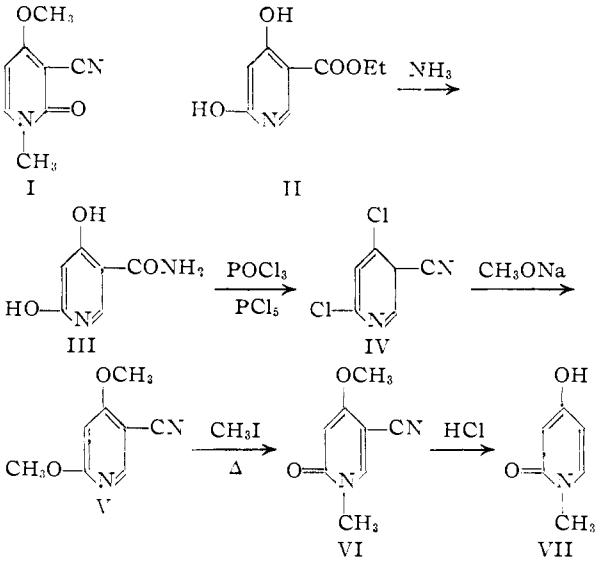
methyl-4-hydroxy-2-pyridone (VII), which also is formed by similar treatment of ricinine itself.[5]

It is of interest that the isomerization of V to VI took place much more readily than the analogous isomerization of 2,4-dimethoxynicotinonitrile to ricinine.[2,6]



Experimental

4,6-Dihydroxynicotinamide (III).—A mixture of 10 g. of ethyl 4,6-dihydroxynicotinate[3] and 70 ml. of liquid ammonia was sealed in a steel bomb and heated at 150° for a period of 3 hours (the internal pressure at this temperature is 1900 lb./sq. in.). Removal of excess ammonia gave 8.4 g. of colorless solid which darkens above 300° and has an indistinct decomposition point about 353°. Since no satisfactory method could be found for recrystallization of this material, it was used directly without further purification in the next step.

4,6-Dichloronicotinonitrile (IV).—A mixture of 5.5 g. of crude 4,6-dihydroxynicotinamide, 20 g. of phosphorus pentachloride and 75 ml. of phosphorus oxychloride was heated under reflux for 3 hours. The resulting red solution was concentrated under reduced pressure to remove excess phosphorus oxychloride and the residual sirup was poured with vigorous stirring onto ice. After this aqueous mixture had stood at 0° for 1 hour, it was extracted thoroughly with ether and the ether extracts washed with water, dried over anhydrous sodium sulfate and concentrated to give 5.23 g. of a white, crystalline solid. Sublimation of this material at 70° (0.5 mm.) gave 4.11 g. (67%, based on II) of pure 4,6-dichloronicotinonitrile, m.p. 134–136°.

Anal. Calcd. for $C_6H_2N_2Cl_2$: C, 41.6; H, 1.2; N, 16.2. Found: C, 41.3; H, 1.2; N, 15.9.

4,6-Dimethoxynicotinonitrile (V).—Into a 1-l. 3-necked round-bottom flask, to which was attached a liquid sealed stirrer and a reflux condenser provided with a drying tube, was placed a solution of 3.0 g. of sodium in 500 ml. of absolute methanol and 7.0 g. of 4,6-dichloronicotinonitrile. The mixture was heated under reflux with stirring for 5 hours, the excess methanol was removed by distillation until solid product started to crystallize from the solution, and 250 ml. of cold water then was added. The cooled mixture was filtered and the collected solid washed with water and dried at 100° to give 6.22 g. (94%) of colorless crystals, m.p. 153.7–155.7°. The analytical sample was prepared by recrystallization from absolute ethanol followed by sublimation *in vacuo*, m.p. 154.7–155.7°.

Anal. Calcd. for $C_8H_8N_2O_2$: C, 58.5; H, 4.9; N, 17.1. Found: C, 58.9; H, 4.9; N, 17.3.

