nated as below: *BAW*—butanol: acetic acid: water—4:1:5. The upper phase was used: *BAm*—butanol: 1.5*N* ammonium hydroxide—1:1. The upper phase was used. *MPW*—methyl ethyl ketone: pyridine: water—4:1:1.6. The compounds were located on the paper by means of ninhydrin (N) or diazotized sulfanilic acid (P). A compound which has an R_5 value of 0.5 in the MPW system and was located with ninhydrin reagent is reported as R_f^{MPW} 0.5 (N).

*N-Carbobenzyloxy-*L*-valyl-*L*-tyrosyl-*L*-tyrosine methyl ester* (IIIa). A solution of 12.5 g. of L-tyrosine methyl ester[2] in 92 ml. of *N,N*-dimethylformamide was cooled to about 0° in an ice bath and 13.5 g. of *N,N'*-dicyclohexylcarbodiimide was added. A cold solution of 26.6 g. of *N*-carbobenzyloxy-L-valyl-L-tyrosine[1] in 180 ml. of ethyl acetate was added dropwise during a period of 30 min. while the reaction mixture was stirred. After 2 hr. at 0°, the mixture was kept at room temperature for 16 hr. The *N,N'*-dicyclohexylurea (12 g.; 82%) was removed and the filtrate was concentrated at reduced pressure. The residual oil (55 g.) was dissolved in 150 ml. of warm ethyl acetate and kept at 0° for 2 hr. About 0.2 g. of additional *N,N'*-dicyclohexylurea was removed. The ethyl acetate solution was washed with 100 ml. of saturated sodium bicarbonate solution and 100 ml. of saturated sodium sulfate solution. The product (23 g.) crystallized rapidly from the ethyl acetate layer. Recrystallization from 75 ml. of methanol gave 16.5 g. of *N*-carbobenzyloxy-L-valyl-L-tyrosyl-L-tyrosine methyl ester, m.p. 173–176°. A 0.5-g. sample of the product, recrystallized from 5 ml. of methanol, gave 0.38 g. of *N*-carbobenzyloxy-L-valyl-L-tyrosyl-L-tyrosine methyl ester, m.p. 175–177°; $[\alpha]_D^{25}$ −6.0° (*c*, 2.02 in *N,N*-dimethylformamide).

Anal. Calcd. for $C_{32}H_{37}N_3O_8$ (591.6). C, 64.96; H, 6.30; N, 7.10. Found: C, 65.27; H, 6.73; N, 6.97.

*N-Carbobenzyloxy-*L*-valyl-*L*-tyrosyl-*L*-tyrosine* (IIIb). *N*-Carbobenzyloxy-L-valyl-L-tyrosyl-L-tyrosine methyl ester (9.4 g.; 15.9 mmoles) was dissolved in a mixture of 100 ml. of methanol, 50 ml. of acetonitrile and 32.7 ml. of 1.0*N* sodium hydroxide. An additional 32.7 ml. of 1.0*N* sodium hydroxide and 50 ml. of water were added and the solution was kept at room temperature for 1.5 hr. The reaction mixture was diluted with 250 ml. of water and acidified (*p*H 1) with concentrated hydrochloric acid. After being cooled in an ice bath for 1 hr., the mixture was filtered. The dried product, m.p. 175–205°, weighed 9.7 g.

The product above was combined with similar material from another preparation giving a total of 11.9 g. which was dissolved in 250 ml. of boiling acetone. The cooled solution was chromatographed on a column of 300 g. of acid-washed alumina. Elution with 1200 ml. of acetone gave 1.5 g. of recovered starting ester. Elution with 1 l. of methanol–formic acid (9:1) gave, after concentration of the eluate to a volume of 100 ml., 7.2 g. of *N*-carbobenzyloxy-L-valyl-L-tyrosyl-L-tyrosine, m.p. 214–217°.

A 0.5-g. sample was dissolved in 15 ml. of acetone and 50 ml. of water was added; 0.45 g. of crystalline product was obtained, m.p. 215–217°, $[\alpha]_D^{25}$ +1.7° (*c*, 2.33 in *N,N*-dimethylformamide); R_f^{BAm} 0.5–0.7(P).

