# Synthesis of 3-Indolyl and 5-Bromo-3-indolyl Phosphate for Histochemical Demonstration of Alkaline Phosphatase[1]

K. C. Tsou, Helen C. F. Su, Dorothy J. Rabiger,

*Harrison Department of Surgical Research, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania*

Hans Heymann, and Arnold M. Seligman

*Departments of Surgery, Sinai Hospital of Baltimore and Johns Hopkins University, Baltimore, Maryland*

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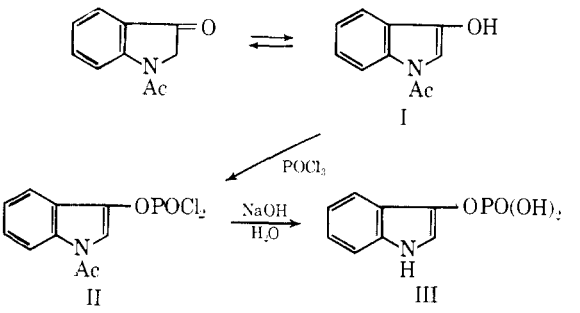
A synthesis of 3-indolyl and 5-bromo-3-indolyl phosphate is given. These substrates are useful for the colorimetric and histochemical demonstration of alkaline phosphatase activity in tissue, respectively.

The use of 3-indolyl acetate for the histochemical demonstration of esterase was first reported by Barrnett and Seligman[2] and Holt.[3] Enzymatic hydrolysis of this ester results in the liberation of indol-3-ol which is then readily oxidized to form an insoluble blue indigo at the site of enzymatic activity. In other synthetic studies designed to arrive at better substrates for histochemistry, we found that introducing a bromine atom at the 6 position of naphthol improves the substantivity of the naphthol dye.[4] A practical synthesis of bromo-substituted 3-indolyl esters was therefore developed in order to improve the indol-3-ol method for esterase.[5] Independent of our work, a thorough study of the requirements for indigogenic substrates was carried out by Holt and his associates[6] who concluded that 5-bromo- and 5-bromo-4-chloro-3-indolyl acetate represent the most useful substrates for esterase histochemistry. To extend this then new principle of histochemistry, Seligman, *et al.*, reported the use of 3-indolyl phosphate for alkaline phosphatase,[7] and Holt reported the use of 5-bromo-3-indolyl phosphate as a better substrate for alkaline phosphatase.[8] However, the synthesis of both these substrates has never been reported, and no detailed work has appeared since.

In the course of our continuing interest in correlating biochemical findings with histochemical methods, we undertook at first a study of the kinetics of hydrolysis of 3-indolyl phosphate by alkaline phosphatase and recently extended its use as a chromogenic substrate in a new colorimetric method for the determination of this enzyme.[9] Incidental to this work, it was also found that both 3-indolyl phosphate and 5-bromo-3-indolyl phosphate are useful substrates for the demonstration of alkaline phosphatase by gel electrophoresis.[10] It is thus appropriate for us to make available the synthesis and interesting observations on the properties of these 3-indolyl phosphates and their salts, related to these studies.[11]

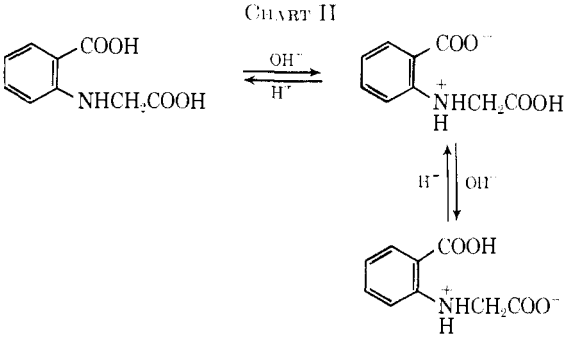
The synthetic scheme of 3-indolyl and 5-bromo-3-indolyl phosphate is illustrated in Chart I.