N-Methyl-3-cyano-4-methoxy-6-pyridone (VI).—A mixture of 8.2 g. of 4,6-dimethoxynicotinonitrile and 83 ml. of methyl iodide was heated in a sealed tube at 130° for 5 hours and then evaporated to dryness. The crude product (8.04 g.) was recrystallized from water to give 7.65 g. (93%) of colorless needles, m.p. 241–242°. The analytical sample was prepared by sublimation at 175° (0.5 mm.).

Anal. Calcd. for $C_8H_8N_2O_2$: C, 58.5; H, 4.9; N, 17.1. Found: C, 58.7; H, 4.9; N, 16.9.

In a separate experiment, 0.1 g. of 4,6-dimethoxynicotinonitrile was heated alone in a sealed tube at 200–225° for 10 hours, and the resulting brown solid (0.094 g., m.p. 215–230°) was recrystallized from water and then sublimed to give VI, identical with the material prepared as described above.

1-Methyl-4-hydroxy-2-pyridone (VII).—The method used here was adapted from procedures reported for the hydrolysis of ricininic acid[7] and ricinine.[5] A mixture of 2.0 g. of N-methyl-3-cyano-4-methoxy-6-pyridone and 10 ml. of concentrated hydrochloric acid in a sealed glass tube contained in a steel pressure bomb was heated at 150° for 4.5 hours. The cooled reaction mixture was diluted with 40 ml. of water, filtered, and the filtrate evaporated to dryness. The residue was extracted with boiling absolute ethanol, the extracts evaporated to dryness, the residue triturated with dilute ammonium hydroxide and the mixture again taken to dryness. The resulting residue again was extracted with ethanol, the extracts evaporated to dryness and the residue recrystallized from water to give a white crystalline solid which melted at 60–70°, then solidified and remelted at 160–165° After sublimation at 150° (0.5 mm.), the product melted at 171–172°.

Anal. Calcd. for $C_6H_7NO_2$: C, 57.6; H, 5.6; N, 11.2. Found: C, 57.8; H, 5.6; N, 11.2.

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Synthesis and Properties of High Specific Activity DL-α-Lipoic Acid-S_2^{35}

BY RICHARD C. THOMAS AND LESTER J. REED

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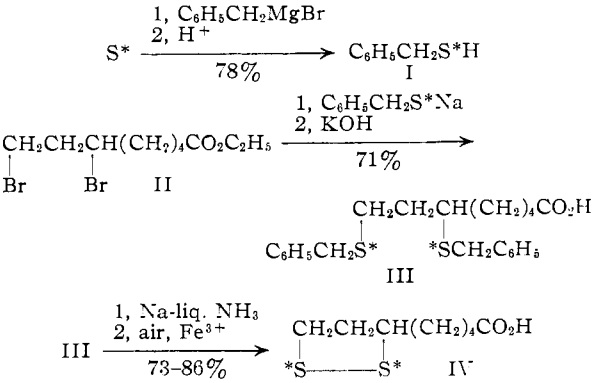
To facilitate studies of the metabolism of the biocatalyst, α-lipoic acid, in this Laboratory, a semimicro method for the synthesis of high specific activity DL-α-lipoic acid-S_2^{35} has been developed. The sequence of reactions employed in the synthesis is shown below.



Benzyl mercaptan-S^{35} was prepared by modification of the method of Wood, *et al.*[1] The recent syn-

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thesis of DL-ethyl 6,8-dibromoöctanoate[2] (II) has made possible the introduction of sulfur 35 into the lipoic acid molecule on a semi-micro scale with good isotopic conversion efficiency (41–48% based on amorphous sulfur-35).

DL-6,8-Dibenzylmercaptoöctanoic acid-S_2^{35} (III) was obtained in crystalline form and was shown by means of one-dimensional paper chromatography to contain no radioactive impurities. With either 2,6-lutidine–water or *n*-butanol–water as solvent systems[3] a single radioactive spot was observed on radioautographs of the chromatograms at R_f values of 0.86 and 0.95, respectively.

