was placed in the first 9 tubes of an all-glass countercurrent distribution apparatus[31] and distributed for 1450 transfers in the system *sec*-butyl alcohol–0.08 *M* *p*-toluenesulfonic acid.[17] The pressor activity was concentrated in a single peak (K 0.64). The modified Folin color curve[32] approximated the activity curve closely, except for the indication of the presence of a small amount of other material, possibly due to decomposition. The contents of 36 tubes included in the peak were placed in a separatory funnel, and ether was added to facilitate separation of the phases. The aqueous layer was withdrawn, the organic phase was washed twice with water and the washings were added to the aqueous phase. The *p*-toluenesulfonic acid was removed by passage of the solution through a column of Amberlite IR-45 (acetate).[17] The eluate (approximately 16,000 pressor units) was concentrated on a rotary evaporator[30] and lyophilized. The weight of the dry residue was 69 mg.; 1.16 mg. of this material was dissolved in 0.25% acetic acid (50 ml.) and tested for pressor activity. The activity was found to be approximately 230 units per mg. A sample was redistributed in *sec*-butyl alcohol–0.08 *M* *p*-toluenesulfonic acid for 235 transfers (K 0.57). The biologically active product was isolated in the same way and the specific activity was found to be approximately the same as before the additional distribution step.

In another purification, 2.0 g. of crude material (approximately 150,000 pressor units) resulting from the sodium–liquid ammonia reduction of the protected nonapeptide was used. After distribution between *sec*-butyl alcohol and 0.08 *M* *p*-toluenesulfonic acid for 250 transfers (K 0.82), the material (1.2 g.) had a pressor activity of 125 to 135 units per mg. and contained considerable inorganic contamination. A portion of this material (170 mg., 21,000–23,000 pressor units) was subjected to electrophoresis on a cellulose-supporting medium in pyridine–acetate buffer (*p*H 5.6; ionic strength, 0.4 *M*).[17,19] The material was dissolved in 1 ml. of buffer and placed 2 cm. from the anode end of a previously prepared cellulose block (10 cm. × 46 cm. × 10 cm.). A current of 400 volts and 70–80 milliamperes was applied for 43 hr. The block was then cut into segments (2 cm.) starting from the origin. The buffered solution was pressed from each segment and the cellulose was washed once with 1% acetic acid (7 ml.). The pressor activity, modified Folin color and weight of aliquots of each segment were determined. The activity was found to be concentrated in segments 11, 12 and 13. A second compound having no pressor activity appeared in small quantities in segments 2 to 9 with a peak in segments 6 to 7. The solutions from segments 11, 12 and 13 were lyophilized separately, the weight of material isolated being 14, 24 and 14 mg., respectively. Assays on a sample from segment 12 gave an activity of approximately 280 pressor units per mg. Samples from the other two segments were found to have approximately the same activity.

The electrophoresis of the material previously distributed between *sec*-butyl alcohol and 0.08 *M* *p*-toluenesulfonic acid (250 transfers) was repeated on a larger scale (60,000 pressor units). Material (55 mg.) having a pressor activity of approximately 280 units per mg. was obtained from the peak segments. The material obtained from the solution extracted from the trailing side of the peak was found to have a somewhat lower specific activity and was purified by countercurrent distribution in *sec*-butyl alcohol–0.1% acetic acid. Two components separated after 600 transfers, one having no pressor activity (K 0.017) and the other (K 0.66) containing all the activity and about 70% of the weight of the material before distribution. Pressor assays indicated an activity of approximately 280 units per mg.

Amino acid analysis[33] of an hydrolysate of the material showed the following amino acid content, expressed in molar ratios (with the ratio for phenylalanine arbitrarily taken as 1): phenylalanine 1.00, tyrosine 0.88, proline 0.91, glutamic acid 1.03, aspartic acid 1.08, glycine 1.05, lysine 0.98, cystine 0.68 and ammonia 3.28.

The specific rotation of the synthetic material was $[\alpha]^{20}D$ −47.5° (c 0.99, H_2O).

A sample of the hygroscopic product was dried at room temperature *in vacuo* over P_2O_5 for 18 hr.

Anal. Calcd. for $C_{46}H_{65}N_{13}O_{12}S_2 \cdot (C_2H_4O_2)_2$: C, 51.0; H, 6.25; N, 15.5. Found: C, 50.5; H, 6.27; N, 14.9.