Chart I




N-Carboxymethylanthranilic acid was treated with acetic anhydride to yield 1-acetyl-3-indolyl acetate. Hydrolysis of this compound with aqueous sodium sulfite led to the formation of 1-acetylindol-3-ol (I).[12] N-Carboxymethylanthranilic acid was obtained in three different melting forms which were dependent on the pH of the solution from which the material was obtained. The structural assignments of these forms were based on their infrared spectra and are shown in Chart II.

Chart II




The selective hydrolysis of the O-acetyl group in 1-acetyl-3-indolyl acetate should be carried out cautiously and the optimal time of hydrolysis was established by taking out samples in different time intervals and check-

(1) This research was supported by U. S. Public Health Research Grants CA-07339-03 and CA-02478 from the National Cancer Institute, National Institutes of Health.

(2) R. J. Barrnett and A. M. Seligman, *Science*, **114**, 579 (1951).

(3) S. J. Holt, *Nature*, **169**, 271 (1952).

(4) K. C. Tsou and A. M. Seligman, *J. Am. Chem. Soc.*, **74**, 3066, 5605 (1952).

(5) H. C. F. Su and K. C. Tsou, *ibid.*, **82**, 1187 (1960).

(6) S. J. Holt and D. G. O'Sullivan, *Proc. Roy. Soc.* (London), **B148**, 465 (1958).

(7) A. M. Seligman, H. Heymann, and R. J. Barrnett, *J. Histochem. Cytochem.*, **2**, 441 (1954).

(8) S. J. Holt, *Proc. Roy. Soc.* (London), **B142**, 160 (1954).

(9) K. C. Tsou and H. C. F. Su, *Anal. Biochem.*, **11**, 54 (1965).

(10) K. C. Tsou, H. C. F. Su, H. Gimpelson, and H. Rawnsley, *J. Histochem. Cytochem.*, **13**, 10 (1965).

(11) While this manuscript was in preparation, J. P. Horwitz, J. Chua, M. Noel, G. T. Donatti, and J. Freisler [*J. Med. Chem.*, **9**, 447 (1966)] reported a preparation of *p*-toluidinium 5-bromo-4-chloro-3-indolyl phosphate.

(12) A. Etienne, *Bull. Soc. Chim. France*, 651 (1948).

ing the infrared spectra of this crude mixture. 1-Acetylindol-3-ol (I) was found to assume the keto form rather than the enol form as shown by the presence of a 5.80-$\mu$ band in its infrared spectra. The phosphorylation of I was carried out in benzene solution and $\beta$-picoline or pyridine was used both to convert the keto form of the indol-3-ol liberated *in situ* to the enol form, and as catalysts for phosphorylation. When phosphorylation of indol-3-ol was substantially complete, the hydrolysis of the mixture resulted in very little indigo formation. Otherwise, the isolation of 3-indolyl phosphate became difficult and the yield was drastically reduced. Attempts to distil the intermediate 1-acetyl-3-indolylphosphoryl chloride were not possible even at pressures below 1 mm without danger of explosion, but the existence of this intermediate was confirmed by the preparation of a dianilide as a derivative.

The synthesis of the 5-bromo derivative was essentially carried out by a similar route. In spite of the fact that they could both, in theory, be made also by the dibenzyl chlorophosphonate method, this method offered no advantage over that of direct phosphorylation by $POCl_3$ in the 3-indolyl compound and yielded a debrominated product in the attempted 5-bromo-3-indolyl compound.

Although 3-indolyl phosphate has been reported to be useful for both colorimetric and histochemical demonstration of alkaline phosphatase, we are in agreement with Holt[8] that the 5-bromo derivative is definitely preferred for histochemical experiments.[13] One must, however, point out that the original procedure of Holt[8] is not recommended. When the disodium salt of 5-bromo-3-indolyl phosphate is used, no additional oxidant other than air is necessary. The reaction proceeds reasonably fast at pH 9.2 to give good localization of an amorphous deposit of 5,5′-dibromoindigo. Examples of the localization along the brush borders of human duodenum and seminal vesicles can be seen in Figure 1.