The crystalline DL-α-lipoic acid-S_2^{35} (IV) obtained (specific activity 68 μc./mg.) was shown to possess the physical constants and biological activity reported[2,4] for the non-radioactive substance. When the purity of IV was checked by one-dimensional paper chromatography, three radioactive spots were detected. The relative amounts varied slightly with the solvent system used. With 2,6-lutidine–water, 2.5% of the total radioactivity was located at the origin, 2.1% at R_f 0.60, and the remainder at R_f 0.80. The latter two spots correspond[3] to β-lipoic acid (a sulfoxide of α-lipoic acid) and α-lipoic acid, respectively. Whether the two radioactive impurities observed on the paper chromatograms were originally present in the crystalline IV or were artifacts produced during preparation of the paper chromatograms, has not been ascertained. Evidence obtained previously by means of bioautographs[3,5] indicated that β-lipoic acid (Protogen-B) is produced from α-lipoic acid (Protogen-A) after the latter is applied to the paper. In the present investigation we have observed that the percentages of both radioactive β-lipoic acid and the unidentified material which remains at the origin increased when the paper to which IV had been applied was allowed to stand in air before introduction of the solvent.

Samples of compounds III and IV were checked for evidence of radiation decomposition 10 weeks after storage in dry, solid form in a refrigerator. Paper chromatograms of the stored III showed no radioactive impurities. However, evidence was obtained which indicated that radiation decomposition, to the extent of approximately 10%, had occurred to IV during storage. The ϵ_{max} (332 mμ) of IV decreased from a value of 147 to 133 during the 10-week period, whereas that of a sample of non-radioactive DL-α-lipoic acid (ϵ_{max} 147), prepared and stored in an identical manner, remained unchanged. Paper chromatograms of the stored IV showed 9.7% of the radioactivity at the origin, 4.9% as β-lipoic acid, and the remainder as α-lipoic acid, with no other radioactive impurity. It is interesting to note that solutions of IV (approximately 1 mg./ml.) in 95% ethanol or 1% aqueous sodium bicarbonate, which were stored in a refrigerator during the 10-week period, underwent little if any decomposition, as indicated by paper chromatograms.

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Experimental

Benzyl Mercaptan-S^{35} (I).—A solution of elemental sulfur-35 (2.2 mg., 20 mc.)[6] in benzene was transferred to a 40-ml. centrifuge cone containing 17.8 mg. of non-radioactive amorphous sulfur (diluted sulfur-35, 0.65 mmole). The contents of the tube were warmed to dissolve the added sulfur and then cautiously evaporated to a volume of 5 ml. A nitrogen atmosphere was maintained in all subsequent operations. Five milliliters of a 0.5 *M* solution of benzylmagnesium bromide in benzene was added. The tube was stoppered and allowed to stand overnight at room temperature. The resulting suspension was centrifuged and the excess Grignard solution was withdrawn by means of a pipet. The solid was washed with three 5-ml. portions of Skellysolve B[7] by centrifugation and removal of the liquid with a pipet. The solid was suspended in 5 ml. of ice-cold Skellysolve B. One milliliter of 6 *N* hydrochloric acid was added and the mixture was stirred until free of solids. The aqueous layer was separated by means of a pipet and the organic layer was washed with two 1-ml. portions of water. The combined aqueous layers were extracted with 1-ml. portions of Skellysolve B until a negative nitroprusside test was obtained. The organic layers were combined and a small aliquot was analyzed for —SH by micro-titration with a standard iodine solution. The yield of benzyl mercaptan-S^{35} was 78%.

