trans-bromobutene gives primarily *dl*-dibromide. These experiments were repeated several times and found to be reproducible. Increasing the reaction (irradiation) time to one hour did not result in any significant changes.

That the above additions are radical rather than ionic is clear from the orientation. When a mixture of *cis*-2-bromo-2-butene and liquid hydrogen bromide was allowed to stand in the dark at −80° for one hour the reaction mixture was found to contain 8.5% of bromobutene (mostly *trans* isomer) and 91.5% 2,2-dibromobutane. Under similar conditions using *trans*-2-bromo-2-butene the reaction mixture consisted of 10% of unreacted bromobutene, 87% 2,2-dibromobutane and 3% *dl*-2,3-dibromobutane (presumably formed by radical addition). These experiments also were found to be reproducible.

The present work demonstrates clearly that under the present conditions the radical addition involves a stereospecific *trans* addition. Assuming that the chain process involves two steps, (1) addition of a bromine atom to form an intermediate radical followed by (2) transfer with the addendum,[3,7] it is clear that different intermediate radicals are formed from the isomeric bromobutenes which undergo the transfer step faster than they are interconverted. Clearly the time interval between the two steps must be very short. A concerted process involving attack of a bromine atom on a substrate molecule complexed with hydrogen bromide has been suggested previously[3] and may be involved in the present case.

(7) M. S. Kharasch, J. V. Mansfield and F. R. Mayo, THIS JOURNAL, **59**, 1155 (1937).

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ISOPROPOXYFLUOROMETHYLENE[1]

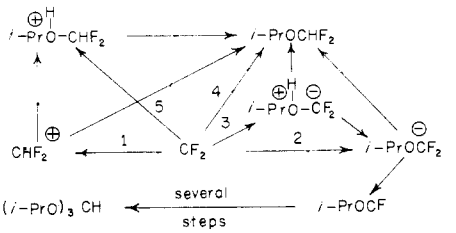
Sir:

Methylene is formed in such homolytic reactions as the decomposition of diazomethane and the photolysis of ketene,[2] and some of its derivatives apparently are formed in analogous reactions. Many of the polar reactions claimed to involve methylenes have been shown not to,[3] but the basic hydrolysis of chloroform, the reaction for which such a mechanism was first suggested,[4] does proceed through dichloromethylene.[5] The *dihalomethylenes*[6] appear to be the only ones that have been established as intermediates in polar reactions, since it is not clear just how concerted the observed α-eliminations with rearrangement are.[7] We now present evidence for the intermediate, isopropoxyfluoromethylene.

Chlorodifluoromethane reacted rapidly ($k = 8 \times 10^{-3}$ l. mole^{-1} sec.$^{-1}$) with potassium isopropoxide in dry isopropyl alcohol at 0° to give isopropyl difluoromethyl ether (b.p. 44.2–44.5°, d^{25}_4 0.97604, n^{25}D 1.3204, molecular refraction calcd. 22.115, found 22.404), triisopropyl orthoformate and about 1% fluoroform as the only detected organic products (no carbon monoxide or propylene[8]). The yields of the two major products were calculated from the base and chloride concentrations by stoichiometric considerations. The fraction of isopropyl orthoformate produced increased from 0.15 with 0.02 *M* isopropoxide to 0.30 with 0.10 *M* isopropoxide. The isopropyl difluoromethyl ether was inert under the reaction conditions and reacted very slowly even at 50°.

The basic decomposition of chlorodifluoromethane in methanol must involve difluoromethylene since with sodium thiophenolate the formation of phenyl difluoromethyl sulfide is powerfully catalyzed by sodium methoxide.[9] Under the more strongly basic conditions of the present case the α-dehydrochlorination is even better facilitated. The competing initial removal of hydrogen fluoride must be a negligible side reaction. While fluorine atoms increase the reactivity of haloforms from which other halogens may be removed to give fluoromethylenes, the initial removal of fluoride ions is very difficult, fluoroform being inert even to potassium *t*-amyl oxide at 50°.[10] This rules out the possibility that the orthoester is produced from chlorofluoromethylene, a hypothesis that is also incompatible with the change in orthoester yield with changing isopropoxide concentration.

