# The Mechanism of Action of Aldolases
## III. Schiff base formation with lysine[1]

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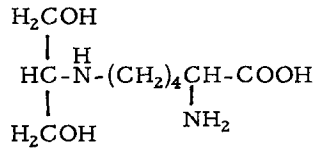
In previous communications (Horecker et al., 1961, Grazi et al., 1962), we have described the stabilization of the transaldolase-dihydroxyacetone and aldolase-dihydroxyacetone phosphate complexes by reduction with borohydride. It was suggested (Grazi et al., 1962) that the linkage of the substrate to the enzyme might be through Schiff base formation with the ε-amino group of lysine. On reduction with borohydride the Schiff base intermediate would be converted to a stable secondary amine. We have now isolated this amine from acid hydrolysates of the reduced protein complexes and have obtained evidence for the following structure:[4]



We may therefore conclude that the active site of aldolase contains lysine, which combines with the carbonyl group of the substrate to

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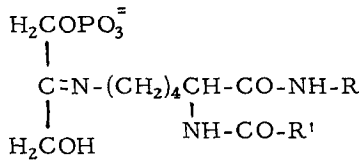
[4] The products obtained from transaldolase and aldolase were found to be indistinguishable by chromatographic analysis and in chemical tests (Horecker et al., 1962).

form an active intermediate having the following structure:



when R and R' represent the groups attached to lysine in the protein chain. This formulation is consistent with the isotope exchange data for aldolase reported by Bloom and Topper (1956), Rose and Rieder (1955, 1958), and Rutter and Ling (1958) and with the spectral changes observed by Topper et al. (1957). The transaldolase complex would have a similar structure but would lack the phosphate group.

It has been shown that primary and secondary amines catalyze dealdolization reactions (Westheimer and Cohen, 1938; Speck and Forist, 1957), and that Schiff base intermediates are possibly involved (Hamilton and Westheimer, 1959). It would appear that the Schiff base mechanism does indeed operate in enzyme-catalyzed aldol condensation and transfer reactions.

The respective complexes with transaldolase and aldolase were prepared and reduced with borohydride as previously described (Horecker et al., 1961; Grazi et al., 1962), using $C^{14}$-fructose 6-phosphate and $C^{14}$-dihydroxyacetone phosphate as substrates. When the radioactive protein complexes were hydrolyzed with 6 N HCl at $110^{\circ}$ for 22 hours, identical radioactive compounds were obtained from the two proteins; these compounds were isolated by paper electrophoresis or chromatography. Parallel experiments with aldolase using $P^{32}$-labeled dihydroxyacetone phosphate revealed that the phosphate group was completely removed during the acid treatment. The details of these procedures and analyses will be reported elsewhere.

In our previous report of the transaldolase-dihydroxyacetone complex (Horecker et al., 1961), we had stated that the product was not susceptible to oxidation by periodate. We have now found that it is attacked by periodate at higher pH (8.6) where secondary amino alcohols such as diethanolamine are oxidized. This class of compounds is only very slowly oxidized at pH 4.5. Oxidation of the $C^{14}$-labeled compound can be followed by isolation of labeled formaldehyde or by measurement of the radioactivity adsorbed to Dowex 50 ($H^+$) columns. Following exposure to periodate at pH 8.6 all of the radioactivity is removed from the amino acid and is no longer adsorbed by this resin (Table I).

To identify the amino acid to which the radioactive group is attached, the reaction mixture before and after treatment with periodate was subjected to electrophoresis at pH 1.8. The results are shown in Fig. 1. Before periodate treatment the samples (I) show faint spots corresponding to glycine, alanine, and serine or valine, which are close to the radioactive area in the first electropherogram (see Table I). There are also strong ninhydrin spots (A and A') just above glycine which coincide with the radioactivity. After periodate oxidation (II), the patterns are similar except that the spots containing radioactivity have disappeared and are replaced by strong ninhydrin positive spots (B and B') corresponding to lysine. These are no longer radioactive. The identification of lysine in the products following treatment with periodate has been confirmed by paper chromatography in 2,4-lutidine saturated with M/50 phosphate buffer, pH 6.2 (McFarren, 1951), by

TABLE I

Adsorption of Radioactivity on Ion Exchange Resin Before and After Treatment with Alkaline Periodate

| | Aldolase Product | | Transaldolase Product | |
|---|---|---|---|---|
| | Before $HIO_4$ | After $HIO_4$ | Before $HIO_4$ | After $HIO_4$ |
| | cpm | cpm | cpm | cpm |
| Placed on column | 8,300 | 16,000 | 2,240 | 244,000 |
| Unadsorbed | 2,000* | 16,000 | 0 | 209,000 |
| Eluted with 1 M $NH_4OH$ | 6,300 | 0 | 2,140 | 24,600 |

*This column was overloaded.