Anal. Calcd. for $C_{31}H_{35}N_3O_8$ (577.61): C, 64.46; H, 6.11; N, 7.28. Found: C, 64.57; H, 6.26; N, 7.15.

*N-Carbobenzyloxy-*L*-valyl-*L*-tyrosyl-*L*-tyrosyl-*L*-isoleucyl-*L*-histidyl-*L*-prolyl-*L*-phenylalanine methyl ester* (V). A solution of 4.0 g. of L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanyl methyl ester dihydrobromide[4] in 100 ml. of water was washed with 50 ml. of ethyl acetate. The aqueous phase plus 200 ml. of chloroform was cooled and adjusted to *p*H 10 with saturated potassium carbonate solution. The chloroform layer was separated and the aqueous layer was extracted with an additional 100 ml. of chloroform. The dried chloroform solution was concentrated at reduced pressure. About 2.2 g. of L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine methyl ester was obtained as an oil.

This tetrapeptide methyl ester was dissolved in 20 ml. of ethyl acetate; 0.9 g. (4.3 mmoles) of *N,N'*-dicyclohexylcarbodiimide was added. The mixture was cooled to about 0° and a solution of 2.4 g. (4.2 mmoles) of *N*-carbobenzyloxy-L-valyl-L-tyrosyl-L-tyrosine in 15 ml. of *N,N*-dimethylformamide was added dropwise. Stirring was continued at room temperature for 16 hr.

The precipitated *N,N'*-dicyclohexylurea (0.42 g.) was removed and the filtrate was concentrated at reduced pressure. The residue was dissolved in 100 ml. of ethyl acetate-*n*-butyl alcohol (3:1) and the solution was washed with two 50-ml. portions of 1*N* hydrochloric acid, two 50-ml. portions of saturated aqueous sodium bicarbonate and 25 ml. of water. The organic phase was concentrated at reduced pressure and a 4.7-g. residue was obtained. This was dissolved in 50 ml. of warm acetonitrile. When the solution was cooled, 2.2 g. of crude substituted heptapeptide precipitated. For further purification, it was dissolved in 20 ml. of isopropyl alcohol and chromatographed on 150 g. of acid-washed alumina. Elution with isopropyl alcohol containing increasing amounts of methanol gave a total of 0.46 g. of product containing impurities. Finally, elution with methanol gave 1.3 g. of product. This material was dissolved in 100 ml. of acetone, 400 ml. of water was added, and the solution was concentrated to 250 ml., giving 0.95 g. of *N*-carbobenzyloxy-L-valyl-L-tyrosyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine methyl ester, m.p. 205–210°, $[\alpha]_D^{25}$ −59° (*c*, 2.0 in methanol). A sample after treatment with hydrogen bromide in acetic acid showed R_f^{BAW} 0.78 (N).

Anal. Calcd for $C_{58}H_{71}N_9O_{12}$ (1086.2). C, 64.13; H, 6.59; N, 11.61. Found. C, 63.85; H, 6.79; N, 12.27.

L-Valyl-L-tyrosyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine methyl ester dihydrochloride (I). A mixture of 817 mg. of *N*-carbobenzyloxy-L-valyl-L-tyrosyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine methyl ester, 400 mg. of 10% palladium on charcoal and 50 ml. of acetic acid was shaken with hydrogen at low pressure for 16 hr. The catalyst was removed and the filtrate was concentrated at reduced pressure. The product showed R_f^{BAW} 0.80 (N) and R_f^{MPW} 1.0 (N), while L-valyl-L-tyrosyl-L-tyrosine showed R_f^{BAW} 0.70 (N) and R_f^{MPW} 0.80 (N) and L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine showed R_f^{BAW} 0.70 (N) and R_f^{MPW} 0.90 (N).

The product was dissolved in 6 ml. of methanol and treated with dry hydrogen chloride. When ether was added, the hydrochloride precipitated. This material was reprecipitated from 3 ml. of methanol with 50 ml. of acetone. The yield of L-valyl-L-tyrosyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine methyl ester dihydrochloride, $[\alpha]_D^{25}$ −34.3° (*c*, 1.05 in 0.1*N* hydrochloric acid) was 630 mg.

