The Enzymatic Significance of S-Acetylation and N-Acetylation of 3-Phosphoglyceraldehyde Dehydrogenase

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SUMMARY

Specific S- and N-acetylations of the protein by two model substrates, acetyl phosphate and p-nitrophenyl acetate, have been related to the catalytic activity of 3-phosphoglyceraldehyde dehydrogenase in the following manner.

1. DPN facilitates the S-acetylation of the dehydrogenase with acetyl phosphate but inhibits the reaction with p-nitrophenyl acetate.

2. The S-acetyl enzyme compound is more labile at pH 7.0 than simple thioesters such as S-acetyl glutathione or S-acetyl coenzyme A. Denaturation increases the stability of the S-acetyl enzyme, suggesting that labilizing groups such as histidine have been removed from the active center.

3. In the presence of DPN the S-acetyl enzyme is rapidly deacylated by transfer of the acyl group to P1 or arsenate. Neither DPN nor P1 alone induces this rapid deacylation.

4. At pH 8.5, a specific lysine residue is acetylated by an S-N transfer reaction. The N-acetyl group is enzymatically inert in that it cannot be removed by addition of DPN and P1 or arsenate.

5. The lysine moiety is not readily acetylated in the presence of DPN, and conversely the N-acetylated enzyme binds less DPN. This suggests that the lysine moiety is involved in the coenzyme-enzyme interaction.

6. A number of experiments involving S-N transfer and dual acetylation of both the cysteine and lysine residues indicates that the S and N sites are in close proximity, although not near neighbors on a peptide chain.

7. The above observations have been fitted into a general proposal for the mechanism of catalysis by this enzyme.

The substrates acetyl phosphate (1) and p-nitrophenyl acetate (2) acetylate a specific cysteine residue (3) and a specific lysine residue (4) of 3-phosphoglyceraldehyde dehydrogenase from rabbit muscle. The sequence of the octadecapeptide containing the S-acetyl cysteine is shown (3).

Although 14C p-nitrophenyl acetate and 14C-acetyl phosphate label the same cysteine residue, the properties of the acetylation reactions are shown in this paper to differ considerably. As would be expected, however, the deacylation reactions of the acetyl enzyme complexes prepared with the two acetylating compounds are identical. The relationship of the S- and N-acetyl protein complexes to each other and to the mechanism of the enzymatic catalysts will also be considered in this paper.

MATERIALS AND METHODS

Crystalline 3-phosphoglyceraldehyde dehydrogenase from rabbit muscle, 14C-p-nitrophenyl acetate, and 14C-acetyl phosphate were prepared as described in a previous paper (2). The various reagents used in these experiments and the analytical procedures are also detailed in prior publications (2, 3, 6) or in the text.

RESULTS

Effects of DPN and pH on Acetylation and Carboxymethylation of 3-Phosphoglyceraldehyde Dehydrogenase

The data in Table I show the effect of DPN on the acetylation and carboxymethylation of the dehydrogenase. At pH 4.6, acetyl phosphate reacts with the enzyme, whereas p-nitrophenyl acetate is inert. DPN facilitates the acetylation with acetyl phosphate. At pH 7.0 and 8.0, however, the DPN-free enzyme...
can be acetylated with both substrates. DPN inhibits rather than facilitates the acetylation reaction for reasons which are given in Tables II and III above. At pH 8.5, with p-nitrophenyl acetate, considerably more 14C-acetyl groups are bound and there is partial labeling even in the presence of DPN.

The carboxymethylation with iodoacetic acid has a different pH profile than either of the substrates. At pH 4.6, carboxymethylation is slow unless DPN is present. In contrast to the substrates, at pH 7.0 and 8.5 the extent of carboxymethylation is the same in the absence or presence of DPN. Rate studies have shown that the coenzyme facilitates carboxymethylation.1

The products of the acetylation reaction were determined by examining the radioactive peptides obtained after pepsin digestion of the labeled enzyme and electrophoresis at pH 3.5. The distribution of the radioactive peptides was the same whether the DPN-free dehydrogenase was labeled with p-nitrophenyl acetate at pH 7.0 or acetyl phosphate at pH 4.5 (Fig. 1, Panel 1 and 2, bands S1 to S7). These bands, which are mainly peracid labile, were shown in previous studies (3) to be peptides derived from the octapeptide which contains a 14C-S-acetyl group at position 8

\[
14COCH_3\]

\[
\text{Lys-Leu-Val-Ser-Asn-Ala-Ser-Cys-Thr}\]

When the enzyme free of DPN was labeled with 14C-acetyl phosphate at pH 7.0 and room temperature, there appeared three performic acid-stable bands (Fig. 1, Panel 3, bands N1, N2, N3) in which the acetyl group was attached to the ε-amino group of lysine (4). On addition of DPN, the enzyme was rapidly deacetylated at pH 7.0 and neither N-acetyl nor S-acetyl bands could be detected (Fig. 1, Panel 4). The S-acetyl protein was stable, however, in the presence of DPN at pH 4.5, conditions under which the enzyme cannot catalyze transacylase reactions (Fig. 1, Panel 5).

