However, the rate of hydrolysis of fluoroacetylcholine alone was 2–3 times that of acetylcholine and, like that of acetylcholine, was suppressed to spontaneous nonenzymatic levels by neostigmine. Thus, the limited duration of the cholinergic effects of both the substituted pyruvic acids and fluoroacetylcholine *in vivo* may be due to hydrolysis of the haloacetylcholines by cholinesterases.

Although the possibility exists that the substituted pyruvic acids form short acting anticholinesterases, it would seem more likely that the cholinergic effects of these compounds are due to their ready utilization by nerve cells and nerve ending as a source of haloacetyl or glycolyl fragments used for the rapid synthesis of substituted acetylcholines, which are then released at the nerve endings. The inactivity of the corresponding two carbon analogs, glycolic, fluoroacetic, and chloroacetic acids suggests that the acetate-activating enzymes at the nerve endings are absent or inactive.

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Mechanism of Action of Aldolase and Phosphotriose Isomerase

The aldolase-catalyzed condensation of dihydroxyacetone phosphate (DHAP) with various aldehydes is an enzymatic equivalent of nonenzymatic condensation reactions which involve labilization of carbon-bound hydrogen situated adjacent (alpha) to carbonyl groups. In alkali-catalyzed reactions of this type the hydrogen is considered to be labilized as a proton from the alpha carbon according to the scheme shown in Eq. 1. In the acid-catalyzed system hydrogen is thought to be labilized as a proton by the enhanced positive character of the carbonyl carbon (Eq. 2), where X^+ may be in any one of a variety of electrophilic reagents. It will be noted that in both formulations the activation consists in the formation of a resonance system consisting of carbanion and enol (C⟷E).

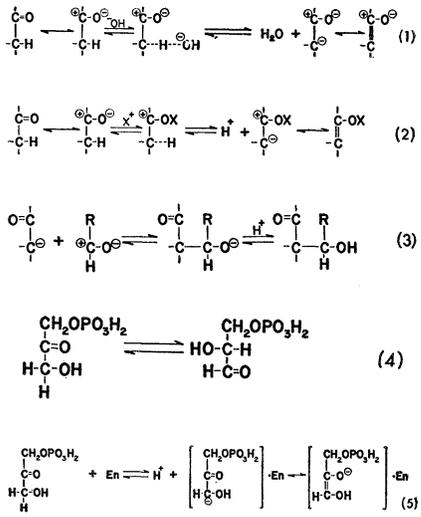
Subsequent reaction of C⟷E with the electrophilic carbonyl carbon of the aldehyde establishes a new carbon-carbon bond (Eq. 3).

Another metabolic reaction of DHAP consists in its transformation into D-glyceraldehyde-3-phosphate as catalyzed by phosphotriose isomerase (Eq. 4).

Details of the mechanism of these enzyme-catalyzed reactions have been sought through the following inquiries. (i) Is alpha hydrogen of DHAP labilized by aldolase in the absence of acceptor aldehyde? (ii) Is aldolase stereospecific with respect to the two hydrogen atoms residing on the carbon bearing the primary hydroxyl group? (iii) Is the polarization of the carbonyl group of the aldehyde intensified by aldolase? (iv) Does the triose-isomerase reaction involve the migration of a hydride ion or activation of a proton (*1*)? (v) Is phosphotriose isomerase stereospecific with respect to the two hydrogen atoms residing on the carbon bearing the primary hydroxyl group?

While these investigations were in progress preliminary reports (*2, 3*) by Rose and Rieder related to some of these inquiries have appeared.

The observation (*2*, and Table 1) that approximately one atom of carbon-bound hydrogen per mole of DHAP exchanges with the aqueous solvent on incubation with aldolase in the absence of acceptor aldehyde suggests that the mechanism shown in Eq. 5 is operative. It is thus apparent that the over-all condensation reaction can be broken down into at least two steps—namely, C⟷E formation from DHAP (Eq. 5) followed by the formation of a new carbon-carbon bond (Eq. 3). In other words, the presence of aldehyde on the enzyme surface is not a prerequisite for the labilization of alpha hydrogen in DHAP. Rose and Rieder (*2*) suggest a mechanism wherein the enzyme functions in a manner comparable to that described for hydroxyl ion catalysis. It should, however, be pointed out that the protonated enzyme could not dissociate from the C ⟷ E, since otherwise a loss of the stereospecific character of the reaction would result (see later). Other alternatives that should be considered are (i) that the functional center on the enzyme responsible for the activation of DHAP is an electrophilic site which enhances the polarization of the carbonyl group (Eq. 2), (ii) that *both* nucleophilic and electrophilic sites on the enzyme, in a "concerted" fashion, are involved in the activation of the substrate. The latter concept, that of



the enzyme acting as a polyfunctional catalyst, has been put forward by Swain and Brown (*4*).

