and phenylpyruvate. The synthesis of D-valine, D-methionine, D-pipecolic acid, and D-leucine was observed in similar studies. In an experiment with D-methionine, Δ^1-pyrroline-2-carboxylic acid,[4] and D-amino acid oxidase, D-proline formation was shown to be accompanied by stoichiometric ammonia formation and D-methionine disappearance (Table I). In these experiments, the formation of α-keto-γ-methiolbutyric acid was demonstrated by preparation of the corresponding 2,4-dinitrophenylhydrazone and catalytic reduction of this derivative to methionine.[5] D-Methionine could be replaced by a number of other D-amino acids which are known to be oxidized by D-amino acid oxidase, but not by amino acids (*e.g.*, D-glutamic acid) which are not appreciably susceptible to oxidation. The formation of new amino acid was dependent upon the presence of flavin adenine dinucleotide (FAD). Formation of D-proline in reactions involving Δ^1-pyrroline-2-carboxylic acid, which may be regarded as a substituted imino acid, proceeded more rapidly, under the conditions employed, than did reactions with α-keto acids and ammonia.

Analogous studies have been carried out with purified L-amino acid oxidase[6]; for example, L-methionine was formed when L-leucine, L-amino acid oxidase, and α-keto-γ-methiolbutyric acid were incubated. The latter reaction may be represented as

$$\text{L-Leucine} + \text{Enzyme–FAD} + H_2O \rightleftharpoons \alpha\text{-Ketoisocaproate} + NH_3 + \text{Enzyme–FADH}_2$$

$$\text{Enzyme–FADH}_2 + NH_3 + \alpha\text{-Keto-}\gamma\text{-methiolbutyrate} \rightleftharpoons \text{L-Methionine} + \text{Enzyme–FAD} + H_2O$$

$$\text{Sum: L-Leucine} + \alpha\text{-Keto-}\gamma\text{-methiolbutyrate} \rightleftharpoons \text{L-Methionine} + \alpha\text{-Ketoisocaproate}$$

Although the over-all reaction is identical with a transamination reaction, it is clear that this reaction does not represent amino group transfer, but involves the intermediate participation of ammonia. Findings consistent with this interpretation are given in Table II. In these experiments, the purified oxidases were incubated with an amino acid substrate, its α-keto acid analog, and N^{15}-ammonia. The N^{15} content of the amino acid after incubation is recorded in the table[7]; isotope was not incorporated in control experiments in which the enzymes were inactivated by treatment with ethanol. These results conclusively demonstrate the reversibility of the amino acid oxidase reactions.

TABLE I

SYNTHESIS OF D-PROLINE BY D-AMINO ACID OXIDASE[a]

	D-Methionine utilized (μmoles)	D-Proline formed (μmoles)	NH_3 formed (μmoles)
Complete system[b]	0	0	0
Complete system	2.4	2.6	2.6
FAD omitted	0	0	0

[a] The reaction mixtures consisted of purified-D-amino acid oxidase preparation (10 mg.), D-methionine (25 micromoles), Δ^1-pyrroline-2-carboxylate (10 micromoles), and sodium pyrophosphate buffer of *p*H 8.3 (60 micromoles) in a final volume of 0.6 ml.; incubated at 37° for 180 minutes; gas phase, nitrogen. Disappearance of methionine was determined as described by K. V. Giri, A. N. Radhakrishnan and C. S. Vaidyanathan, *Anal. Chem.*, **24**, 1677 (1952); proline formation was determined by the procedure of W. Troll and J. Lindsley, *J. Biol. Chem.*, **215**, 655 (1955), as modified by A. Meister, A. N. Radhakrishnan and S. D. Buckley, *J. Biol. Chem.*, in press. [b] The enzyme was inactivated by treatment with 3 volumes of ethanol, or by heating at 100° for 5 minutes.

TABLE II

INCORPORATION OF N^{15}-AMMONIA INTO AMINO ACIDS CATALYZED BY AMINO ACID OXIDASES

Amino acid	Enzyme	Atom % excess N^{15} in amino acid
D-Alanine[a]	D-Oxidase	0.53
L-Leucine[b]	L-Oxidase	0.37

[a] Reaction mixture contained amino acid (50 micromoles), sodium pyruvate (25 micromoles), $N^{15}H_4NO_3$ (25 micromoles, 63 atom % excess $N^{15}H_4$), enzyme preparation and sodium pyrophosphate buffer of *p*H 8.3 (100 micromoles) in a final volume of 1.0 ml.; incubated at 37° for 120 minutes, under nitrogen. [b] Reaction mixture contained amino acid (10 micromoles), sodium α-ketoisocaproate (20 micromoles), $N^{15}H_4NO_3$ (20 micromoles, 63 atom excess $N^{15}H_4$), enzyme preparation (1.37 mg.), and tris-(hydroxymethyl)-aminomethane buffer of *p*H 7.2 (100 micromoles) in a final volume of 1.0 ml.; incubated at 37° for 180 minutes, under nitrogen. After incubation, the mixtures were made alkaline by addition of Na_2CO_3 and the free NH_3 was removed by exhaustive aeration. The α-amino nitrogen was subsequently released as NH_3 by treatment with the appropriate amino acid oxidase in air after readjustment of *p*H.