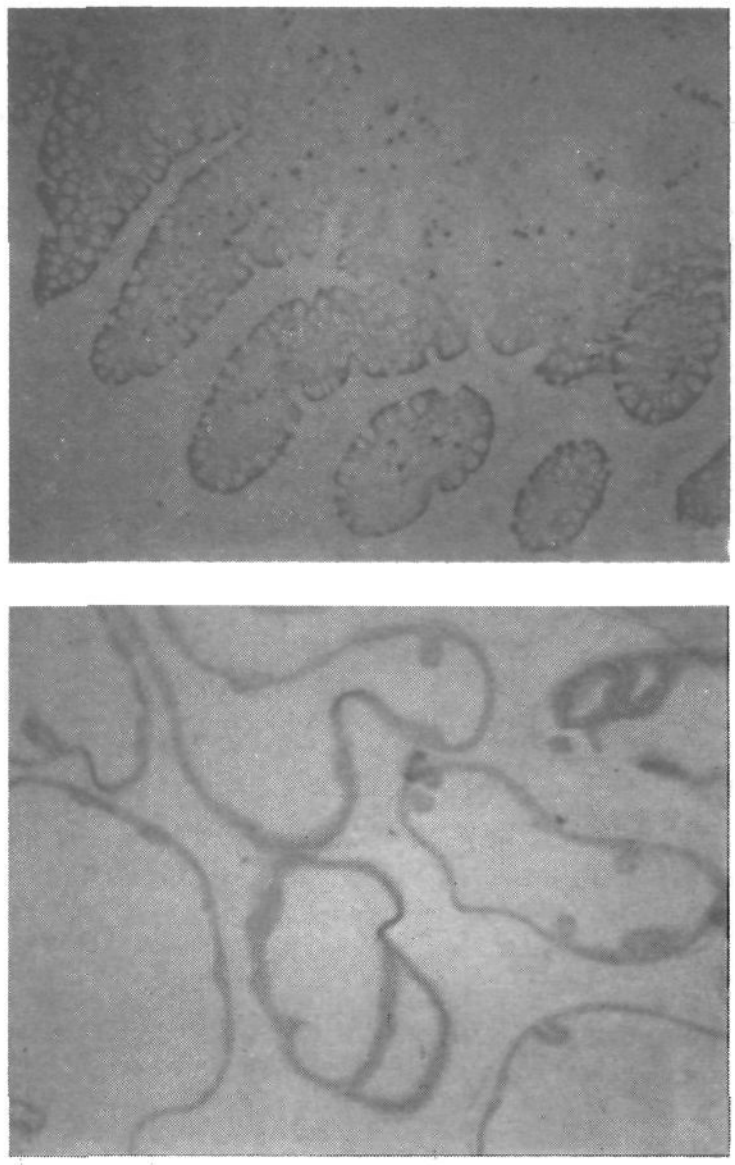


Figure 1.—Alkaline phosphatase in human small intestine, 90× (top), and seminal vesicles, 90× (bottom).

## Experimental Section[14]

**3-Indolyl phosphate (III)** can be prepared by either of following two methods.

**A. Direct Phosphorylation with $POCl_3$. 1. Calcium Salt.**—1-Acetylindol-3-ol (5 g, 0.0286 mole) suspended in 50 ml of dry benzene was heated to boiling for a few minutes. Dry pyridine (2.5 ml, 0.031 mole) was added followed by $POCl_3$ (2.65 ml, 0.029 mole). The mixture was left stirring at 60° for 24 hr and filtered while hot. The filtrate was then added dropwise into a solution of NaOH (5.9 g, 0.143 mole) in 25 ml of water with vigorous stirring while the temperature was kept below 30°. This procedure has been found to minimize pyrophosphate formation. After stirring at this temperature for 1.5 hr, the solution was filtered to remove the disodium di(3-indolyl) pyrophosphate. The aqueous layer from the filtrate was adjusted to pH 9 (originally pH 11–12) by addition of dilute acetic acid, then a solution of 10 ml of 10% $Mg(NO_3)_2$ and 10% $NH_4Cl$ was added. After standing in the refrigerator overnight the precipitated $MgNH_4PO_4$ was removed by filtration. The solution was heated rapidly to 70° and a solution of 10 g of calcium acetate (hydrate) in 25 ml of water was added. The calcium 3-indolyl phosphate collected was washed with water, ethanol, and acetone successively. The yield was 6.3 g (88%). It gave a negative Beilstein flame test for halogen. Its infrared spectrum showed the absence of C=O. Potentiometric titration of a 0.2417-g sample required 9.85 ml of 0.1 *N* HCl as compared to the calculated amount of 9.61 ml.

