plotted in terms of 1/v with respect to 1/[s] a straight line is not obtained (Fig. 29a). The relationship of these two reciprocal entities is a curve. This type of non linear relationship is indicative of the presence of separate binding sites for activator and substrate molecules, and this has been interpreted as an indication of enzyme kinetics of "allosteric" type (115-117).

As noted by a number of authors (52,144,145) the Hill plot (146) is a useful tool for analyzing some allosteric effects, and the values of the slopes (n) obtained are considered to be a measure of the number of interacting sites as well as the strength of their interaction. When the interaction is very strong, the value of the slope of the Hill plot approaches the number of binding sites for the ligand.

The kinetic data of 2-epimerase action substrate can be fitted to the empirical Hill equation. By plotting \( \log \frac{V}{V_{\text{max}} - V} \) against \( \log [s] \) as shown in Fig. 29a, one gets a straight line whose slope is equal to 1.66. This slope is a measure of an interaction coefficient, n, between sites. Even though n is not an elementary kinetic parameter of the enzyme, the fact that the value of n is greater than 1 implies that the number of "interacting active sites is at least 2".
The Hill equation also can be applied to the kinetics of activation by ATP. The value of $n = 2.6$ for the slope of the Hill plot suggests that at least three binding receptor sites for ATP exist per molecule of enzyme.

The first indication that the two sites are distinct was the observation that treatments which remove the sensitivity of the enzyme to ATP simultaneously normalize the kinetics of N-AcMm epimerization.

When the specific activating effect of ATP on the 2-epimerase is destroyed by heating or PCMB treatment of the enzyme, a concomitant abolition of the cooperativity of substrate binding and enhancement of the apparent Km value is observed (Table 14). PCMB or heat treatment might have weakened or damaged the quaternary bonds leading to a simultaneous loss of the homotropic interactions (of substrate ligands) and the heterotropic interactions without destroying the ATP-independent catalytic activity of the 2-epimerase. On the basis of the Monod's model (53), conservation of the of the interactions should depend upon the integrity of the whole native structure, including in particular the inter-proto-mer binding, whereas conservation of the activity should depend only on the integrity of the active site. According to the model, loss of the interactions may follow from any structural alteration of protein.
A distinctive kinetic feature of the 2-epimerase is the inverse relationship between the cooperativity of substrate binding (Hill coefficient) and the apparent Km value for the substrate. As can be seen from Table 14 any process that weakens the substrate interaction also weakens the substrate affinity (i.e. increases the concentration of substrate required for the half maximal velocity) for the enzyme. The conformation of the 2-epimerase in the active state not only promotes cooperativity of substrate binding but it also leads to a greater affinity of the enzyme for its substrate. In other words, 'conformational constraints' acting on the 'protomers' is a necessary condition for higher catalytic activity and higher substrate affinity in the case of the allosteric protein, N-AcGm-2-epimerase. Obviously kinetics of the allosteric effect of the 2-epimerase show the inadequacy of the mathematical model of allosteric proteins described by Monod et al. (53).
Summary
Ghosh and Rosenberg (65) reported in 1965 that N-acetylglucosamine 2-epimerase isolated from hog kidney showed an absolute requirement for a catalytic quantity of ATP. The epimerase activity of the enzyme even in the absence of ATP has now been demonstrated by using high enzyme and substrate concentration. The ATP-dependent and ATP-independent epimerase activities could not be separated, and the ratio of the specific activities remained nearly constant throughout the range of 1500-fold purification of the enzyme. We have now observed that ATP is not absolutely essential for the activity of 2-epimerase, but it exerts a stimulatory effect on the enzyme causing approximately a 20-fold increase of its activity.

2-epimerase has now been purified by about 1500-fold from the crude extracts of hog kidney by employing the following steps:

(i) Precipitation of the enzyme from the crude extracts by protamine sulphate solution, (ii) solubilization of the enzyme by phosphate buffer, (iii) removal of inactive proteins by bentonite treatment, (iv) DEAE-cellulose column chromatography and (v) further adsorption of inactive proteins by calcium phosphate gel.

The following significant observation on the properties of the enzyme have been made:

Km value for N-AcMm determined in the absence of ATP was $9 \times 10^{-3}$M; whereas the same, in the presence of saturating concen-
- 100 -

tration of ATP, was $1.7 \times 10^{-3}$ M. Optimum pH for ATP-dependent and ATP-independent activity was approximately 7.6 in both the cases.

The ATP saturation curve of the 2-epimerase did not conform to Michaelis-Menten kinetics. The curve obtained was sigmoidal in shape indicating cooperative binding of ATP molecules. Hill plot yielded an interaction coefficient of $n = 2.6$.

Other kinetic evidences indicate the presence of homotropic interactions between substrate molecules.

The allosteric effect of ATP is now further confirmed by the specific disruption of the ATP binding site without affecting the ATP-independent catalytic activity of the enzyme. The desensitization of 2-epimerase to ATP could be brought about in two ways - controlled heat treatment and PCMB treatment. The selective destruction of ATP effect (on 2-epimerase) strongly suggests that the site for binding the stimulatory is largely independent of the site for binding the substrates.

All these results clearly demonstrate that the specific stimulatory action of ATP on the enzymatic epimerization is due to an allosteric effect.