The configuration of the amino acids formed in these systems was established by determining the effect of purified L- and D-amino acid oxidases on the enzymatically synthesized amino acids. The α-keto acid analogs of amino acids which were not susceptible substrates were inactive in these systems; for example, α-ketoglutarate did not yield glutamic acid with either oxidase. L-Amino acids were not active in the D-amino acid oxidase system and vice versa.

Further studies on these systems may yield additional information concerning the mechanism of the amino acid oxidase reaction. It is possible that under appropriate physiological conditions, reactions of this type catalyzed by amino acid oxidases may represent a pathway alternative to transamination for interconversion of amino and keto acids.

(7) The authors thank Dr. S. Hartman and Dr. J. M. Buchanan for the isotope analyses.

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(4) A. Meister, *J. Biol. Chem.*, **206**, 577 (1954).

(5) A. Meister and P. A. Abendschein, *Anal. Chem.*, **28**, 171 (1956).

(6) Prepared from snake venom as described by T. P. Singer and E. B. Kearney, *Arch. Biochem.*, **29**, 190 (1950).

THE REACTION OF TRIVALENT ORGANOPHOSPHORUS COMPOUNDS WITH POLYHALOMETHANES

Sir:

In view of the current interest in the reactions of tertiary phosphines,[1] of trialkyl phosphites,[2a] and

(1) R. N. Haszeldine and B. O. West, *J. Chem. Soc.*, 3631 (1956).

(2) (a) G. Kamai and L. P. Egoroda, *J. Gen. Chem. USSR.*, **16**, 1521 (1946); *C. A.*, **41**, 5439h (1947); (b) Z. L. Khisamova and G. Kamai, *J. Gen. Chem. USSR*, **20**, 1162 (1950); *C. A.*, **45**, 1531*d* (1951).

of phosphonites[2b] with polyhalomethanes and with mercaptans,[3] disulfides[4] and alkylsulfenyl halides[5] (RSCl), we wish to describe some of our observations.[6]

Triphenylphosphine did not react appreciably with purified **bromoform** at room temperature. Reaction was observed under the following conditions: (1) near the boiling point of bromoform, when an 85% yield of **triphenyl-(dibromomethyl)-phosphonium bromide (II)** was obtained in a few seconds; (2) at room temperature under irradiation with a Hanovia ultraviolet lamp (80% yield of II[7]); (3) at *ca.* 80° in benzene solution containing traces of benzoyl peroxide (over 80% of II[7] in 30 minutes); in the absence of peroxide, under comparable conditions, no reaction was observed.

This reaction clearly involves free radicals[8] and probably constitutes a chain process. These steps are proposed

$$(C_6H_5)_3P + \cdot CHBr_2 \longrightarrow \underset{\text{I}}{(C_6H_5)_3\dot{P}\text{-}CHBr_2} \quad (1)$$

$$\underset{\text{I}}{(C_6H_5)_3\dot{P}\text{-}CHBr_2} + BrCHBr_2 \longrightarrow \underset{\text{II}}{[(C_6H_5)_3\overset{(+)}{P}\text{-}CHBr_2]\overset{(-)}{Br}} + \cdot CHBr_2 \quad (2)$$

An intermediate with pentacovalent phosphorus may be involved in step (2). The fate of the postulated neutral *phosphoranyl radical*, I, with an expanded phosphorus valence shell, could depend on the nature of the groups attached to the phosphorus atom[9]; for instance, the process $R_3\dot{P}\text{-}X \rightarrow R\cdot + R_3P\text{-}X$ is conceivable. Propagation steps with R· radicals may then be involved. Haszeldine[1] recently has suggested an initial nucleophilic attack by phosphorus on halogen in order to explain the formation of dimethyl-(trifluoromethyl)-phosphine, $(CH_3)_2P\text{-}CF_3$, and tetramethylphosphonium iodide $(CH_3)_4P^+I^-$, in the reaction of trimethylphosphine with iodotrifluoromethane.

The reaction of triphenylphosphine with polyhalomethanes seems to be a case in which a salt is formed in a free radical process.

Triphenyl-(dibromomethyl)-phosphonium bromide (II) had m.p. 235° (from methanol–ethyl acetate), was moderately soluble in water, had ionic bromine and exhibited a band at 3.6μ characteristic[10] of structure $R_3P^+\text{-}C\text{-}H$. Calcd. for $C_{19}H_{16}Br_3P$: C, 44.3; H, 3.1. Found: C, 43.9; H, 2.8. The phosphonium bromide II is of interest in connection with our studies on stable phosphine-methylenes[10,11] $R_3P^+\text{-}C^-XY$.