The radioactive complexes were isolated from the acid hydrolysates by paper electrophoresis. In the case of aldolase, hydrolysate from 18 mg of protein (58,000 cpm) was placed in a 13 cm band on Whatman 3 MM paper and subjected to electrophoresis at pH 9.7 and 3,600 volts for 2 hours. The radioactivity was located by autoradiography and eluted with 0.01 N HCl. Of the 30,000 cpm eluted one-third was tested directly and two-thirds was treated at pH 8.6 with periodate. Excess periodate was destroyed with glycerol before this aliquot was tested on the Dowex 50 $H^+$ column (4 cm x 0.7 cm).

In the case of transaldolase, hydrolysate from 12.9 mg of protein (332,000 cpm) was placed in an 8 cm band on 2 strips of Whatman 3 MM paper and subjected to electrophoresis at pH 5.8 and 5,000 volts for 2 hours. The radioactive area was located as before and eluted. A total of 246,000 cpm were recovered.

electrophoresis at pH 5.8, and by bioassay with a lysine-less mutant strain of Escherichia coli.[5/]

It is thus established that the active site of aldolase and of transaldolase contains a lysine residue which reacts with the substrate, dihydroxyacetone phosphate, to form a Schiff base intermediate. It is

[5/] Strain 4-106 derived from E. coli K12 was kindly provided by Dr. Werner Maas of this Department.

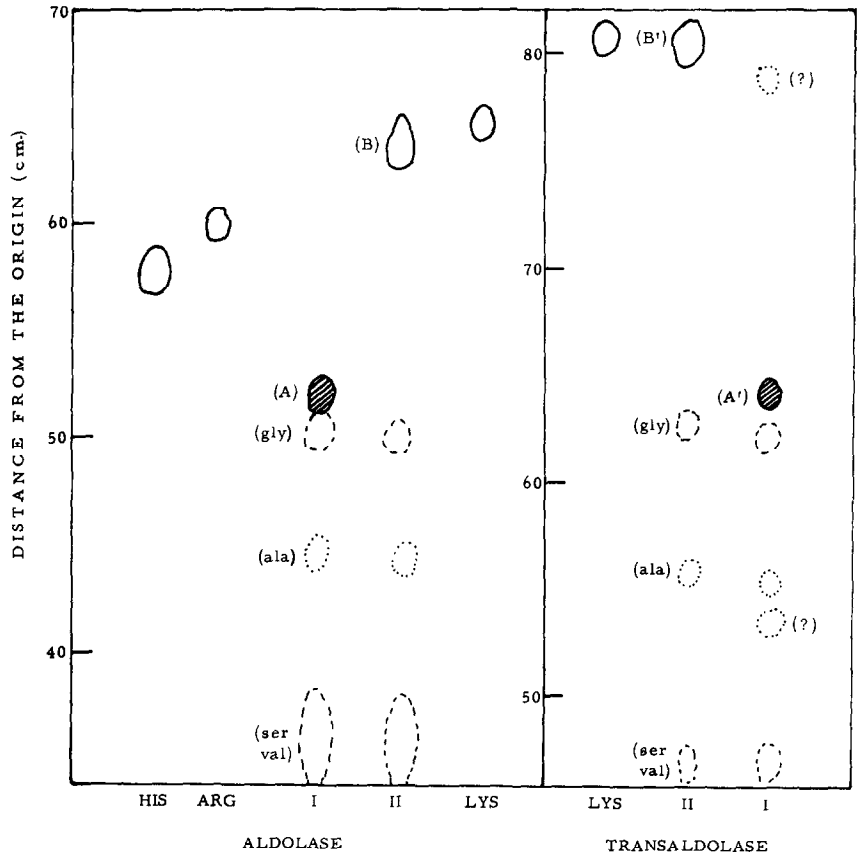



Figure 1. Electrophorogram of the compounds recovered from hydrolysates of aldolase and transaldolase before (I) and after (II) treatment with periodate. The radioactive areas eluted from the electrophoresis runs in Table I were divided into two portions, one of which was treated at pH 8.6 with excess periodate. This was then placed on a Dowex 50 ($H^+$) column (see Table I) and eluted with 1 M $NH_4OH$. The first alkaline fractions, containing the bulk of the ninhydrin reactive material, were concentrated and applied to Whatman 3 MM paper (II).

An equivalent quantity which had not been treated with periodate was similarly adsorbed on Dowex 50 ($H^+$) and eluted and applied to the paper (I). The electrophorogram was run in 4% acetic acid-4% formic acid (pH ~ 2) for 2 hours at 5,000 volts. The paper was sprayed with ninhydrin reagent and the radioactivity located by autoradiography. Lysine, histidine and arginine standards were run as shown.

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probable that this involves the $\varepsilon$-amino group of lysine, since Udenfriend and Velick (1951) have found only proline as N-terminal amino acid in aldolase. Furthermore, experiments carried out by Stark and

Smyth at the Rockefeller Institute[6/] have shown that transaldolase does not contain a terminal amino lysine residue.

Studies are now in progress to determine the sequence of amino acids around lysine in the region of the active site and in particular to provide information on the nature of the differences in activity and substrate specificity exhibited by aldolase and transaldolase.

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6/ G.R. Stark and D.E. Smyth, submitted for publication.