The formation of a new carbon-carbon bond in the aldolase reaction is a consequence of electron sharing between C ⟷ E derived from DHAP and the electrophilic carbonyl-carbon of the aldehyde. In spite of the fact that this reaction is quite nonspecific with respect to the aldehyde moiety, it was conceivable that the condensation is promoted by enzymatic enhancement of the polarization of the carbonyl group. Such activation of the aldehyde, insofar as it would lead to increased positive character of the carbonyl-carbon, might be expected to result in labilization of alpha hydrogen. How-

Table 1. Incorporation of carbon-bound tritium into dihydroxyacetone phosphate.*

Expt. No.	Enzyme	Tritium uptake (μatoms/μmole DHAP)
1a	Boiled aldolase	0.05
1b	Active aldolase	1.04
2a	Boiled aldolase	0.07
2b	Active aldolase	0.72
3a	Boiled isomerase	0.42
3b	Active isomerase	0.95

* Incubation conditions were as follows. The reaction mixtures contained per milliliter: from 3.56 to 6.25 μmoles of DHAP (*5*), 5 mg of crystalline aldolase free of phosphotriose isomerase (*6*) or 3.3 mg of crystalline phosphotriose isomerase free of aldolase (*7*), 50 μmoles of triethanolamine-HCl buffer, *p*H 7.0, 0.5 mc of tritium oxide. Incubation was at room temperature for 30 minutes; the reaction was stopped by freezing. The residue obtained after lyophilization was treated with absolute ethanol in order to inactivate the enzyme. Following removal of the alcohol the remaining exchangeable tritium was eliminated by repeated addition and removal of water. The final residue was taken up in 10 ml of 1/19/80 H_2O, absolute alcohol, and 400 mg percent diphenyloxazole in toluene and assayed for tritium by scintillation counting.

ever, when 0.27*M* acetaldehyde in tritium oxide was incubated for 30 minutes at room temperature in the presence of aldolase, no tritium was detected in the isolated dimedon derivative. This finding, taken in conjunction with the observed failure of fructose-1,6-diphosphate to incorporate tritium in the presence of aldolase (*2*), indicates that the enzyme does not, either in the presence or absence of DHAP, significantly exalt the polarization of the aldehyde carbonyl function. From the foregoing considerations it appears that the formation of C ⟷ E from DHAP is the sole detectable activating function of aldolase.

The observation has been made (*1*) that when fructose-6-phosphate is incubated with phosphoglucose isomerase in deuterium oxide the product, glucose-6-phosphate, contains one atom of deuterium per mole. This finding reveals that the isomerization is mediated by labilization of a proton leading to C ⟷ E formation rather than through a hydride ion migration. The data in Table 1 and results reported recently (*3*) demonstrate that approximately one atom of tritium is incorporated per mole of DHAP incubated with phosphotriose isomerase. Thus it would appear that the mechanism of isomerization of triosephosphate (Eq. 4) is identical with that of hexose phosphate. The same stereochemical relationships as described for the hexose isomerase reactions (*1*) would apply here; that is, either the *cis* or the *trans* enediol, but not both, is the intermediate in the interconversion of DHAP and D-glyceraldehyde-3-phosphate. The formation of L-glyceraldehyde-3-phosphate, if it occurs, would presumably involve the other enediol.