(3) F. W. Hoffmann, R. H. Ess, T. C. Simmons and R. S. Hanzel, THIS JOURNAL, **78**, 6414 (1956).

(4) H. I. Jacobson, R. G. Harvey and E. V. Jensen, *ibid.*, **77**, 6064 (1955).

(5) D. C. Morrison, *ibid.*, **77**, 181 (1955).

(6) For earlier work on the reaction of tertiary phosphines with polyhalomethanes: (a) H. Hantzsch and H. Hibbert, *Ber.*, **40**, 1508 (1907); (b) A. W. Hofmann, *Proc. Roy. Soc.*, **11**, 291 (1859). For the action on disulfides: A. Schöenberg, *Ber.*, **68**, 163 (1935).

(7) A second substance (m.p. 314°) was also produced in small yield.

(8) A chain-reaction mechanism has been proposed for the photochemical or peroxide-initiated addition of polyhalomethanes to olefins (M. S. Kharasch, E. V. Jensen and W. H. Urry, THIS JOURNAL, **69**, 1100 (1947); M. S. Kharasch, O. Reinmuth and W. H. Urry, *ibid.*, **69**, 1105 (1947)).

(9) In the related case of the trialkyl phosphites[2a] (and of the phosphonites[2b]) one of the possibilities would be: $(RO)_3\dot{P}\text{-}X \rightarrow R\cdot + (RO)_3P(O)X$ (*cf.* G. Kamai and Z. Kbarrasova, *Zhur. Obshchei. Khim.*, **27**, 953 (1957)). In a reinvestigation of the reaction of trialkyl phosphites with mercaptans, C. Walling and R. Rabinowitz have reached similar conclusions THIS JOURNAL, **79**, 5326 (1957).

(10) F. Ramirez and S. Dershowitz, *J. Org. Chem.*, **22**, 41 (1957).

(11) (a) F. Ramirez and S. Levy, THIS JOURNAL, **79**, 67 (1957); (b) F. Ramirez and S. Dershowitz, *ibid.*, **78**, 5614 (1956).

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IDENTIFICATION OF SELENOMETHIONINE IN THE PROTEINS OF *Escherichia coli* EMPLOYING THE CHROMATOGRAPHIC "FINGERPRINT" METHOD

Sir:

The wide distribution of organic selenium compounds throughout the plant and animal kingdoms has been recognized for many years. It has been frequently suggested that selenium may replace sulfur in the methionine and cystine of proteins, although no selenium containing organic compound obtained from natural sources has been unequivocally identified.[1]

Cowie and Cohen have observed that selenomethionine could completely replace methionine for the normal exponential growth of a methionine-requiring mutant of *Escherichia coli*, and that selenium from radioactive selenite was incorporated into proteins.[2] Recent experiments by Schwarz and Foltz lead to the conclusion that selenium is an essential trace element.[3]

We wish to report the identification of selenomethionine from a hydrolysate of the proteins of *E. coli* grown in the presence of radioactive selenium, supplied as selenite, using the chromatographic "fingerprint" method.[4]

Wild-type *E. coli*, strain B, was grown in a sulfur-deficient glucose-salts medium under conditions which permitted rapid incorporation of selenium into proteins.[5] The cells were harvested and fractionated by the method of Roberts, *et al.*[6] One milligram of the highly radioactive protein fraction was successively hydrolyzed enzymatically with pepsin, pancreatin, and erepsin. In order to purify the hydrolysate and to eliminate peptides and inorganic selenium salts, the hydrolysate was loaded on an eight per cent. cross-linked Dowex 50 cation exchange column in the hydrogen form. The column was then washed with water to remove traces of selenite and selenate. Elution of the column with 1.5 normal hydrochloric acid produced a highly radioactive eluate which, after drying in a vacuum desiccator, was loaded on a 3-ml. Dowex 2 anion exchange column in the hydroxide form. No radioactivity could be removed by washing with water. Most of the activity could be eluted

(1) For a review of the literature, see S. F. Trelease and O. A. Beath, "Selenium," Publ. by the Authors, New York, N. Y., 1949.

(2) D. B. Cowie and G. N. Cohen, *Biochem. Biophys. Acta*, in press.

(3) K. Schwarz and C. M. Foltz, THIS JOURNAL, **79**, 3292 (1957).

(4) R. B. Roberts, P. H. Abelson, D. B. Cowie, E. T. Bolton and R. J. Britten, "Studies of Biosynthesis in *Escherichia coli*," Carnegie Institution of Washington Publ. 607, Washington, D. C., 1955, pp. 190–192.

(5) T. W. Tuve and H. H. Williams, presented before the 132nd Meeting of the American Chemical Society, New York, N. Y., Sept. 8-13, 1957; see Abstracts, p. 10-C.

(6) R. B. Roberts, *et al.*, ref. 4, pp. 13-14.