Higgins and Ralph G. Beaman on direction of this research, by Dr. C. R. Bohn on the infrared analyses and their interpretations and by members of the analytical divisions of the Pioneering Research Laboratory.

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[CONTRIBUTION FROM THE DEPARTMENTS OF BIOCHEMISTRY AND UROLOGY, COLLEGE OF MEDICINE, STATE UNIVERSITY OF IOWA]

Phosphoramides Labeled with P^{32} which also Contain Phosphoric Acid Monoester Groupings

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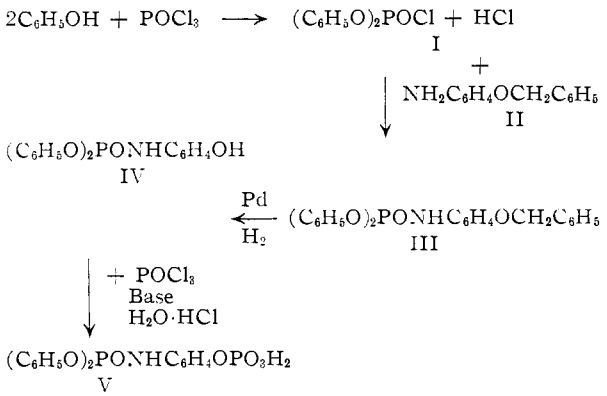
4-(O,O-Diphenylphosphoramido)-phenylphosphoric acid (D4P) in which the phosphoramido group has been labeled with P^{32} has been prepared for the purpose of testing its potential concentration in prostatic tissue.

In order to test the proposition that compounds having an orthophosphoric acid monoester grouping may be hydrolyzed by the acid phosphatase of the human prostate *in vivo*, it was desirable to synthesize phosphorylatable compounds insoluble in biological fluids and labeled with a radioactive element. If the phosphoric acid ester of such a compound were hydrolyzed by prostatic enzyme, the alcohol residue should be preferentially deposited in prostatic tissue. P^{32} was chosen because of its desirable radiation characteristics, chemical properties and ease of analysis. For this purpose it was necessary that the labeled phosphorus be bound in a manner that would make it relatively stable to the enzyme present in the body. Mixed ester amides of orthophosphoric acid were selected as the most readily obtainable compounds which possessed these characteristics. Finally it was necessary for these compounds to possess one or more hydroxyl groups which could be phosphorylated to form an ortho ester capable of being split by acid phosphatase.

The following syntheses have been carried out to produce these compounds



Compound V was first synthesized from unlabeled phosphorus oxychloride and its toxicity was determined. The synthesis was then repeated with P^{32}-labeled phosphorus oxychloride which was prepared from radioactive phosphoric acid by treating it with phosphorus pentachloride as described by Kalinsky and Weinstein.[2] This phosphoric acid ester, V, was insoluble in water and soluble in ethanol. After acidification with strong hydrochloric acid, the gummy acid was washed with water and dissolved in ethanol from which the cyclohexylamine salt was obtained readily. This salt apparently was a mixture of the mono- and diamine salts; therefore it was converted to the barium salt by shaking with barium acetate solution. The barium salt gave good analytical figures which proved the composition of compound V. The soluble sodium salt was easily obtained from the insoluble barium salt by shaking with a slight excess of sodium sulfate solution. The resulting colorless solution, after sterilization by filtration, proved to be satisfactory for intravenous or subcutaneous injections.

The results of the pharmacological and clinical testing of this compound will be described elsewhere. 4-(O,O-Diphenylphosphoramido)-phenylphosphoric acid (D4P) as its sodium salt was given intramuscularly to dogs and also to elderly men just previous to scheduled partial prostatectomies. A transient concentration of this compound was observed in prostatic tissue which was less than that found in liver, kidney, jejunum and lung, but higher than the concentrations which were found in most of the other tissues.

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Experimental

P^{32}-Labeled Diphenyl Phosphorochloridate[3] (I).—Radioactive phosphorus oxychloride (5.49 g.) prepared by the method of Kalinsky and Weinstein[2] was heated with 6.75 g. of phenol in 15 ml. of xylene solution for 36 hours in an oil-bath at 160–170°. The product was transferred by suction

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(2) J. L. Kalinsky and A. Weinstein, THIS JOURNAL, **76**, 5882 (1954).