Mechanistically the aldolase and phosphotriose isomerase reactions have two features in common; that is, both involve C ⟷ E formation and both activate only one hydrogen atom per mole of substrate. The latter observation suggests that both enzymes are capable of distinguishing between the two hydrogen atoms on the primary carbinol. It was of further interest to determine whether the hydrogen which is labilized by aldolase is the same as that labilized by phosphotriose isomerase. When DHAP containing carbon-bound tritium, introduced by the action of aldolase in tritium oxide, was again subjected to the action of aldolase in hydrogen oxide a complete loss of tritium occurred. A similar incubation of labeled substrate with phosphotriose isomerase resulted in no significant loss of tritium. These results and those previously reported (*3*) clearly show that each enzyme acts on a different alpha hydrogen. Here then, are two more examples of substrate-enzyme relationships in which the Ogston concept applies; that is, at least three sites are implicated in complex formation. Two of these sites must involve the hydroxyl and one of the hydrogen atoms of the carbinol.

As is pointed out in a foregoing paragraph, C ⟷ E formation is a common feature of the aldolase and phosphotriose isomerase reactions. This finding, taken together with the observation related to the stereospecific character of these enzymes suggests that in the one case a *cis*-enediol is involved, while in the other a *trans*-enediol is implicated (*1*).

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Plasma 17-Hydroxycorticosteroid Changes Related to Reserpine Effects on Emotional Behavior

Recent studies of adrenal-pituitary activity and behavior have shown that marked elevations of plasma 17-hydroxycorticosteroid (17-OH-CS) levels can be selectively related to conditioned emotional response patterns of the "fear" or "anxiety" type in laboratory animals (*1*). Disruption of lever-pressing behavior for a liquid reward by thirsty monkeys in response to the presentation of an auditory stimulus that had previously been paired with an electric shock was found to be accompanied by plasma 17-OH-CS increase at the rate of 20 to 25 μg percent per hour. A closely related series of experiments has also shown that chronic administration of reserpine can markedly attenuate the suppressing effect of the conditioned anxiety stimulus on lever-pressing behavior (*2*). This series prompted a further analysis of the interrelationships between such drug activity and the adrenalcortical response to emotional conditioning.

Rhesus monkeys that had been deprived of solid food and liquids for 24 hours or more were trained to press a bar for a reward of sugared orange juice. When the response rates had stabilized, the conditioned emotional response was superimposed on the lever-pressing behavior by repeated presentation of a 5-minute clicking noise that was terminated contiguously with a painful electric shock to the feet. After a few such pairings, the anxiety response appeared as a disruption of the stable lever-pressing pattern, accompanied by crouching, trembling, piloerection, and frequently urination and defecation.

Experimental sessions lasting 1 hour consisted of six such 5-minute clicker presentations with 5-minute periods of no clicker between each trial. Blood samples were drawn before and after each session, and the plasma was analyzed for 17-OH-CS concentrations by the Nelson-Samuels method (*3, 4*). Base lines were determined for plasma 17-OH-CS responsiveness to the anxiety stimulus alone and to control procedures including lever-pressing alone after conditioning. Figure 1 shows the selective increases in steroid output related to the anxiety conditioning situation as compared with the control procedures.

Following several such base-line determinations and unequivocal establishment of the relationship between plasma corticosteroid increase and the emotional behavior, daily intramuscular doses of reserpine (0.75 mg/kg) were administered 20 to 22 hours before the time of the experimental sessions (*5*). Figure 2 shows the effects of these treatments on the behavioral response to the conditioned anxiety stimulus and on the corticosteroid response during such experimental sessions for one monkey. The strength of the anxiety response is indicated on the left ordinate of the curve in terms of "inflection ratios" (*6*), a

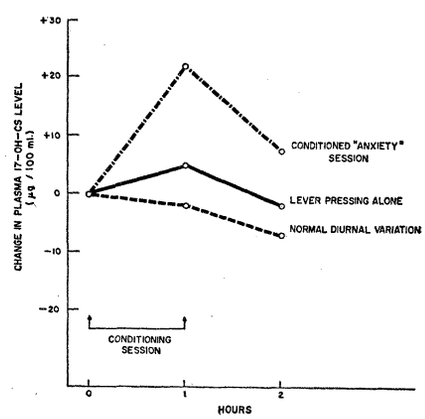

Fig. 1. Plasma 17-hydroxycorticosteroid levels during anxiety conditioning sessions compared with control levels during lever-pressing sessions without the emotional stimulus and compared with normal diurnal levels.