The only subsequent reactions of the difluoromethylene that seem plausible are



protonation (1), coördination with isopropoxide ion (2) or isopropyl alcohol (3), or simultaneous protonation and coördination with isopropoxide (4) or alcohol (5). Reactions 1, 4 and 5 can lead reasonably only to *i*-PrOCHF$_2$, as do 2 and 3 if the carbanions formed thereby are protonated. Hence the isopropyl orthoformate must be formed through reaction 2 and/or 3 followed (or accompanied) by loss of a fluoride ion.[11] Reaction through the *i*-PrOCF$_2^-$ anion seems more reason-

(1) This investigation was supported in part by the Office of Naval Research.

(2) F. O. Rice and A. L. Glasebrook, THIS JOURNAL, **56**, 2381 (1934); T. G. Pearson, R. H. Purcell and G. S. Saigh, *J. Chem. Soc.*, 409 (1938).

(3) F. Adickes, *Ber.*, **60**, 272 (1927); **63**, 3012 (1930); C. R. Hauser, W. R. Brasen, P. S. Skell, S. W. Kantor and A. E. Brodhag, THIS JOURNAL, **78**, 1653 (1956).

(4) A. Geuther, *Ann.*, **123**, 121 (1862).

(5) J. Hine, THIS JOURNAL, **72**, 2438 (1950); J. Hine and A. M. Dowell, Jr., *ibid.*, **76**, 2688 (1954).

(6) We use the term to include only species in which carbon is attached to two other atoms, neglecting isocyanides, for example.

(7) D. G. Hill, W. A. Judge, P. S. Skell, S. W. Kantor and C. R. Hauser, THIS JOURNAL, **74**, 5309 (1952).

(8) *Cf.* J. Hine, E. L. Pollitzer and H. Wagner, *ibid.*, **75**, 5607 (1953).

(9) J. J. Porter, unpublished experiments from this Laboratory. *Cf.* ref. 5.

(10) J. Hine, A. M. Dowell, Jr., and J. E. Singley, Jr., THIS JOURNAL, **78**, 479 (1956); and N. W. Burske, unpublished experiments from this Laboratory.

(11) S$_N$2 attack on the intermediate *i*-PrOCF$_2^-$ anion seems improbable for the same reasons that applied in the case of the trichloromethyl anion.[5]

able since the loss of fluoride ion should be facilitated by the unshared electron pairs of the isopropoxy group. Since isopropoxyfluoromethylene is the only intermediate in the scheme in which the inert end-product, i-PrOCHF$_2$, has been bypassed, it must be an intermediate in the formation of the isopropyl orthoformate.

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GLUTAMIC ASPARTIC TRANSAMINASE

Sir:

Glutamic aspartic transaminase of pig heart has been highly purified and the presence of firmly bound pyridoxal phosphate has been confirmed. The enzyme was purified by heat denaturation (75° for 20 minutes in maleate buffer pH 6.0), ammonium sulfate fractionation (50–67%), adsorption of impurities onto calcium phosphate followed by chromatography on a calcium phosphate column[1] and a repetition of the ammonium sulfate fractionation to give an over-all yield of 30%. Sedimentation and electrophoresis studies indicate that the enzyme is 70% pure; it has an activity of 380 μM glutamate/min./mg. protein using a modification of the assay of Nisonoff, *et al.*,[2] at 37° and pH 8.5. The preparation contains one mole of pyridoxal phosphate per 67,000 grams of protein.

Figure 1 shows that the enzyme is colorless at pH 8.5 (λ_{max} 362 mμ) but turns yellow at pH 4.8 (λ_{max} 430 mμ) and in 0.1 N NaOH (λ_{max} 388 mμ). In the latter solution the prosthetic group is split off and could be isolated by use of a Dowex 1-Cl column. In 0.1 N HCl the chromophore absorbs strongly at 295 mμ. Its 2,4-dinitrophenylhydrazone, which is not extracted from acidic solution by ethyl acetate, absorbs at 478 mμ in alkali and at 415 mμ in acid. These spectra confirm the fact that the prosthetic group is a derivative of pyridoxal phosphate. It should be noted, however, that the pyridoxal phosphate has characteristically different spectra when bound to the enzyme. We believe that these spectra may be due to a pyridoxal phosphate imine, since Metzler[3] has shown that these imines absorb at 360 mμ at high pH and above 410 mμ at lower values. Matsuo and Greenberg[4] have shown that the crystallized cystathionase/homoserine dehydrase has a similar absorption band at 427 mμ.