(3) G. M. Kosolapoff, "Organophosphorus Compounds," John Wiley and Sons, Inc., New York, N. Y., 1950, p. 244.

via an inverted glass U tube to a small Claisen distilling flask. By drawing out the ends of the glass tubing and applying heat to the distilling flask, it was possible to remove by distillation the solvent during the transfer operation. Washings with small portions of xylene removed most of the radioactive product from the reaction flask. By the use of this technique transfer could be effected with a minimum of contamination of the outside of the glassware. The reaction vessels were readily cleaned of radioactive compound by washing with acetone.

A two-pronged adapter with 19/22 glass joints was used to separate the first fraction of dichloridate from the desired second fraction of monochloridate which was collected between 141–155° at 0.7 mm. pressure. Two milliliters (2.55 g.) of non-radioactive diphenyl phosphorochloridate was added to the distillation flask after the completion of the distillation and distilled over into the same receiver. Total yield of product was 6.73 g. or 4.18 g. before carrier was added, giving a yield of 42.5% based on radioactive $POCl_3$ used.

Diphenyl N-(4-Benzyloxyphenyl)-phosphoramidate (III).—Diphenyl phosphorochloridate (unlabeled) (2.68 g., 0.01 mole) was dissolved in 10 ml. of cold purified lutidine together with 2 g. (0.01 mole) of 4-benzyloxyaniline.[4] Lutidine hydrochloride started to crystallize at once. The product also crystallized out of solution while standing at room temperature for four hours. An excess of water was added which dissolved the lutidine hydrochloride and caused the separation of crystalline product. The product was washed with water, 2 *N* HCl and finally with water until free of acid. The final washing was accomplished by heating with 100 cc. of water on a steam-bath until the internal temperature reached 80°, filtered and dried; m.p. 134–136°; yield 3.8 g. (88%). For analysis, 3 g. of the product was dissolved in 50 ml. of hot benzene; filtered and allowed to crystallize in the cold; filtered and dried at 90°; m.p. 137°.

Anal. Calcd. for $C_{25}H_{22}NO_4P$: C, 69.60; H, 5.14; N, 3.25; P, 7.18. Found: C, 69.48, 69.59; H, 5.25, 5.23; N, 3.25; P, 7.26.

The synthesis was repeated using the P^{32}-labeled diphenyl phosphorochloridate. Special precautions and apparatus were used to avoid physical contact with the radioactive materials.

Diphenyl N-(4-Hydroxyphenyl)-phosphoramidate (IV).—Unlabeled diphenyl N-(4-benzyloxyphenyl)-phosphoramidate (3.8 g.) from the previous experiment was dissolved in 200 ml. of hot absolute ethanol and reduced at 40 lb. pressure with 2 g. of 10% $PdCl_2$ on Norite. The reduction was complete in about 2 minutes. The charcoal catalyst was removed by filtration through Super-Cel and the filtrate concentrated under reduced pressure to about 25 ml. when crystallization set in. After standing overnight in the cold, it was filtered and dried; yield 2.66 g., 87%. For analytical purposes a sample was recrystallized from absolute ethanol; m.p. 188–190°.

Anal. Calcd. for $C_{18}H_{16}NO_4P$: C, 63.34; H, 4.72; N, 4.10; P, 9.08. Found: C, 63.33, 63.21; H, 4.81, 4.70; N, 4.19; P, 9.07.

The reduction was repeated using labeled diphenyl-(4-benzyloxyphenyl)-phosphoramidate. The yield was 4.28 g. (80.5%) over two steps from 6.73 g. of diphenyl phosphorochloridate; m.p. 183–185°, mixed m.p. 184–186° with model compound. The specific radioactivity per mg. was 163,830 counts per minute using a thin mica end-window counter.

4-(O,O-Diphenylphosphoramido)-phenylphosphoric Acid (V) Cyclohexylamine Salt.—Unlabeled diphenyl N-(4-hydroxyphenyl)-phosphoramidate (5.25 g.) was added in solid form to 2 ml. (1/3 excess) of $POCl_3$ dissolved in 20 ml. of lutidine while stirring with a magnetic stirrer in an ice-bath. The mixture became too pasty to stir, so 75 ml. of dry benzene was added. An additional 5 ml. of lutidine was used to wash in the remainder of the phenol. After 1 hr. of stirring, the mixture was diluted with 700 ml. of ice and water and then acidified with 25 ml. of concd. hydrochloric acid. The phosphoric acid ester separated as a viscous mass and the mixture was allowed to stand in a refrigerator for 3 hr. The aqueous layer was decanted and the viscous mass was washed several times with water. The aqueous washings were filtered to recover some insoluble material. The viscous mass was dissolved in 75 ml. of ethanol and the solution passed through the above filter. It was again filtered by suction through a little Super-Cel to clarify it.