When the enzyme was acetylated with acetyl phosphate at pH 7.0 with subsequent adjustment to 8.5, N-acetyl groups were obtained predominantly (Fig. 2, Panel 1 compared with Panel 2). High yields of N acetylated enzyme were also obtained when the dehydrogenase was directly acetylated at pH 8.5 without prior pH adjustments. The pattern obtained with 14C-p-nitrophenyl acetate at pH 8.5 is somewhat different (Fig. 2, compare Panels 2 and 3). At the higher pH and 0°, p-nitrophenyl acetate acetylates both cysteine and lysine moieties. This accounts for the increase in 5.6 in the number of acetyl groups bound. The acetylation of the cysteine moiety with p-nitrophenyl acetate at 0° is faster than with acetyl phosphate at room temperature (Table I); however, the formation of the N-acetyl bonds is not as complete with p-nitrophenyl acetate (Fig. 2, Panels 2 and 3). The reduction in the amount of N-acetylation with p-nitrophenyl acetate as substrate is a consequence of the lower temperature which decreases the rate of the S-N transfer.

Close examination of Panel 3, Fig. 2, shows a marked intensity of radioactivity in the area of the S1 band. This 14C-acetyl group is in thioester linkage as indicated by its performic acid lability. The sequence of a heptapeptide containing the reactive cysteine shows that it is neither cysteine number 8 nor 12 in the active center peptide (7, 8)

1 J. H. Park and B. P. Meriwether, unpublished experiments.
Fig. 1. $^{14}$C-Acetyl peptides produced by pepsin digestion of $^{14}$C-acetyl enzymes prepared at pH 7.0 and 4.6. Panel 1, the charcoal-treated DPN-free enzyme was incubated with $^{14}$C-p-nitrophenyl acetate (PNPA) (molar ratio, PNPA-enzyme = 20) for 15 min at 0°, pH 7.0, as specified in Table I. Three moles of acetyl groups were bound per mole of enzyme. Panels 2 and 3, the dehydrogenase was treated with acetyl phosphate (AcP) (molar ratio, AcP-enzyme = 40) for 1 hour at room temperature, pH 4.6 or 7.0, under conditions described in Table I. The number of $^{14}$C-acetyl groups bound per mole of enzyme were 1.2 and 2.5, respectively. Panels 4 and 5, a similar experiment was carried out with acetyl phosphate and the crystalline enzyme-DPN$_1$ complex to which 10 eq of DPN were added. The number of moles of acetyl groups bound per mole of enzyme were 2.5 and 0 at pH 4.6 and 7.0, respectively. The pepsin digestion of the $^{14}$C-acetyl enzymes and the procedures for electrophoresis and radioautography are described in the preceding paper (3). The S-acetyl peptides are designated as S1 to S7 and the N-acetyl peptides as N1 to N3.

Inorganic phosphate in the absence of DPN has no significant effect on the acylation or deacylation reaction (Table II and Fig. 3). However, a combination of DPN and PO$_4$ accelerates deacylation (Table II). This result is a confirmation of previous findings that DPN is essential for the transfer of the acetyl group to phosphate (10, 11).

2. Acetyl Phosphate—With this substrate, DPN does not inhibit acetylation but accelerates deacylation of the S-acetyl enzyme. It is technically more complicated to show the effects of DPN on the acylation and deacylation reactions with acetyl phosphate as the substrate than with p-nitrophenyl acetate.

Examined by adding the coenzyme to preformed $^{14}$C-acetyl enzyme (Table II, column 3). The number of bound $^{14}$C-acetyl groups decreased only slightly from 2.0 to 2.6. This loss is about the same as the breakdown or turnover of the S-acetyl enzyme which can be calculated from the linear portion of the curve in Fig. 3. Thus, DPN does not affect the deacylation of the enzyme, but prevents the reacylation of the enzyme in the reaction mixture.