Carbonyl reagents react with the transaminase to yield the enzyme oxime, hydrazones, cyanhydrin and complexes with thiols and bisulfite, all of which have characteristic spectra.

The addition of glutamate to the enzyme at pH 8.5 causes the absorption peak to shift to 332 mμ (see Fig. 1) a peak which is characteristic of all pyridoxine compounds including the zwitterion

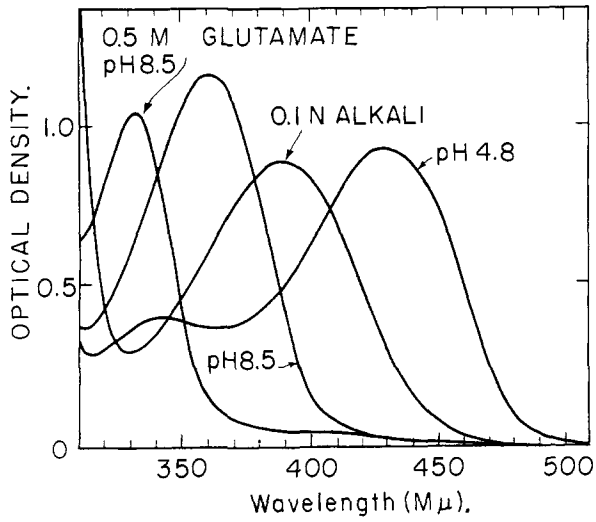

Fig. 1.—Spectra of the enzyme in 0.1 M Tris buffer pH 8.5 alone and with 0.5 M glutamate, in 0.1 N NaOH and in 0.15 M acetate buffer pH 4.8. Each cuvette contained 0.863% protein.

form of pyridoxamine. Isolation of the prosthetic group on Dowex 1-Cl, after the addition of excess glutamate, yielded pyridoxamine phosphate characterized by its absorption bands in acid (λ_{max} 293) alkali (λ_{max} 306) and at pH 5.2 (λ_{max} 326). The addition of less glutamate causes partial disappearance of the pyridoxal phosphate (measured as its 2,4-dinitrophenylhydrazone after cleavage from the enzyme by 0.1 N alkali) and the formation of an equivalent amount of α-ketoglutarate.

The reaction is reversed by increasing concentrations of ketoglutarate added to the enzyme in the presence of a constant amount of glutamate, as shown by an increase in the band at 362 mμ characteristic of the free enzyme and an increase in the band at 435 mμ ascribed to a complex of ketoglutarate with the protonated enzyme. By the use of C^{14}-glutamate rapid transamination has been confirmed in this steady-state system in which the concentrations of glutamate and ketoglutarate do not change. We believe that these experiments essentially confirm the pyridoxal-pyridoxamine hypothesis suggested on structural grounds by Snell.[5]

Dicarboxylic acids combine with the protonated form of the enzyme to produce an absorption maximum at about 435 mμ. The order of effectiveness is: glutarate > maleate > adipate > malate > ketoglutarate > oxalacetate > succinate. The fact that oxalate, malonate, pimelate, suberate, glycolate and fumarate do not interact suggests that a specific orientation of both carboxyl groups is required and that this specificity may be related to the substrate specificity of the enzyme.

These complexes may explain the apparent stabilizing effect of maleate in this purification and that of ketoglutarate in the purification of kynurenine transaminase.[6] This type of complex formation results in a new type of inhibition,[7] in which the in-

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(2) A. Nisonoff, S. S. Henry and F. W. Barnes, Jr., *ibid.*, **199**, 699 (1952).

(3) D. E. Metzler, THIS JOURNAL, **79**, 485 (1957).

(4) V. Matsuo and D. M. Greenberg, *Federation Proc.*, **16**, 218 (1957).

(5) E. E. Snell, *J. Biol. Chem.*, **154**, 313 (1944).

(6) M. Mason, *Federation Proc.*, **15**, 310 (1956).

(7) A. E. Braunstein, *Nature*, **143**, 609 (1939).