to reach a volume of 0.2 cm^3 was plotted on a probability scale and compared with the curves of the treated groups, it was found that the curves were almost parallel. The curves of the two treated groups overlapped. The mean time for the tumors in the treated animals to reach this volume was again about double that of the controls.

The gamma globulin appears to act in one of several ways. By itself, in the dose used, it has no effect on tumor growth, although larger doses have a stimulating effect on the growth. When it is given in conjunction with guinea pig serum, it may suppress the growth of the tumor so that it does not appear at all, or it may have no added effect on the inhibitory action of guinea pig serum. Here also there is an optimum dose. The course taken is probably dependent on the tumor-host relationship.

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Cholinergic Effects of β-Substituted Derivatives of Pyruvic Acid

As part of a continuing search for inhibitors of lactic dehydrogenase, fluoropyruvic acid (*1*) was studied *in vitro* in systems containing lactic dehydrogenase and then *in vivo* in rats (*2, 3*). Within 5 minutes after intraperitoneal administration of 150 mg/kg of fluoropyruvic acid, rats exhibited sialorrhea, lacrimation, and rhinorrhea, accompanied by wheezing and audible rhonchi. These effects were followed by lethargy, head drop, a decreased sensitivity to pain, occasional clonic movements, hyperpnea, labored breathing, and ultimately death of the animals in periods ranging from 30 minutes to 12 hours. In doses of 1 mg/kg, atropine suppressed the muscarinic effects of these compounds.

The specificity of the compounds causing these effects was investigated.

Table 1. Specificity of β-substituted pyruvic acids in producing cholinergic effects.

Positive effects	No cholinergic effects
Fluoropyruvic acid	Pyruvic acid
Chloropyruvic acid	α-Ketobutyric acid
Bromopyruvic acid	Oxaloacetic acid
Hydroxypyruvic acid	Fluoroacetic acid
	Chloroacetic acid
	Glycolic acid
	α-Chloropropionic acid
	Sodium fluoride
	Potassium oxalate
	Potassium oxamate
	Acetic acid

Chloropyruvic acid was synthesized by the method of Garino and Muzio (*4*), and bromopyruvic and hydroxypyruvic acids were synthesized by the procedure of Sprinson and Chargaff (*5*). As is indicated in Table 1, each of these β-substituted derivatives of pyruvic acid induced cholinergic effects in rats following intraperitoneal injection of 75–150 mg/kg. The effects of 2 and 3 carbon analogs of these pyruvic acid congeners were also studied (Table 1). The convulsant effects of fluoroacetic acid are well known, but this compound, like chloroacetic acid, does not induce cholinergic effects in the rat. Pyruvic acid itself and its β-methyl analog, α-ketobutyric acid (Sigma Chemicals Company), did not induce cholinergic responses.

The effects of β-substituted pyruvic acids were studied in other animals. In the mouse, chloropyruvic acid subcutaneously caused death in 1.5 to 12 hours and was found to have an LD_{50} of 200 mg/kg, closely approximating the value of 250 mg/kg reported by Blank *et al.* (*1*) for fluoropyruvic acid. Chloropyruvic acid produced analgesia in the mouse in less than 30 minutes, and the AD_{50} by the method of Haffner (*6*) was also found to be 200 mg/kg. As in the rat, the spontaneous activity of the mouse decreased, but activity could be readily evoked by sensory stimulation.

When 50 mg/kg of chloropyruvic acid was injected intravenously into spinal vagotomized cats, there was an initial fall followed by an increase in blood pressure and pulse pressure. These immediate effects were followed by a prolonged depression of blood pressure and pulse pressure. The cat also exhibited marked salivation, lacrimation, and rhinorrhea. Although instillation of 0.2 ml or 5-percent bromopyruvic or chloropyruvic acid in the conjunctival sac did not induce miosis in the rabbit, miosis was generally observed following intravenous injection of these compounds. Administration of acetylcholine in doses of 2 μg/kg to the cat resulted in a depressor response without an accompanying bradycardia prior to the administration of chloropyruvic acid. Following the administration of chloropyruvic acid, a marked bradycardia accompanied the depressor response to acetylcholine. Approximately 0.5 hour later, the depressor response to acetylcholine was noted again, but the bradycardia failed to recur. Flexion and extension of rear extremities with superimposed tremor were also observed following administration of chloropyruvic acid to the spinal cat. Administration of 1 μg/kg of norepinephrine after the administration of chloropyruvic acid did not result in a pressor effect.

Immediately after injection of 150 mg/kg of chloropyruvic acid in the cat, the EEG, which had shown frequent spindle activity, became activated and remained so for 8 minutes. During this time, there was a gradual diminution of the amplitude of the high-frequency activity. After 10 minutes, the EEG was characterized by spikes, abortive spindles, and slow wave activity. The activating response to auditory stimuli was still present and was longer than in the control period. The amplitude continuously decreased, and within an hour the cortex was isoelectric; however, single auditory stimuli produced evoked cortical potentials in the frontal, parietal, and occipital leads. These potentials were not artifact and are presumptive evidence that the activating system was still capable of mediating impulses to the cortex.

To test the possibility that the effects of the halogenated pyruvic acids were due to the formation *in vivo* of haloacetylcholines, which might be the direct stimulatory compounds, fluoroacetylcholine was synthesized by the method of Gryszkiewicz-Trochimowski *et al.* (*7*). Fluoroacetylcholine, in doses of 50 μg/kg, in the unanesthetized cat produced the cholinergic effects observed with chloropyruvic acid as well as bradycardia and motor activity of the rear extremities. After intravenous administration to rats of 15 mg/kg of fluoroacetylcholine, hemodacryorrhea was observed in the first 15 seconds and was followed within a minute by the other cholinergic effects observed with β-substituted pyruvic acids. The same effects in the rat were noted with acetylcholine at the same dose levels (*8*).

Inasmuch as the possibility existed that these compounds functioned as anticholinesterases, their effects were determined on the rate of hydrolysis of acetylcholine by purified bovine erythrocyte cholinesterase (Winthrop-Stearns). Neither fluoropyruvic acid, chloropyruvic acid, nor fluoroacetylcholine significantly altered the rate of enzymatic hydrolysis of acetylcholine.

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).

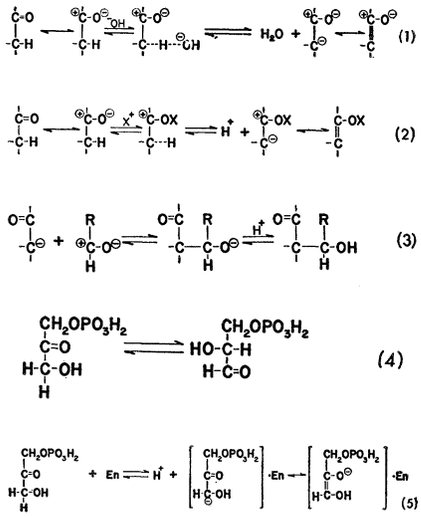
Subsequent reaction of C⟷E with the electrophilic carbonyl carbon of the aldehyde establishes a new carbon-carbon bond (Eq. 3).

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.