Inorganic phosphate in the absence of DPN has no significant effect on the acylation or deacylation reaction (Table II and Fig. 3). However, a combination of DPN and PO$_4$ accelerates deacylation (Table II). This result is a confirmation of previous findings that DPN is required for the transfer of the acetyl group to phosphate (10, 11).

The first line of Table III shows the control values of 2.0 moles of DPN on the acylation and deacylation reactions with acetyl phosphate as the substrate than with p-nitrophenyl acetate. First, the effects of DPN alone could not be tested because inorganic phosphate is always present in the reaction mixture as a contaminant of the $^{14}$C-acetyl phosphate and as a reaction product of the acetylation. The phosphate contamination is about equivalent to the amount added in the p-nitrophenyl acetate experiments of Table II. Second, there is no convenient spectrophotometric method for measuring acylation. Consequently, one must look at the overall disappearance of acetyl phosphate and infer the extent of acetylation by the hydroxamate method (12). Third, the dehydrogenase has a low phosphatase activity (13) which, at the high enzyme concentrations used in these experiments, causes some hydrolysis of acetyl phosphate. Insofar as possible, these factors are taken into account in the experiments described in Table III.

The first line of Table III shows the control values of 2.0 moles...
of 14C-acetyl groups bound per mole of enzyme for the 30- or 40-min periods of acylation. Under the given conditions, about 80% of the acetyl groups are in thioester linkage with cysteine residues. During the incubation, 1.2 μmoles of acetyl phosphate disappeared.

In the presence of DPN there is apparently a minimal acetylation of 0.3 mole of 14C-acetyl groups bound per mole of enzyme. As indicated in the next column, the addition of DPN to the preformed 14C-acetyl enzyme complex promotes the deacylation of complex in a transfer of the acetyl group to inorganic phosphate. There is an overall disappearance of 3.2 μmoles of acetyl phosphate. The increased disappearance of acetyl phosphate is due in part to the DPN-dependent acetyl phosphatase activity, mentioned above, and could be inhibited by the addition of cyanide (13). Clearly, cyanide (0.002 M) does not effect the acylation or deacylation of the enzyme. With a combination of CN and DPN, the acylation and deacylation values, when measured as bound 14C-acetyl groups, are the same as those with

Table II

<table>
<thead>
<tr>
<th>Additions</th>
<th>[14C-acetyl] groups bound per mole of enzyme</th>
<th>Spectrophotometric determination of bound acetyl residues</th>
<th>Acetylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PO₄</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPN + PO₄</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The next set of experiments on the arsenolysis of acetyl phosphate (Table III) definitely shows that DPN does not inhibit acylation but rather accelerates deacylation. Low concentrations of arsenate (2.0 μmoles) did not significantly affect the acylation or deacylation of the enzyme. However, with arsenate and DPN there was no apparent acylation and definite deacylation of the preformed 14C-acetyl enzyme complex. The acetyl phosphate (4.0 μmoles) was completely hydrolyzed during the incubation. In order to rule out the possibility of phosphatase activity, the same experiment was performed in the presence of cyanide, and the results were identical. In the absence of any measurable acylation there is still a total disappearance of the added acetyl phosphate. Since the possibility of phosphatase activity is ruled out by cyanide the only explanation for the disappearance of the acetyl phosphate is the rapid formation of the acetyl enzyme followed by an irreversible arsenolysis reaction. There is, therefore, a marked difference between the acetyl phosphate acetylation which occurs in the presence or absence of DPN and the p-nitrophenyl acetate acetylation which is strongly inhibited by DPN.
enzymes were prepared with acetyl phosphate at pH values from 4.6 to 9.0. The ^14C-acetyl phosphate (0.004 m) was selected so that the acetylation was predominantly S-acetyl in the lower pH range and N-acetyl in the higher pH range. An

### Comparative Reactivity of ^14C-S-Acetyl and ^14C-N-Acetyl Groups

1. **Arsenolysis and Phosphorolysis**—In contrast to the S-acetyl groups, the lysine-bound N-acetyl moiety does not appear to participate in acetyl transfer reactions (Table IV). ^14C acetyl groups were measured as described above.

| Acetyl enzyme complex—An S-acetyl protein was formed by incubating the DPN-free enzyme (0.1 pmole) with ^14C-acetyl phosphate (4.0 pmoles) for 30 min at room temperature, pH 7.0. The additions were then made and mixed for 10 min. The reaction was then terminated and bound acetyl groups were measured as described above.

| Measurement of combined processes of acetylation and deacetylation—The determination of the disappearance of acetyl phosphate under appropriate conditions represents the acetylation-deacetylation of the enzyme. For this determination the DPN-free dehydrogenase (0.1 pmole) was incubated for 10 min with compounds listed in the table. Acetyl phosphate (4.0 pmoles) was then added to make a total volume of 1.5 ml. After 30 min, 1 ml of 2 N hydrazine, pH 6.4, was added to convert the remaining acetyl phosphate to acetyl hydroxamate. Ten minutes later, 1 ml of FeCl₃, Na hydroxyacetone, was added, the precipitate was centrifuged, and the supernatant was read in the Klett colorimeter (12). Suitable zero time blanks were run with the dehydrogenase and the various reagents of this experiment.