(31) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *Anal. Chem.*, **23**, 1236 (1951).

(32) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(33) S. Moore and W. H. Stein, *ibid.*, **178**, 53 (1949).

NEW YORK 21, N. Y.

COMMUNICATIONS TO THE EDITOR

ACTIVATION OF A FERRICYANIDE LINKED PYRUVATE OXIDASE BY α-TOCOPHEROL ESTERS

Sir:

A purified soluble enzyme and particulate fraction obtained from cell extracts of an acetate requiring mutant of *Escherichia coli* combine to catalyze the ferricyanide linked oxidative decarboxylation of pyruvate to acetate and CO_2 according to the equation[1,2]

$$CH_3—CO—COO^- + 2Fe^{+++}(CN)_6 + H_2O \xrightarrow[\text{pyrophosphate}]{\text{Thiamin}} CH_3—COO^- + CO_2 + 2Fe^{++}(CN)_6 + 2H^+$$

In an attempt to obtain soluble enzyme preparations from the particulate fraction, acetone-dried particles were prepared by extracting aqueous suspensions of the particles with 20 volumes of acetone at −20°. The dried particles obtained by this procedure were essentially inactive; however, the acetone residue obtained by removal of the acetone under reduced pressure, activated the soluble enzyme in the absence of the particulate fraction. By analogy to the work of Nason and Lehman[3,4] on the activation of the diphosphopyridine nucleotide–cytochrome C reductase system by α-tocopherol, α-tocopherol and various vitamin E derivatives, as well as other fat soluble vitamins and oxidation–reduction coenzymes were tested for their ability to replace the factor or factors present in the acetone residue. Among a large number of compounds tested, only α-tocopherol phosphate and α-tocopherol succinate were active. These results are summarized in Table I. The rate of pyruvic acid oxidation is a function of the concentration of the α-tocopherol ester or acetone residue as shown in Fig. 1.

(1) L. P. Hager, *Federation Proc.*, **16**, 190 (1957).

(2) L. P. Hager, *J. Biol. Chem.*, in press.

(3) A. Nason and I. R. Lehman, *Science*, **122**, 19 (1955).

(4) A. Nason and I. R. Lehman, *J. Biol. Chem.*, **222**, 54 (1956).

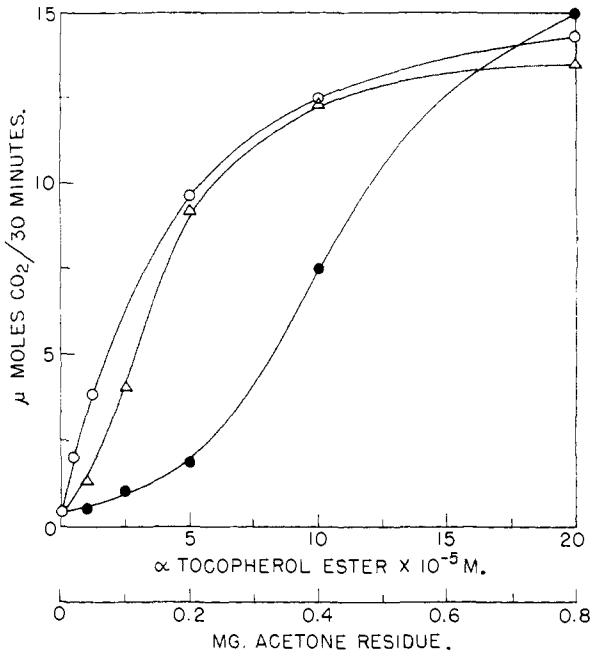

Fig. 1.—The rate of pyruvate oxidation as a function of the concentration of the α-tocopherol ester or acetone residue. The complete system contained the same additions listed in Table I plus the indicated amount of α-tocopherol phosphate (△—△—△), α-tocopherol succinate (●—●—●) or acetone residue(O—O—O).

α-Tocopherol and α-tocopherol acetate when added to the enzyme assay either from a concentrated alcoholic solution or "suspended" in bovine serum albumin[4] were not active. Other compounds which were tested and found to be without activity were vitamin K_1, menadione, vitamin A, carotene, vitamin C, vitamin D, lipoic acid, ethylenediamine tetraacetate, phenylphosphate, di- and triphosphopyridine nucleotides, flavin adenine dinucleotide, riboflavin, riboflavin monophosphate and Tween 80. Bovine serum albumin and *n*-butyl stearate, both of which activate the diphosphopyridine nucleotide–cytochrome C reductase system in the absence of α-tocopherol,[7,8] do not activate this pyruvate oxidation system.

