ture. A solution of 11 g. (0.275 mole) of sodium hydroxide in 40 cc. of water was then added dropwise over a period of twenty minutes. The stirring was continued for a total of one hour, and the product was collected by filtration. The mother liquor was cooled overnight in a refrigerator and a small additional amount of product was obtained. The combined crops of the product weighed 13.5 g. (100%). After one recrystallization from ethanol, the methionine anhydride weighed 10.1 g. (75%) and melted at 226–227.5°. A mixed-melting point determination with an authentic sample of methionine anhydride was not depressed. It was of sufficient purity to give *dl*-methionine in 85–95% yields. Direct hydrolysis[1] of the crude product gave 10 g. (65%) of crude *dl*-methionine.

Summary

3,6-Bis-(β-chloroethyl)-2,5-diketopiperazine is converted quantitatively to the bis-isothiouronium salt by treatment with thiourea in boiling ethanol.

Treatment of the bis-isothiouronium salt with dilute alkali, followed by oxidation with air in the presence of ferric chloride and hydrolysis with acid, provides a convenient synthesis of homocystine.

Decomposition of the bis-isothiouronium salt with alkali in the presence of methyl sulfate produces *dl*-methionine. This method of converting the dichlorodiketopiperazine to *dl*-methionine is superior to that previously described.

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[FROM THE DEPARTMENT OF BIOCHEMISTRY AND PHARMACOLOGY, THE UNIVERSITY OF ROCHESTER SCHOOL OF MEDICINE AND DENTISTRY]

Metabolism of Phosphoryl Choline. I. Synthesis of Calcium Phosphoryl Choline Chloride Containing the Radioactive Isotope, P^{32} [1]

BY RICHARD F. RILEY

Although monophosphoric acid esters of both choline[2] and ethanolamine[3,4] have been isolated from normal tissues, their place in metabolism has not been adequately described. An extension[5] of available information on phosphoryl choline (Smith,[6] Welch and Welch,[7] and Taurog[8]) therefore appeared of interest. Since an undue increase in tissue concentration of any substance is to be avoided where its normal metabolism is to be followed (ref. 9, p. 292) and since labeling this molecule with radiophosphorus would provide a convenient means of following the metabolic path of that portion of the ester, a highly radioactive preparation was desirable.

The published procedures for the synthesis of the monophosphoric acid ester of choline are not well suited to preparation of the ester containing radiophosphorus. They permit extensive isotope dilution[2,10,11] or yield products contaminated by the diester and inorganic phosphate.[12] Accordingly, the method of preparation has been adapted to efficient use with radioactive phosphorus and/or choline containing tracer elements, and to elimination of contaminating compounds. The phosphoryl choline containing radiophosphorus, of which the preparation is described here, was of satisfactory purity and, allowing for decay, possessed the same activity per mg. of phosphorus as the starting material.

The ester was prepared in most satisfactory yields by heating equimolar quantities of 100% phosphoric acid with dry choline chloride under reduced pressure for twelve hours at 165°, and isolating the product as the calcium chloride salt. In preliminary attempts at improvement of the synthesis, several new derivatives of the mono- and dicholine esters of phosphoric acid were prepared for purposes of identification and comparison. An attempt to form the ester by treating bromocholine bromide and trisilver phosphate or disilver monophenyl phosphate gave instead excellent yields of neurine. It was also found that choline hydroxide and phosphoric acid may be esterified in refluxing toluene to give a satisfactory product, although in rather low yield and with considerable decomposition.

Experimental

Calcium Phosphoryl Choline Chloride Containing P^{32}.—150 mg. of phosphoric acid containing P^{32} in 6 ml. of water was introduced into a pear-shaped flask of 35 ml. capacity. The flask was fitted with a standard taper 24 stopper and two outlets with glass stopcocks. The dilute acid was evaporated to 100% phosphoric acid by immersion in an oil-bath which was gradually raised to 180° as a stream of dry air was passed through the flask. Only 0.005% of the activity was lost by this evaporation. When complete, 214 mg. of pure dry choline chloride was well mixed with the phosphoric acid and about 1 g. of phosphorus pentoxide mixed with asbestos contained in a Soxhlet thimble,

(1) The substance of this paper was presented in part before the American Chemical Society at Pittsburgh, September, 1943. This investigation was supported by a grant from the Nutrition Foundation, Inc., of New York City.

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supported over the reactants. The contents were maintained for twelve hours at 165° under vacuum. On cooling, the products were taken up in a few ml. of water, 100 mg. of calcium chloride added and the solution brought to the phenolphthalein end-point with saturated calcium hydroxide solution and worked up by the method of Plimmer and Burch[11]; yield 306 mg. or 63% of thin colorless rectangular plates. Unreacted phosphate, recovered as calcium phosphates during the isolation, was salvaged for further use by conversion to magnesium ammonium phosphate. The total recovery of radiophosphorus as the ester and magnesium ammonium phosphate was 96%. *Anal.* Calcd. for $C_5H_{13}O_4NPCaCl\cdot 4H_2O$: C, 18.2; H, 6.37; N, 4.25. Found: C, 18.11; H, 6.42; N, 4.19.

By isotope dilution, the product was found to contain 0.02% contaminating inorganic phosphorus and < 0.05% by colorimetric analysis. Calcium phosphoryl choline chloride prepared in this fashion contains negligible amounts of the diester, as indicated by analysis and the fact that measurable quantities of the insoluble diaurichloride could not be obtained from larger scale preparations utilizing non-isotopic phosphorus.