**2. Disodium Salt.**—Calcium 3-indolyl phosphate (4 g) and anhydrous $Na_2CO_3$ (1.66 g) were mixed and suspended in 32 ml of water. This suspension was heated to boiling for 5 min and then kept stirring for 1.5 hr while the solution was cooled to room temperature under $N_2$. The solution was then treated with charcoal. The yellow solution obtained was concentrated in a vacuum oven at 40° over anhydrous $CaCl_2$ to about 2–3 ml. Boiling ethanol (100 ml) was added. The precipitate collected was washed with hot ethanol to obtain fine white crystals. The yield was 2.8 g (68%). This sample when titrated with 0.1 *N* HCl was often found to be contaminated with $Na_2CO_3$. Further purification could be made by dissolving in 20 ml of water and glacial acetic acid (equivalent to the contaminated $Na_2CO_3$) and then adding 400 ml of boiling ethanol. The pure white leaflets collected were dried *in vacuo* at 40° for 3 hr. This sample was titrated with 0.1 *N* HCl and found to be 100% pure.

*Anal.* Calcd for $C_8H_6NNa_2PO_4$: C, 37.37; H, 2.35; N, 5.44; P, 12.04. Found: C, 37.30; H, 2.34; N, 5.38; P, 11.96. (Hydrate: C, 33.00; H, 2.38; N, 4.93; P, 9.71.)

The purified product was stable for months in a dry atmosphere; in the air a blue tinge gradually developed. Neutral or slightly basic solutions turn blue gradually; acidic solution deposits copious amounts of indigo overnight. For biochemical purposes, the best assay for the purity of the material is potentiometric titration. The product exhibits a $pK_{a1}$ and $pK_{a2}$ of 2.70 and 6.72, respectively, at 25° for the protolytic dissociation.

**3. Acid Form and Bis(cyclohexylamine) Salt.**—About 15 g of Dowex 50W-X8 cation-exchange resin was washed free from acid. The 3-indolyl phosphate disodium salt (4 g) in 40 ml of water was added. The mixture was stirred under $N_2$ for 2 hr and filtered. The yellow filtrate was freeze-dried to obtain yellow 3-indolyl phosphate crystals. Due to the instability of this phosphate, it was isolated as the bis(cyclohexylamine) salt

(13) The barium salt of 5-bromo-3-indolyl phosphate has been used successfully in the detection of intestinal alkaline phosphatase by A. Przelecka, H. Dominal, and M. G. Scerzola, *Folia Morphol.*, **13**, 371 (1962). However, this procedure gives a coarser precipitate of the dye than is obtained by our method.

(14) All melting points are corrected. Analyses were performed by Dr. S. M. Nagy, Belmont Rd., Belmont, Mass. Infrared spectra of compounds were obtained in potassium bromide disks on a Perkin-Elmer 137 spectrophotometer.

by dissolving it in 90 ml of ethanol and adding an ethanolic solution of cyclohexylamine (3.9 g in 20 ml). A jellylike precipitate formed immediately. The solid collected was dissolved in 125 ml of hot water, then reprecipitated by addition of 500 ml of acetone. Fine, white crystals were obtained. The yield was 2 g (48.4%).

*Anal.* Calcd for bis(cyclohexylamine)salt, $C_{20}H_{34}N_3O_4P \cdot 3.5H_2O$: C, 50.58; H, 8.71; N, 8.85. Found: C, 50.32; H, 8.76; N, 9.06.