DL-6,8-Dibenzylmercaptoöctanoic Acid-S_2^{35} (III).—To the solution of benzyl mercaptan-S^{35} in Skellysolve B was added 214 mg. of non-radioactive benzyl mercaptan (diluted benzyl mercaptan-S^{35}, 2.2 mmoles). The resulting solution was transferred to a 5-ml. round-bottomed flask containing 330 mg. (1 mmole)[8] of DL-ethyl 6,8-dibromoöctanoate.[2] The solvent was removed by evaporation with a stream of nitrogen at room temperature. A solution of 2.2 mmoles of sodium ethoxide in 2 ml. of absolute ethanol was added to the flask. The mixture was stirred by means of a magnetic stirrer and was heated under reflux in an atmosphere of nitrogen for 5 hours. At the end of this period the reaction mixture was cooled to room temperature and 132 mg. (2 mmoles) of potassium hydroxide was added. The flask was stoppered and stirring was continued for 20 hours at room temperature. The reaction mixture was poured into 10 ml. of water and the resulting solution was extracted with two 2-ml. portions of peroxide-free ether. The aqueous phase was acidified with 6 *N* hydrochloric acid and the product was extracted with five 2-ml. portions of peroxide-free ether. The combined ether extracts were washed successively with 2 ml. of water, two 1-ml. portions of ice-cold 5% sodium bicarbonate solution,[9] and 2 ml. of water. The ether solution was dried over anhydrous sodium sulfate and the solvent was removed with a stream of nitrogen and finally *in vacuo* (oil pump). The resulting solid was dissolved in 1 ml. of warm benzene and 4 ml. of hot Skellysolve B was added gradually. The clear solution was seeded with a small crystal of non-radioactive 6,8-dibenzylmercaptoöctanoic acid and allowed to stand overnight at room temperature and then in a refrigerator for 4 hours. The liquid was removed by means of a pipet and the crystals were washed with three 2-ml. portions of cold Skellysolve B. The yield was 304 mg. (71%, based on benzyl mercaptan-S^{35}), m.p.[10] 68.5–69.5° (uncor.).

(6) Elemental sulfur-35 (P-3) obtained on allocation from the Isotopes Division, United States Atomic Energy Commission.

(7) A *n*-hexane fraction, b.p. 60–68°, obtained from the Skelly Oil Co., Kansas City, Missouri.

(8) In preliminary experiments with non-radioactive benzyl mercaptan it was found that a 10% excess of the latter substance resulted in a higher yield of crystalline 6,8-dibenzylmercaptoöctanoic acid, based on the amount of benzyl mercaptan employed, than did the theoretical amount of benzyl mercaptan, or a 10% excess of the dibromo ester.

(9) In trial runs with non-radioactive materials it was found that the extraction with sodium bicarbonate solution removed unidentified impurities which otherwise interfered with the crystallization of 6,8-dibenzylmercaptoöctanoic acid. The latter substance is only very sparingly soluble in 5% sodium bicarbonate solution.

(10) Melting points were determined on a hot stage viewed with a microscope equipped with polaroid discs.

DL-α-**Lipoic Acid-S_2^{35}** (IV).—To a 15 × 180 mm. tube, equipped with a Hershberg-type stirrer and immersed in a Dry Ice–isopropyl alcohol mixture, was added in succession 5 ml. of anhydrous liquid ammonia, 16 mg. of sodium wire, and a solution of 50 mg. (0.13 mmole) of DL-6,8-dibenzylmercaptoöctanoic acid-S_2^{35} in 2 ml. of peroxide-free, anhydrous ether. The mixture was stirred until decolorized and small pieces of sodium wire were added until a permanent blue color resulted. The blue color was discharged with ammonium chloride, the cooling bath was removed, and the liquid ammonia and ether were allowed to evaporate under a slow stream of nitrogen. To the residue was added 4 ml. of water and the *p*H was adjusted to 9 with 2 *N* hydrochloric acid. Two drops of 1% ferric chloride solution were added and a stream of air was bubbled through the solution from a capillary tube until the reddish color changed to pale yellow (approximately 15 minutes). The mixture was extracted with two 2-ml. portions of peroxide-free ether to remove some suspended solid and then acidified with 6 *N* hydrochloric acid. The product was extracted with three 1-ml. portions of chloroform. The combined chloroform extracts were dried over anhydrous sodium sulfate and then carefully evaporated in a small sublimation apparatus with a stream of nitrogen. The last traces of solvents were removed at 10^{-4} mm. The solid residue of crude DL-α-lipoic acid-S_2^{35} was distilled[11] at 10^{-4} mm. and 90° onto the cold finger which was cooled with powdered Dry Ice. The solid product adhering to the cold finger was rinsed with anhydrous benzene into a small weighing tube. The solvent was removed with a slow stream of dry nitrogen and finally *in vacuo* (oil pump). The yield of microcrystalline product was 19.5–22.8 mg. (73–86%), m.p.[10] 60.5–61.5° (uncor.), ϵ_{max} 147 (332 mμ), specific activity 68 μc./mg.