<table>
<thead>
<tr>
<th>Type of reaction and additions</th>
<th>^14C-acetyl bound per mole of enzyme</th>
<th>Disappearance of acetyl phosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acylation</td>
<td>Decylation</td>
</tr>
<tr>
<td>Phosphorolysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>DPN</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>CN</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>DPN + CN</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Arselenolysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AsO₄</td>
<td>1.5</td>
<td>1.6</td>
</tr>
<tr>
<td>DPN + AsO₄</td>
<td>0.0</td>
<td>0.2</td>
</tr>
<tr>
<td>DPN + AsO₄ + CN</td>
<td>0.0</td>
<td>0.4</td>
</tr>
<tr>
<td>Iodoacetic acid inhibition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IAA</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>IAA + CN</td>
<td>0.1</td>
<td>0.2</td>
</tr>
</tbody>
</table>

### Table IV

**Effect of pH on formation of ^14C-acetyl enzyme complex and its susceptibility to arsenolysis**

The charcoal-treated, DPN-free dehydrogenase (0.1 pmole) was incubated with ^14C-acetyl phosphate (4.0 pmoles) in a volume of 1.0 ml at the designated pH for 20 min. The acetyl phosphate concentration and incubation time were selected so that the acetylation at pH 7.0 would be predominantly on the cysteine. Arsenolysis was then induced by the addition of DPN (1.0 pmole) and arsenate (2.0 pmole). Phosphorolysis took place on addition of DPN because of the inorganic phosphate present as a contaminant of ^14C-acetyl phosphate (approximately 0.8 pmole). Ten minutes later the protein was precipitated, and the specific activity of the labeled enzyme was determined (2).
residues. The addition of DPN and arsenate removes 60% of the \(^{14}C\)-label by arsenolysis of the \(S\)-acetyl bonds, and thereby lowers the value of the enzyme-bound \(^{14}C\)-acetyl groups to 1.47. The radioautograph (Fig. 4, Panels 5 and 6) shows that the thioester bond was susceptible to arsenolysis but the \(N\)-acetylated site was unaffected. At pH 8.5 the control enzyme bound 3.43 moles of \(^{14}C\)-acetyl groups which could not be split from the enzyme by arsenolysis (Table VI). The stability of the \(^{14}C\)-N-acetyl group in the presence of DPN and AsO\(_4\) is shown in Fig. 4, Panels 1 and 2. When the enzyme is acetylated at pH 8.5 and then the pH lowered to 7.0, there is a small increase in the number of bound \(^{14}C\)-acetyl groups to a value of 4.2. The extra \(^{14}C\)-label was predominantly on the cysteine site since the added radioactivity could be removed by arsenolysis. The increase in \(S\)-acetyl peptides after the pH adjustment is verified in Panel 3 of the radioautograph. Upon addition of DPN and AsO\(_4\), the \(S\)-acetyl peptides disappear, and only the \(N\)-acetyl bands remain (Panel 2). These data indicate that very little, if any, of the \(N\)-acetyl group was transferred back to the cysteine moiety since there is no diminution in the amount of \(N\)-acetyl peptides as the pH is lowered.

When the pH is lowered from 8.5 to 4.6 there is a significant increase in the specific activity of the enzyme, and 5.25 to 5.40 moles of \(^{14}C\)-acetyl groups are bound to the enzyme. Since the enzyme is inactive as a transferase at pH 4.6, the location of the acetyl groups cannot be ascertained by arsenolysis. However, the radioautograph indicates a substantial increase in the amount of \(^{14}C\)-S-acetyl peptides (Fig. 5, Panels 3 and 4) with no apparent increase in the \(^{14}C\)-N-acetyl peptides (Panel 1).