Phosphoryl Choline Reineckate.—3.37 g. of recrystallized phosphoryl choline aurichloride, prepared according to Jackson,[12] was decomposed with powdered silver by the procedure of Dudley.[13] The dilute aqueous solution of phosphoryl choline chloride so obtained was evaporated to a thin sirup, brought to incipient precipitation with ethyl alcohol and to this added a 100% excess of acidified ammonium reineckate in ethyl alcohol. The precipitated salt was centrifuged and washed twice with 20-ml. portions of alcohol with centrifugation and finally filtered on a sintered glass filter; yield 2.17 g. or 99%.

The compound does not precipitate well from an aqueous or acetone solution (*cf.* choline reineckate) although when once dried it redissolves only slowly in these solvents. It is quite insoluble in ethyl alcohol and dioxane after it has once been obtained in the dry state. *Anal.* Calcd. for $C_{14}H_{36}O_8N_8S_4P_2Cr$: N, 16.37; P, 9.05. Found: N, 16.28; P, 8.54.

Phosphoryl Choline Mercuric Chloride Complex.—0.75 g. of the aurichloride was decomposed with silver as above. The phosphoryl choline chloride, contained in 5 ml. of water, was precipitated as the mercuric chloride complex by the addition of an excess of a saturated alcoholic solution of mercuric chloride. The complex was recrystallized once from warm 80% alcohol, filtered, washed with absolute alcohol and dried; yield 1.08 g. or 76% as beautiful long white needles, singly and in clusters. It is more soluble in water than the mercuric chloride complex of choline; m. p. 180–184° cor. *Anal.* Calcd. for $C_5H_{15}O_4NP\cdot 3HgCl_2$: N, 1.40; P, 3.10. Found: N, 1.45; P, 3.26.

Phosphoryl Choline Phosphotungstate.—A preparation of characteristic composition was not obtained although phosphotungstic acid gave a water-insoluble microcrystalline product which was extremely difficult to filter. It contained 0.91% N; yield 71% based on N analysis.

Dicholine Phosphate Reineckate.—This was prepared by the same procedure given for the monoester: 0.35 g. of the diaurichloride gave 0.23 g. of reineckate; very slightly soluble in water, acetone, ether and chloroform; insoluble in ethyl alcohol. This salt probably is of variable composition. That obtained showed N, 19.70; P, 3.92; yield 79% based on P analysis.

Dicholine Phosphate Mercuric Chloride Complex.—This was prepared by the same procedure given for the monoester complex: 0.30 g. of the diaurichloride gave 0.13 g. of a fluffy crystalline powder on recrystallization of the crude product from hot water; m. p. 202–207°; N, 0.89; yield 13% based on N analysis.

Direct Esterification of Choline Hydroxide by Phosphoric Acid in Toluene.—Ten grams of choline chloride was dissolved in 50 ml. of water and stirred in the dark with a calculated excess of silver carbonate until the filtrate gave a negative test for halogen. It was filtered and the calculated quantity of barium hydroxide added. The solution was filtered from barium carbonate and the filtrate evaporated down under nitrogen with reduced pressure. The sirup obtained was distilled with toluene to remove further water, 3.83 ml. of 100% phosphoric acid added slowly and distillation continued until no more water came over with the toluene, further toluene being added as required. On cooling the toluene was decanted from the residue which was washed with two small portions of absolute alcohol and dried in the air. The residue was taken up in 10 ml. of water, filtered and precipitated by addition of 10 volumes of absolute alcohol. The precipitated sirup was taken up in water, 8.0 g. of calcium chloride added and the solution neutralized to phenolphthalein with saturated calcium hydroxide solution. The filtrate[14] from this solution was evaporated under reduced pressure on the steam-bath to a thin sirup and precipitated by the addition of 200 ml. of absolute alcohol. The crystalline product on drying in air following a second precipitation weighed 3.2 g. or 24%. The product was obtained as thin fragile rectangular plates or needles depending on the rate of crystallization. Analysis showed this to be the calcium chloride salt of Plimmer and Burch.[11] *Anal.* Calcd. for $C_5H_{13}O_4NPCaCl\cdot 4H_2O$: P, 9.46; N, 4.25; H_2O, 21.86. Found: P, 9.2; N, 4.10; H_2O, 21.4; ionic P, 0.07.

Reaction of Bromocholine Bromide and Silver Phosphates.—Five grams of trisilver phosphate suspended in 300 ml. of absolute alcohol reacted with 2.95 g. of bromocholine bromide by refluxing for six hours in the dark. The reaction mixture was cooled, filtered to separate silver bromide and the filtrate, after decolorization with Norite, worked up in two parts to give the picrate and aurichloride of the main reaction product; yield 65%. The neurine picrate melted at 270° and the aurichloride at 243°. *Analysis* of the picrate showed: calcd. for $C_{11}H_{14}O_7N_4$: C, 42.05; H, 4.49; P, 0.00. Found: C, 42.17; H, 4.36; P, 0.00. A similar experiment employing disilver monophenyl phosphate in place of the trisilver phosphate gave an 89% yield of neurine isolated as the picrate.[15]

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Summary

1. An economical method for the preparation of phosphoryl choline containing isotopic elements has been developed and applied to the synthesis of the ester containing highly active radiophosphorus.

2. Several new derivatives of the mono- and dicholine phosphoric acid esters have been described.

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(14) Filtration of these solutions containing calcium phosphates may be difficult due to the gelatinous nature of the suspended material. However, use of filter aids is not recommended as it was found that the desired compound is strongly adsorbed by such preparations.

(15) Disilver monophenyl phosphate was prepared from the acid kindly supplied by the Victor Chemical Company.