Anal. Calcd. for $C_{50}H_{67}N_9O_{10}Cl_2$ (1024.1). C, 58.60; H, 6.59; Cl, 6.92. Found: C, 58.66; H, 6.59; Cl, 7.27; ash, 1.4.

The ratios of the amino acids in this product relative to phenylalanine were Val, 1.04; Tyr, 1.91; Ileu, 0.98; His, 0.96; Pro, 1.05; Phe, 1.00.[6]

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(6) Amino acid analyses were carried out in the laboratories of Prof. M. Brenner of the Organisch-Chemische Anstalt der Universität, Basel, Switzerland by the Moore and Stein method.

Improved Preparation of Phosphorylethanolamine and Phosphorylcholine

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Received April 27, 1960

The cytidine and deoxycytidine diphosphates of choline and ethanolamine occur in living matter

and they are intermediates in the formation of phospholipids.[1] In order to elucidate the mechanism whereby phosphorylethanolamine and phosphorylcholine are incorporated into these natural products, a convenient synthesis of the carbon-14-labeled compounds was required. The available methods leading to phosphorylcholine and ethanolamine involved phosphorylation of the alcohols by phosphoric acid with or without phosphorus pentoxide, by phosphorus oxychloride, by diphenylphosphoryl chloride, or required phosphorylation of an ethylene chlorohydrin followed by amination of the chloro derivative.[2] However, they suffer from various disadvantages in regard to the yields achieved, or the complexity of the procedures required. In addition, the products were often obtained as salts, rather than the free compounds.

This paper describes a simplified methodology giving about 60% yields of C^{14}-labeled, free phosphorylethanolamine or choline starting with these amino alcohols and phosphoric acid. In principle, the amino alcohol or its hydrochloride salt was heated *in vacuo* for twelve hours with 1.5–2 fold molar excess[3] of phosphoric acid, the water evolved being trapped in phosphorus pentoxide, and the hydrochloric acid in solid sodium hydroxide. An important factor was the temperature at which the reaction was performed. With ethanolamine 135–140° was optimal. The recommended temperature[2c] of 190° for three hours led to an 85% loss of authentic phosphorylethanolamine. With choline the best temperature was 155–160°. Lower temperatures gave increased recoveries of starting material, while higher ones led to what is believed to be polyphosphorylated compounds.[4] The products were isolated by ion-exchange chromatography[5,6] which allowed the recovery and recycling of unreacted starting material.

EXPERIMENTAL

Phosphorylethanolamine. To an ice-cold solution of 0.84 mmole of ethanolamine-1,2-C^{14} hydrochloride (3.59×10^8 counts per min., c.p.m.)[7] supplied by the Volk Radiochemical Co., Chicago 40, Ill., and contained in the tube of an Abderhalden type drying apparatus (Kontes Glass Co., Vineland, N. J., No. K-56350) in 3 ml. of water, was added 0.8 ml. of 2*M* phosphoric acid, and most of the water was removed *in vacuo*. The desiccant flask holding phosphorus pentoxide and a small tube with some pellets of sodium hydroxide was attached, and the assembly was evacuated to a pressure of 3–5 mm. The stopcock was closed and the tube immersed in an oil bath kept at 138 ± 2°. After 12 hr. 75 ml. of water was added and the *p*H adjusted to about 9 with 2.1 ml. of 1*N* potassium hydroxide.

The solution was applied to a 1 × 30 cm. Dowex 1 formate column (200–400 mesh, 2% cross linked) and washed in with 100 ml. of water. The effluent (*A*) was ninhydrin-positive and radioactive. The column was transferred to a fraction collector and gradient elution begun with a 300 ml. mixing flask containing water into which 0.045*N* formic acid was fed.[6] Fractions of 20 ml. were collected every 30 min. Phosphorylethanolamine was found in tubes 14 to 17 (effluent distinctly acid). Removal of solvent *in vacuo* by means of a rotating evaporator left 69.2 mg. (58%) of white crystalline material, m.p. 236–237° (lit.[2d] m.p. 237°) with an activity of 2.16×10^8 c.p.m. (60%). Paper chromatography of the product in two dimensions, as described by Smith,[8] gave a single radioactive (autoradiography), ninhydrin-positive spot with the correct mobility.