**Table V**

<table>
<thead>
<tr>
<th>Substrate added</th>
<th>(^{14}C)-acetyl groups bound per mole of enzyme</th>
<th>Dilution of enzyme-bound (^{14}C)-acetyl groups</th>
<th>Theoretically possible dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>ph 7.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(^{14}C)-acetyl phosphate (10 µmoles)</td>
<td>3.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(^{14}C)-acetyl phosphate (30 µmoles) plus (^{14}C)-acetyl phosphate (30 µmoles)</td>
<td>2.1 47 75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ph 8.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(^{14}C)-acetyl phosphate (10 µmoles)</td>
<td>3.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(^{14}C)-acetyl phosphate (30 µmoles) plus (^{14}C)-acetyl phosphate (30 µmoles)</td>
<td>3.3 12 75</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In Table VI, acetylation values as high as 5.25 and 5.45 moles of acetyl groups per mole of enzyme obtained when the pH was adjusted from 8.5 to 4.6. These values are significantly greater than the maximal number of active sulfhydryl sites which has been estimated as 4.9 for the tetramer on the basis of DPN binding (14, 15), acetylation (2), and physical studies (16, 17). The radioautographs of Fig. 5, Panels 3 and 4, confirm that acetyl phosphate under these particular conditions can label the cysteine and lysine moiety in one monomer (5). In the enzyme labeled with 5.25 or 5.45 acetyl groups (Panels 3 and 4) all the peptides can be identified as \(S\)-acetyl peptides from the active center (Panel 5) or the known \(N\)-acetyl peptides (Panel 1). Similar results have also been obtained by adjusting the pH from 8.5 to 7.0, but it is more difficult to produce the dual acetylation at 7.0 than at 4.5 (Fig. 4, Panel 3). At pH 8.5 and 0\(^{\circ}\)C, p-nitrophenyl acetate was also shown to acetylate 5.6 to 7.0 sites on the enzyme (Table I). However, p-nitrophenyl acetate is a strong acetylating reagent and can label cysteine residues outside the active site (7, 8). The specificity of this labeling is presently under investigation.

**DISCUSSION**

The acetylation and deacetylation reactions of the enzyme with p-nitrophenyl acetate and acetyl phosphate have an interesting
E-NHAc is the N-acetyl enzyme complex, PNPA is p-nitrophenyl acetate, and AcP is acetyl phosphate.

The reactions with both substrates are briefly summarized below, where ESH, is a monomer of the DPN free dehydrogenase with reduced sulfhydryl groups, ESAc is the S-acetyl enzyme complex, E-\textit{c}-NHAc is the \textit{N}-acetyl enzyme complex, PNPA is \textit{p}-nitrophenyl acetate, and AcP is acetyl phosphate.

**Reaction Occurring at pH 4.5**

\begin{align*}
\text{PNPA} + \text{ESH} & \rightarrow \text{No acetylation (1)} \\
\text{AcP} + \text{ESH} & \rightarrow \text{ESAc (DPN)} + \text{No deacylation (2)} \\
\text{AcP} + \text{ESH} (\text{DPN}) & \rightarrow \text{ESAc (DPN)} + \text{No deacylation (3)} \\
\text{AcP} + \text{ESH} (\text{DPN}) + \text{AsO}_4 & \rightarrow \text{ESAc (DPN)} + \text{No deacylation (4)}
\end{align*}

**Reaction Occurring at pH 7.0**

\begin{align*}
\text{PNPA} + \text{ESH} & \rightarrow \text{ESAc} + \text{DPN} \text{ (5)} \\
\text{AcP} + \text{ESH} & \rightarrow \text{ESAc (DPN)} + \text{Acetate (6)} \\
\text{PNPA} + \text{ESH} (\text{DPN}) & \rightarrow \text{No acetylation (7)} \\
\text{AcP} + \text{ESH} (\text{DPN}) & \rightarrow \text{ESAc (DPN)} + \text{AsO}_4 \rightarrow \text{Acetate (8)} \\
\text{AcP} + \text{ESH} (\text{DPN}) & \rightarrow \text{ESAc (DPN)} + \text{Pi (9)}
\end{align*}

**Reaction Occurring at pH 8.5**

\begin{align*}
\text{PNPA} + \text{ESH} & \rightarrow \text{ESAc} \rightarrow \text{E-\textit{c}-NHAc (10)} \\
\text{AcP} + \text{ESH} & \rightarrow \text{ESAc (DPN)} + \text{Pi} \rightarrow \text{No N-acetylation (11)} \\
\text{AcP} + \text{ESH} (\text{DPN}) & \rightarrow \text{ESAc (DPN)} + \text{Pi} \rightarrow \text{No N-acetylation (12)}
\end{align*}

The reactions will be discussed in the following order: first, the properties of the S-acetyl enzyme compound which is formed at pH 4.5 and 7.0; second, the characteristics of the N-acetyl enzyme compound prepared at pH 8.5; and third, the correlation of these facts with the catalytic activity of the enzyme.