TABLE I

REQUIREMENTS FOR FERRICYANIDE LINKED PYRUVATE OXIDATION

The complete system contained 100 μmoles of potassium phosphate buffer *p*H 6.0, 10 μmoles of $MgCl_2$, 0.1 μmole of thiamin pyrophosphate, 50 μmoles of potassium pyruvate, 25 μmoles of potassium ferricyanide, 0.7 mg. of the soluble enzyme and 0.75 mg. of an acetone extract of the particulate fraction in a total volume of 1 ml. Incubation was carried out at 30° under nitrogen in standard Warburg vessels for 20 minutes.

Additions	Products: CO_2, μmoles	Products: Ferrocyanide,[a] μmoles/2	Products: Acetate[b] μmoles
1. Complete system	9.5	10.1	8.3
2. 1-Thiamin pyrophosphate	0.3	0.5	0.5
3. 1-Pyruvate	0.2	0.9	0.9
4. 1-Ferricyanide	0.1	0.0	0.0
5. 1-Enzyme	0.2	0.6	0.7
6. 1-Acetone extract of the particulate fraction	0.5	1.2	0.7
7. 6 + 0.2 μmoles of α-tocopherol phosphate	7.9	8.5	7.8
8. 6 + 0.2 μmoles of α-tocopherol succinate	8.2	9.0	7.8

[a] Determined colorimetrically by a modification of the Park and Johnson assay for reducing sugars.[5] [b] Determined enzymatically using the acetokinase assay[6] after steam distillation.

(5) I. A. Rose, "Methods in Enzymology," Vol. I, Academic Press, New York, N. Y., 1955, p. 591.

(6) J. T. Park and M. J. Johnson, *J. Biol. Chem.*, **181**, 149 (1949).

Acknowledgments.—This work was supported by grants from the National Science Foundation and the United States Public Health Service. The author gratefully acknowledges the technical assistance of Mr. William Morris and Mrs. Caroline Fenn.

(7) A. Nason, B. C. Averbach and A. J. Terrell, *Biochem. et Biophys. Acta*, **19**, 395 (1956).

(8) K. O. Donaldson and A. Nason, *Proc. Nat. Acad. Sci.*, **43**, 365 (1957).

CONVERSE MEMORIAL LABORATORY
HARVARD UNIVERSITY
DEPARTMENT OF CHEMISTRY LOWELL P. HAGER
CAMBRIDGE 38, MASSACHUSETTS

RECEIVED AUGUST 2, 1957

INCORPORATION OF ATMOSPHERIC OXYGEN INTO THE CELL CONSTITUENTS OF A GROWING CULTURE OF *Pseudomonas*

Sir:

Studies on various oxygenases with O_2^{18} and H_2O^{18} have recently shown that a number of enzymes from animal, plant and microbial sources catalyze incorporation of atmospheric oxygen into organic substrates.[1,2] However, the significance of these oxygenases in the metabolism of intact cells has not yet been explored. When *Pseudomonas sp.*, a strictly aerobic microörganism, was grown with benzoic acid, phenylalanine or tryptophan as the carbon source, from 4 to 9% of the oxygen of cell constituents was found to be derived from atmospheric oxygen (Table I). CO_2 as well as water, which was in complete isotopic equilibrium with CO_2,[3,4] contained much less O^{18}, indicating that most of the incorporation of O^{18} into the cell material did not involve conversion of the atmospheric oxygen to either CO_2 or water. On the other hand when a more oxygenated substrate such as glucose was used as a carbon source, O^{18}-enrichment was about 0.4% of that of the atmospheric oxygen used in the experiment under similar growth conditions. These observations are consistent with the previous findings that these aromatic compounds are degraded by enzymatic reac-

(1) O. Hayaishi, S. Rothberg and A. H. Mehler, Abstract of Papers, 130th American Chem. Soc. Meeting, Atlantic City, N. J., 1956.

(2) H. S. Mason, *Science*, **125**, 1185 (1957).

(3) M. Cohn and H. C. Urey, THIS JOURNAL, **60**, 679 (1938).

(4) S. Rothberg and D. Steinberg, *ibid.*, **79**, 3274 (1957).