**B. Phosphorylation *via* Dibenzyl Chlorophosphonate.**—NaH (1.2 g of 50% oil dispersion, 0.025 mole) was suspended in 30 ml of dried and redistilled diethylene glycol dimethyl ether (diglyme) under an Ar atmosphere. A solution of 1-acetylindol-3-ol (3.35 g, 0.019 mole) in 60 ml of diglyme was added slowly. After the addition was complete, the mixture was stirred for 30 min when the color changed from yellow to brown and then to green (no blue). A solution of dibenzyl chlorophosphonate (from 6.4 g of dibenzyl hydrogen phosphite) in $CCl_4$ (70 ml) was added dropwise. The solution changed from green to brown, violet, and finally deep red. Then the reaction mixture was stirred overnight at room temperature under an Ar atmosphere and filtered. The filtrate was washed with water after the addition of 200 ml of $CCl_4$. The organic layer collected was washed with 2 *N* NaOH several times and finally again with water. From the alkali washings, 0.23 g of indigo was obtained. The organic layer was dried ($Na_2SO_4$) overnight and concentrated *in vacuo*. The residual red oil was dissolved in a minimum amount of $CHCl_3$ and chromatographed on activated alumina. The dark red eluate was concentrated *in vacuo* to obtain the dark red oil. The oil was dissolved in 100 ml of methanol and refluxed with Raney Ni for 30 min and filtered. The filtrate was hydrogenated at atmospheric pressure in the presence of N-methylmorpholine (5 ml) and Pd–C (0.3 g, 10%). A total of 330 ml of $H_2$ was absorbed. The solution was filtered and evaporated *in vacuo*. The residue was again evaporated twice with water, then dissolved in water, which was extracted with ether to remove the impurities. The water layer was freeze-dried to obtain 1.8 g of brown solid. This solid was dissolved in 40 ml of ethanol and a solution of cyclohexylamine (3 g) in 10 ml of ethanol was added. A gelatinous precipitate formed and was collected by filtration. The precipitate was redissolved in water and decolorized with charcoal. On freeze-drying, fine white crystals were obtained, mp 134–137°. The yield was 1.21 g (20.4%). Analysis and infrared indicated the compound to be the 1-acetyl bis(cyclohexylamine) salt.

*Anal.* Calcd for $C_{32}H_{56}N_3O_6P \cdot 1.5H_2O$: C, 54.98; H, 8.18; N, 8.75. Found: C, 54.84; H, 8.16; N, 8.75.

This compound is relatively stable to keep and can also be used conveniently for biochemical study by dissolving in an equivalent amount of NaOH solution for 30 min and then mixing with the required buffer for enzyme experiments.

**1-Acetyl-3-indolylphosphoric Acid Dianilide.**—1-Acetylindol-3-ol (530 mg) was converted to the phosphoryl chloride by a 1-hr treatment as given above. After filtration from pyridine hydrochloride, the solution was treated with 1 ml of dry aniline in 5 ml of benzene for 3 hr at 60°. Aniline hydrochloride was soon deposited. The mixture was treated sequentially with water, dilute HCl, and dilute alkali, dried, and concentrated to a glassy solid. Trituration of this residue with alcohol deposited a solid which on recrystallization from benzene gave 130 mg of colorless rosettes of the anilide, mp 160–162°. Two more recrystallizations from the same solvent gave mp 161.9–162.6°.

*Anal.* Calcd for $C_{22}H_{20}O_5P$: N, 10.37; P, 7.64. Found: N, 10.34; P, 7.07.

**Disodium Di(3-indolyl) Pyrophosphate.**—The benzene solution of II was added to a calculated amount of water containing more than enough pyridine to bind the acids generated. A sludge settled out, which by treatment with methanolic $CH_3ONa$ was converted into a mixture of sodium salts. Part of this crystallized from the methanol and the rest was precipitated by addition of ether to the methanolic filtrate. Deacetylation took place simultaneously. The material so obtained contained considerable amounts of disodium 3-indolyl phosphate, but that was not isolated in this preparation. Instead, the salts were dissolved in a minimum of hot water and decolorized with charcoal; addition of an equal volume of saturated NaOAc solution precipitated disodium di(3-indolyl) pyrophosphate as a crystalline precipitate. The pyrophosphate resisted hydrolysis by 0.05 *N* alkali at 90°; at pH 2 only a small change occurred during 12 hr at room temperature and any orthophosphate arising under these conditions suffered further hydrolysis as it was being formed. Alkali fusion yielded the 3-indolyl phosphate salt. This compound exhibited neither acidic nor basic properties.