**S Acetyl Enzyme Complex**

Acetylation at pH 4.5—At low pH, p-nitrophenyl acetate cannot acetylate the reactive cysteine moiety of the dehydrogenase or even cysteine itself. On the other hand, acetyl phosphate forms an acetyl enzyme complex in the absence or presence of DPN. Acetyl phosphate carries one negative charge at pH 4.5, and the attack of the cysteine on the carbonyl carbon is facilitated as follows:

Thus, acetyl phosphate, in accordance with the increasing lability of the phosphate bond at low pH, acetylates the enzyme.

Acetylation at pH 7.0—At neutrality and 0°, p-nitrophenyl acetate acetylates three to four sites on the enzyme, whereas acetyl phosphate at the same concentration labels approximately one site. At neutrality DPN prevents the isolation of the S-acetyl enzyme complex (Tables II and III). In the case of p-nitrophenyl acetate, DPN inhibits the formation of the acetyl enzyme complex. DPN may sterically inhibit the acetylation with p-nitrophenyl acetate because the active center cannot spatially accommodate both the large p-nitrophenyl group and the coenzyme. With acetyl phosphate, the DPN does not act as an inhibitor of the acetylation reaction, but rather as a cofactor which facilitates a rapid deacylation reaction by transferring the acetyl group from the enzyme to the acceptor, inorganic phosphate. In considering the general problem of glucose utilisation via the Embden-Meyerhof cycle, it seems reasonable that the DPN, which is more tightly bound to the dehydrogenase than DPNH (18), should promote the release of the acyl phosphates from the enzyme, and thereby accelerate the oxidation of the aldehyde substrates. It is difficult to test the effect of DPNH on deacylation because it is oxidised to DPN by acetyl phosphate (1) or acetyl enzyme (19).

**Transfer Reactions of S-Acetyl Enzyme**—The transfer reactions of the common S-acetyl enzyme intermediate have been studied at pH 4.5 and 7.0. At the lower pH, the S-acetyl group cannot be transferred to either phosphate or arsenate in the presence of DPN. This may be due to the fact that the required DPN is not bound to the enzyme in the same manner at pH 4.5 and 7.0 (20) or that the postulated labilising imidazole groups in the active center are positively charged (21). However, at pH 7.0 the combination of DPN and phosphate promoted an exchange reaction in which the acetyl group is transferred to the acceptor, inorganic phosphate or arsenate. The data are consistent with the earlier findings that DPN is required for the 32P exchange and arsenolysis reactions with acetyl phosphate and 1,3-diphosphoglyceric acid (11, 22). A DPN-independent transfer reaction has only been observed at very high arsenate concentrations (6).

**Stability of S-Acetyl Enzyme**—The thioester bond of the S-acetyl enzyme complex is far less stable than that of S-acetyl glutathione or S-acetyl coenzyme A. These model thioesters are stable for 30 min in 0.1 M Tris, pH 7.0, at room temperature. The S-acetyl enzyme, like N-acetyl imidazole, is 30% hydrolyzed under these conditions. When the S-acetyl dehydrogenase is denatured with acetone, the S-acetyl bond acquires the typical stability of thioester bonds, suggesting that labilising groups have been removed from the active center. An interaction between cysteine and the imidazole ring of histidine has been proposed by Olson and Park to explain the reactivity of the thioester bond in the dehydrogenase (21). The interaction of these two residues in promoting the hydrolysis of p-nitrophenyl acetate is shown in Fig. 6. The same type of mechanism has also been applied to transfer reactions with acetyl phosphate (21).

Owing to the instability of the S-acetyl enzyme, there is a slow...
FIG. 4. The effect of a pH adjustment from 8.5 to 7.0 on the N-acetylated enzyme. The $^{14}C$-acetyl enzymes which were used for this radioautograph were selected directly from the experiment of Table VI. The procedures for acetylating the enzymes with acetyl phosphate ($AcP$), adjusting the pH, and adding the DPN and arsenate to the $^{14}C$-acetyl enzymes are explained in the legend of Table VI. The pepsin digestion of the labeled enzymes and the electrophoresis was carried out as described in Table I and Fig. 1. The S-acetyl peptides are designated as S1 to S7 and the N-acetyl peptides as N1 to N3. The significance of the peptide patterns is explained in the text. The procedures for the pepsin digestions and isoelectrophoresis at pH 3.5 are outlined in Table I and Fig. 1.