(11) In trial runs with non-radioactive materials attempts to crystallize the crude product from Skellysolve B[3] produced varying amounts of a Skellysolve B-insoluble viscous material and, consequently, low yields of crystalline product. However, when the crude product was distilled as described, only traces of residue remained.

BIOCHEMICAL INSTITUTE AND THE
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Biosynthesis of Phenylalanine in Bakers' Yeast[1,2]

BY C. R. THOMAS[3], B. E. CHRISTENSEN, V. H. CHELDELIN AND C. H. WANG

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In contrast to the numerous reports on the biosynthesis of tyrosine in microörganisms, the analogous formation of phenylalanine has not been thoroughly investigated. Davis has shown that in *Escherichia coli*, phenylalanine is not a normal precursor of tyrosine.[4] This supports the observation of Simmonds, *et al.*,[5] that tyrosine does not exert a sparing action on the quantitative requirement of a phenylalanine requiring mutant strain of *E. coli*.

On the other hand, the accumulation of prephenic acid by certain tyrosine and phenylalanine auxotrophs[4] points to a possible common biosynthetic pathway leading to these two amino acids. Studies carried out by Gilvarg and Bloch[6] with yeast grown on glucose-1-C^{14} revealed a close resemblance of the labeling pattern of phenylalanine to that of tyrosine from the same yeast. This also suggested that these two amino acids may have been synthesized from glucose by the same general mechanism.

In this Laboratory, isotopic distribution patterns of phenylalanine have been obtained from yeast[7,8] grown on $CH_3C^{14}OCOOH$ and $CH_3C^{14}OOH$. Comparison of these patterns with those in tyrosine isolated from the same yeast[9] have revealed that whereas from pyruvate the isotopic distributions are practically identical, sharp divergences occur when acetate is the substrate. Thus, from acetate, carbons 2 + 4 + 6 of the tyrosine ring account for over 70% of the ring activity,[9] while in phenylalanine the bulk of the ring activity is located in carbons 1 + 3 + 5.

Experimental

Use was made of the yeast samples grown on $CH_3C^{14}OCOOH$ and $CH_3C^{14}OOH$ as described previously.[7]

After microbiological assay of the phenylalanine in the neutral amino acid fraction of the defatted yeast hydrolysate,[8] this amino acid was diluted ninefold with inert (carrier) phenylalanine. The diluted sample was separated from other amino acids by Dowex-50 column chromatography, using a modified version of the method of Stein and Moore.[10] Isolation was completed by the method of Cutinelli, *et al.*[11] The identity and purity of the final product were established by paper chromatography. The quantities of diluted phenylalanine obtained in this manner from three yeast samples were: from acetate, 244 mg.; from pyruvate (aerobic), 337 mg.; and from pyruvate (anaerobic), 284 mg.

The phenylalanine samples thus obtained were degraded according to the method of Gilvarg and Bloch.[6] The results of these degradation studies on phenylalanine are given in Table I, with the earlier data on tyrosine[9] included for comparison. All samples were counted as $BaCO_3$, using a windowless gas-flow Geiger–Müller counter. Counting data were corrected for background and self-absorption in the conventional manner. Since the previous observations on tyrosine were made with the use of a mica end-window counter, the present figures have been reduced by an appropriate factor to render the phenylalanine and tyrosine data comparable. All data are given for the undiluted amino acids.

Discussion

The labeling patterns of the *side chains* of phenylalanine (Table I) from either acetate or pyruvate are in good agreement with those reported in the corresponding tyrosine experiments,[9] hence give additional support to the suggestion[6,9,12] that the side chains of both amino acids originate from an intact C_3 unit such as pyruvate. The mechanism is also in line with the recent findings,[13,14] that prephenic acid, formed by condensation of shikimic

(1) This research was supported by Contract No. AT(45-1)-301 from the Atomic Energy Commission. Published with the approval of the Monographs Publications Committee, Research paper No. 276, School of Science, Department of Chemistry. Presented before the Northwest Regional Meeting of the American Chemical Society, Richland, Washington, June, 1954.

(2) Taken in part from the Ph.D. thesis of R. C. T., Oregon State College, 1954.

(3) National Science Foundation Predoctoral Fellow.

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