Cyclohexylamine was added dropwise to the above solution until it gave a slightly alkaline reaction to wet Alkacid paper. In about a minute crystalline cyclohexylamine salt separated and was allowed to stand at 3° for several hours. The first crop melted at 200–201° and the second crop from concentration of the filtrate melted at 198–200°; yield 4.19 g. (52.9%) based on a monocyclohexylamine salt. After recrystallization from water the product melted at 198–202° and analyzed best for a monocyclohexylamine salt.

Anal. Calcd. for $C_{24}H_{30}N_2O_7P_2$: N, 5.38; P, 11.92. Found: N, 5.48; P, 11.66.

The neutralization equivalent of the unrecrystallized salt was usually between the calculated figures for the mono and the di salts. Although this salt was excellent for purification purposes, it did not prove suitable for analytical purposes.[5]

The cyclohexylamine salt of P^{32}-labeled 4-(O,O-diphenylphosphoramido)-phenylphosphoric acid was prepared from labeled diphenyl N-(4-hydroxyphenyl)-phosphoramidate. This salt was converted to the soluble sodium salt by dissolving in an excess of 0.1 *N* NaOH, extracting the cyclohexylamine with six portions of ether and back titrating with 0.1 *N* HCl to *p*H 7.2. It was necessary to remove the ether from this solution by vacuum distillation. The product was then sterilized for intravenous use by filtration through a Millipore filter. If the temperature was not carefully controlled during the alkaline phase of this conversion, some decomposition and darkening of the solution occurred.

Unlabeled sodium salt was hydrolyzed 1.36 times more rapidly than glycerophosphoric acid by human prostatic phosphatase at *p*H 5.0. It was hydrolyzed 0.59 as rapidly as glycerophosphoric acid by swine kidney phosphatase at *p*H 9. Its LD_{50} in mice was 720 mg./kg.

4-(O,O-Diphenylphosphoramido)-phenylphosphoric Acid Barium Salt.—One gram of the cyclohexylamine salt of unlabeled 4-(O,O-diphenylphosphoramido)-phenylphosphoric acid was shaken for 1 hr. with 0.6 g. of barium acetate in 60 ml. of water in the presence of glass beads. The barium salt was filtered off, washed with water and alcohol and dried in an oven at about 100°, yield 0.6 g. One and a half grams of the barium salt was recrystallized by dissolving in 1.5 l. of boiling water. After filtration, the solution was concentrated to 100 ml. yielding 1.3 g. of crystalline barium salt.

Anal. Calcd. for $C_{18}H_{15}NO_7P_2Ba$: C, 38.84; H, 2.72; N, 2.51; P, 11.13; Ba, 24.68. Found: C, 39.12; H, 2.93; N, 2.60, 2.53; P, 11.29; Ba, 25.26, 24.85.

P^{32}-Labeled 4-(O,O-Diphenylphosphoramido)-phenylphosphoric Acid Sodium Salt.—Labeled barium salt (2.44 g.) was shaken in presence of glass beads in 100 ml. of water containing 1.4 g. of sodium sulfate for 2 hr. It was filtered through Super-Cel and washed with water. The total solution of 170 ml. was filtered through a Millipore filter for sterilization to give a colorless clear solution which was subdivided in 20-ml. ampules for injection purposes. The specific radioactivity per ml. was 1,286,900 counts per minute using a thin mica end-window counter. The theoretical mole equivalent of the phenol IV per ml. was 8.8 mg. giving therefore 146,238 c.p.m./mg. of phenol. This represents an 89% conversion of barium salt to sodium salt.

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(4) Prepared by catalytic reduction of 4-nitrobenzyloxynitrobenzene with PtO_2 catalyst in ethyl alcohol solution, *Beilstein*, **13**, 439 (1947).

(5) Similar phenomena were reported by C. E. Ballou and H. O. L. Fischer, THIS JOURNAL, **77**, 3329 (1955), and R. W. McGilvery, *J. Biol. Chem.*, **200**, 835 (1953).