FIG. 5. The effect of a pH adjustment from 8.5 to 4.6 on the N-acetylated enzyme. The $^{14}C$-acetyl enzymes of this radioautograph are those which were analyzed in Table VI. The methods for acetylating the dehydrogenase with $^{14}C$-acetyl phosphate ($AcP$), adjusting the pH, and carrying out the arsenolysis reactions are explained in the legend of Table VI. The experimental procedures for pepsin digestion of the acetyl enzymes and electrophoresis at pH 3.5 are given in Table I and Fig. 1. The S-acetyl peptides and N-acetyl peptides are labeled S1 to S7 and N1 to N3, respectively.

FIG. 6. Proposed mechanism for the hydrolysis of p-nitrophenyl acetate. At pH 7.0, the formation of a hydrogen bond between the sulfhydryl group and the unchanged imidazole ring of histidine may facilitate the nucleophilic attack of the sulfhydryl group on the positively charged carbonyl carbon of p-nitrophenyl acetate. The subsequent hydrolysis of the S-acetyl enzyme involves a second nucleophilic attack by the imidazole group which is a good nucleophil for thioesters. The hydrolysis of the S-acetyl enzyme could also occur by a general base catalysis. Recent experiments of Drs. Judy Bond and Jane H. Park have shown that photooxidation of histidine residues inhibited the deacylation of the S-acetyl enzyme but did not effect the acetylation of the active site cysteine with p-nitrophenyl acetate.

hydrolysis of acetyl phosphate during the preparation of the acetyl enzyme complex with DPN-free dehydrogenase (Table III). This breakdown of acetyl phosphate should be distinguished from the acetyl phosphatase activity described by Park and Koshland (13). The latter phosphatase activity is DPN-dependent (13) and is observed with catalytic amounts of an oxidized enzyme which is treated with iodosobenzoate (23). Since an S-acetyl enzyme cannot be formed in the presence of
iodosobenzoate or iodoacetate (2, 3, 10), it is clear that there are two routes for the decomposition of acetyl phosphate. On a rate basis, the decomposition of the S-acetyl enzyme is quite slow (0.3 μmole acetyl phosphate hydrolyzed per 1.0 μmole of enzyme per min) and, perhaps, more appropriately related to the facilitation of transfer reactions than to hydrolysis of acyl phosphates (13, 24).

**N-Acetyl Enzyme Complex**

*S-N Transfer Reaction*—The acetylation of the cysteine residue proceeds by a direct nucleophilic attack of the sulfhydryl group on the carbonyl carbon of the acetate moiety (21). The major route for the *N*-acetylation is an *S-N* transfer reaction of the acetyl group from the active cysteine to the lysine (4, 5). This *S*-N transfer reaction occurs more readily at pH 8.5 than at pH 7.0 or 4.5, and has the chemical characteristics of the intramolecular *S-N* transfer observed with the model compounds, S-acetyl-β-mercaptoethylamine and S-acetyl-γ-mercaptoethylamine (25, 26). The rate of the *S*-N transfer reaction on the dehydrogenase is approximately the same as that observed with S-acetyl-γ-mercaptoethylamine (25). The *S*-N transfer reaction of the enzyme indicates that the cysteine and lysine moieties are in close proximity, although not near neighbors on a given peptide chain (3, 4). Harbaum has shown that the cysteine and lysine moieties, which are involved in the *N*-acetylation (27) and *S*-N transfer in the pig dehydrogenase (28), are separated by 33 amino acid residues (29).

**Dual Acetylation of Cysteine and Lysine Residues**—The acetyl enzyme ratio of the complexes formed with acetyl phosphate is rarely greater than 4:1; however, the experiments in Table VI forced the dual labeling of the cysteine and lysine moiety of one monomer (mol wt 35,000) (5). An enzyme was predominately *N*-acetylated at pH 8.5 with 3.6 moles of bound acetyl groups and the pH was then lowered to 7.0 or 4.6 to provide maximum stability for thioester bonds. At pH 7.0 it was difficult to obtain dual labeling; however, at pH 4.6, the *N*-acetyl enzyme was further acetylated on the cysteine moiety as indicated by radioactivity determinations and radioautography. There may be a correlation between this dual labeling of the two sites at pH 4.6 and the fact that this was the only pH at which acetyl phosphate and DPN could be simultaneously bound. At the lower pH the enzyme configuration may be altered so that the *S* and *N* sites are no longer sterically hindered. In fact, Astrachaan et al. (20) have shown that there are differences in the mode of binding of the DPN to the enzyme at pH 7.0 and 4.5. For example, in the lower pH range the enzyme-bound DPN is more accessible to attack by DPNase (20).