To effluent *A* above, containing the unchanged C^{14}-ethanolamine, was added 0.6 mmole of unlabeled ethanolamine and 1.2 mmoles of phosphoric acid and the solution was reduced to a small volume *in vacuo*. After the addition of 3 ml. of 1*N* potassium hydroxide the mixture was distilled *in vacuo* with a 90° bath temperature and a receiver cooled in Dry Ice. After transfer of the distillate to the reaction vessel and acidification with 1.2 mmoles of phosphoric acid the phosphorylation was repeated, yielding 42.5 mg. of phosphorylethanolamine with an activity of 5.15×10^7 c.p.m. Thus, the total recovery of radioactivity was 2.68×10^8 c.p.m. or 75%.

Phosphorylcholine. A solution of 0.521 mmole of choline-1,2-C^{14} chloride (1.88×10^6 c.p.m., Volk Radiochemical Co.) in 4 ml. of water and 0.5 ml. of 2*M* phosphoric acid was taken to dryness *in vacuo* in the tube of the drying apparatus as in the previous experiment. The assembly was kept in an oil bath at 160 ± 2° for 12 hr. Then, a solution of the reaction mixture in 50 ml. of water, adjusted to *p*H 9 by means of 1.5 ml. of 1*N* potassium hydroxide, was applied to a 1 × 30 cm. column of Dowex 1 formate and washed in with 35 ml. of water. The eluate containing the unchanged choline accounted for 6.54×10^5 c.p.m. (35%). Gradient elution, as described for ethanolamine, gave radioactivity in tubes 11–13 (24 ml. per tube) which contained 1.19×10^6 c.p.m. (63%).

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(3) Slightly lower yields (45–50%) of phosphorylated products were obtained with equimolar amounts of amino alcohol and phosphoric acid, but the yields dropped sharply if a fourfold excess of acid was used.

(4) In one experiment in which the temperature rose to 178° for the major part of the reaction period, 22% of the radioactivity was accounted for as unchanged choline, 38% as phosphorylcholine, 5% as material eluted from the Dowex 1 column with 5*N* formic acid, and 13% as material eluted with 1*N* sodium hydroxide. Paper chromatography of the latter two fractions gave a number of spots, different from choline, or phosphorylcholine. Hydrolysis of these two materials for 24 hr. in refluxing 6*N* hydrochloric acid yielded mainly choline, and small amounts of phosphorylcholine.

(5) We are grateful to Drs. E. A. Peterson and H. A. Sober for valuable discussion on procedures.

(6) E. P. Kennedy, *J. Biol. Chem.*, **222**, 185 (1956).

(7) Determinations of radioactivity were performed by direct plating of aliquots of solutions on glass disks, followed by counting in a gas-flow counter with a 48% efficiency for carbon-14. In the case of ethanolamine, choline, and the corresponding column eluates a few drops of 2*M* phosphoric acid were added to the aliquots in a volumetric flask in order to avoid loss of material by volatilization during the plating and counting operations.

(8) I. Smith, *Chromatographic Techniques*, W. Heinemann, London, 1958, Fig. 4.3.

After removal of the solvent a noncrystalline material resulted at first. Upon drying *in vacuo* over magnesium perchlorate 69.5 mg. (66%) of phosphorylcholine was obtained as a white solid,[9] m.p. 235–240° with decomposition.

Anal. Calcd. for $C_5H_{16}NO_5P$: N, 6.96; P, 15.40. Found: N, 7.10; P, 15.56.

The solution of the unchanged choline, above, was acidified with hydrochloric acid, the solvent evaporated *in vacuo*, and choline hydrochloride extracted from the residue with absolute ethanol. After dilution with unlabeled choline chloride, addition of phosphoric acid and removal of the solvent, another reaction cycle was performed. This gave a 57% yield and a total recovery of 75% of the radioactivity.