Although it is difficult to label simultaneously the *S* and *N* sites with acetyl phosphate, an *N*-acetylated enzyme was readily carboxymethylated with iodoacetic acid at pH 8.5 (5). Carboxymethylation does not remove the *N*-acetyl groups from the lysine. With *p*-nitrophenol acetate it may also be possible to acetylate the two sites in one monomer. Higher acetyl-enzyme ratios of 4:7.1 to 7.0:1 were obtained by labeling the enzyme at pH 8.5 and 6° (Table I). These high ratios along with radioautographs indicate that there is labeling of both the lysine and active site cysteine. However, recent investigations have shown that there is also nonspecific acetylation involving a second *COCH₃* cysteine residue in a sequence Asp-Gln-Val-Val-Cys-Asp.

*Fig. 7.* The correlation of the acetylation and transfer reactions of the dehydrogenase with the catalytic processes. The double dotted line indicates that a given reaction is inhibited by DPN. *AcP*, acetyl phosphate; *PNPA*, *p*-nitrophenyl acetate.
however, could not be displaced by exposure to acetyl phosphate. These observations along with the data in Table I suggest a competition between acetyl phosphate and DPN for the lysine residue. Alternative explanations such as conformational changes in the enzyme are also possible. The unusually high affinity of this dehydrogenase for DPN may protect the enzyme in vivo against inactivation by N-acetylation (4, 28). With the use of the dehydrogenase from pig muscle, Polgar has confirmed the observation that DPN prevents the S-N transfer reaction (29). However, he has not been able to show that N-acetylation effects DPN binding. The failure to show this DPN effect may be due: (a) to species difference in the enzyme; (b) to the incomplete N-acetylation of the pig enzyme which was only 50% acetylated with approximately 2 moles of bound acetyl groups per mole of enzyme; (c) to the fact that the acetyllating substrate, p-nitrophenyl acetate, may show nonspecific labeling at pH 8.0 and room temperature (6, 8); (d) to very specific requirements of the assay techniques for demonstrating the impairment of both DPN binding and dehydrogenase activity of the N-acetylated enzyme, i.e. cysteine concentration.

There are several precedents for the binding of cofactors to the lysine moiety of enzymes. Pyridoxal 5'-phosphate forms a complex with the e-amino group of a lysine residue of the CoA- and DPN-linked pyruvate and α-ketoglutarate dehydrogenation systems (35). Röntgen is firmly bound to the e-amino group of a lysine moiety of three enzymes, propionyl carboxylase (36, 37), oxaloacetate transcarboxylase (38), and β-methylcrotonyl carboxylase (39). Thus, a DPN-lysine interaction appears to be a reasonable proposal for 3-phosphoglyceraldehyde dehydrogenase both on a chemical and biological basis.

Correlations with Enzymatic Activity

The findings of these papers and previous publications (1-3, 7, 10, 11) are shown together in Fig. 7.

The active S-acetyl enzyme complex can be prepared by oxidation of acetaldehyde with DPN or by incubation with acetyl phosphate or p-nitrophenyl acetate. DPN facilitates the acetyl phosphate reaction but inhibits the acetylation reaction with p-nitrophenyl acetate.

The active acetyl enzyme complex, in the presence or absence of DPN, can then be converted to the hypothetical N-acetyl histidyl enzyme (23). In the presence of DPN and acceptor, the acetyl group can be transferred to substrate to form radioactive acetyl phosphate or to arsanate in the arsenolysis reaction which yields acetylenedicarboxylic acid. In the absence of phosphate or arsanate there is a slower hydrolysis of the acetyl enzyme, as indicated by the bottom arrow in Fig. 7.

There are two other possible intraenzymatic transfers. The acetyl group may be transferred by an S-N transfer reaction to the dead end, ε-NH₂-lysine moiety or to an incorrect cysteine moiety by an S-S transfer reaction (6, 8) 1. Both these reactions are prevented by the coenzyme DPN, which has the unexpected function of channeling the movement of the substrate on the enzyme surface. This dehydrogenase is unique among the dehydrogenases in having the DPN bound more tightly than DPNH.

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The Enzymatic Significance of S-Acetylation and N-Acetylation of 3-Phosphoglyceraldehyde Dehydrogenase

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