Addition of hydrochloric acid to a solution of phosphorylcholine in water, followed by removal of the solvent gave a white powder of *phosphorylcholine chloride*, m.p. 108–111° dec., soluble in water and ethanol. The compound readily lost hydrogen chloride upon drying at temperatures of 60–100°.

Anal. Calcd. for $C_5H_{15}ClNO_5P$: Cl, 14.86. Found: Cl, 15.15.

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(9) This compound showed an infrared spectrum identical to that of a product obtained by passing a solution of commercial calcium phosphorylcholine hydrochloride (California Foundation for Biochemical Research, Los Angeles 63) successively through Dowex 50 in the hydrogen form (elution with a hydrochloric acid gradient), and through Dowex 1 formate, as described above.

On the Reaction of Dihydroxypurines with Phosphorus Pentasulfide

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Received September 19, 1960

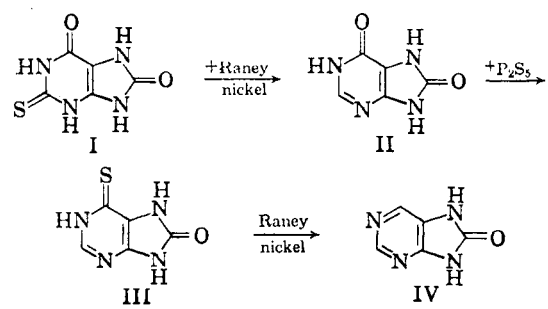
It is known that in xanthine and 4,5-diaminouracil reaction with phosphorus pentasulfide exchanges selectively the oxygen atom at C-6.[1,2] On the other hand, in the reaction with uric acid, one obtains—in addition to the main product, 6-thiouric acid—products thiated at position 8.[3] In order to determine the directive influence of oxygen in various positions of the purine ring on the thiation process, we have studied the behavior of the isomeric dihydroxypurines.

2,8-Dihydroxypurine was not attacked by phosphorus pentasulfide under a variety of conditions, using either pyridine or tetraline as solvent. However, 6,8-dihydroxypurine (II) reacted smoothly to give a 75% yield of pure 8-hydroxypurine-6-thione (III). No other purine derivative was found in the reaction mixture. As II is accessible from 2-thiouric acid (I), the thio derivative III has now become easily available.

For the synthesis of II, it has been found preferable to desulfurate 2-thiouric acid instead of 2-mercapto-6-hydroxy-4,5-diaminopyrimidine.[4] Although the latter procedure gives an 89% yield of the 6-hydroxy derivative, subsequent cyclization with urea[5] or phosgene[6] leaves much to be desired. On the other hand, direct cyclization of the above mercaptopyrimidine gives 2-thiouric acid in almost quantitative yield[7,8] and subsequent catalytic desulfuration results in a 64% yield of 6,8-dihydroxypurine (I).

8-Hydroxypurine-6-thione (III) in its turn may serve as a source of 8-hydroxypurine (IV). Desulfuration of III was tried under a variety of conditions. Even the best yields, obtained by carrying out the reaction in concentrated ammonia, were only 30%. Therefore, this route to IV has no advantage over the conventional method of cyclization of 4,5-diaminopyrimidine.[5]

In view of the low yields of IV, and taking into account previous experience with 3,7-dimethyl-2-hydroxypurine-6-thione,[9] desulfuration of the *S*-methyl ether (IIIa) of III was also tried. IIIa can not be prepared by methylation in the presence of sodium hydroxide because of the great sensitivity of the ether to alkali. By using pyridine instead, a 41% yield of IIIa was secured. Conversion of IIIa into IV proved even less satisfactory than the desulfuration of III. With IIIa, the reaction cannot be carried out in the presence of sodium hydroxide or ammonia. When working in aqueous suspension at a temperature of 70–80°, a large portion of the starting material was recovered. At higher temperatures, a considerable part of IV was apparently converted into a dihydro derivative as judged by the fact that the optical density of the solution at 280 mμ, the λ_{max} of IV, first increased, but then diminished again. Similar un-



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