For an analytical sample, the disodium salt was reprecipitated once more with NaOAc solution, washed alternately *twice* with a little water and with alcohol, and dried *in vacuo* for 3 hr.

*Anal.* Calcd for $C_{16}H_{12}Na_2N_2O_7P_2$: C, 42.49; H, 2.67; N, 6.19; P, 13.09. Found: C, 42.37; H, 2.89; N, 6.08; P, 13.80.

By means of KCl solution, the dipotassium pyrophosphate could also be prepared as a crystalline powder which has a higher solubility temperature coefficient than the disodium salt. This salt was purified by dissolving in water and reprecipitated by the addition of reagent grade *acetone* as a globular aggregate, which was ground and dried *in vacuo* for 3 hr.

*Anal.* Calcd for $C_{16}H_{12}K_2N_2O_7P_2$: C, 39.67; H, 2.49; N, 5.78. Found: C, 40.18; H, 2.66; N, 5.68.

The Mg salt forms tiny cubes; also, it is very sparingly soluble in water.

**5-Bromo-3-indolyl Phosphate.**—Similar procedures were used with 1-acetyl-5-bromoindol-3-ol[4] as starting material.

**A. With $POCl_3$.**—The calcium salt had yields which varied from 54 to 66%; the disodium salt had yields which varied from 61 to 87%. The product was found to be a dihydrate. *Anal.* Calcd for $C_8H_5BrNNa_2PO_4 \cdot 2H_2O$: C, 25.82; H, 2.43; Br, 21.48; P, 8.32. Found: C, 26.01; H, 2.43; Br, 21.37; P, 8.48. When 0.2394 g of this sample was dried *in vacuo* at 100° for 30 hr, 23.07 mg was lost as compared to the 25.07 mg of water content calculated in the dihydrate. When $CHCl_3$ was used instead of benzene as solvent, the over-all yield of disodium salt (from 1-acetyl-5-bromoindol-3-ol) was reduced to 15%.

**B. By Dibenzyl Chlorophosphonate.**—When a similar procedure was used, the phosphate was isolated as the bis(cyclohexylamine) salt of 5-bromo-3-indolyl phosphate instead of the 5-bromo-3-indolyl phosphate as white, fine crystals, mp 190°. The yield was 17%.

*Anal.* Calcd for $C_{20}H_{33}BrN_3PO_4$: N, 8.57. Found: N, 8.71.

*Anal.* Calcd for $C_{20}H_{34}N_3PO_4 \cdot 3.5H_2O$: N, 8.85. This compound had an infrared spectrum identical with a known sample of the hydrated bis(cyclohexylamine) salt of 3-indolyl phosphate. It contained no Br and gave a negative Beilstein test. Debromination must therefore occur in the reaction with NaH.

**Histochemical Methods.**[15]—In a recent report, we presented evidence that the use of $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ as oxidants in the originally proposed procedure of Holt will not allow conversion to the corresponding indigo of all the indol 3-ol that is liberated by enzymatic hydrolysis.[16] We therefore proposed to use 5-bromo-3-indolyl phosphate in the original procedure as recommended by Barnett and Seligman, *i.e.*, only air or $O_2$ is needed. A brief procedure is given here as follows. Tissue is fixed in calcium–formalin overnight at 0° and stored in 0.25 *N* sucrose in a refrigerator. Sections were cut on a cryostat and mounted on cover slips for incubation. The incubation mixture consists of 5 ml of 0.1 *M* barbital buffer–HCl (pH 9.2) and 5 ml of distilled water containing 0.1 ml 2 *M* NaCl solution. After 30 min of incubation at 37°, the sections were rinsed quickly in 0.1 *N* HCl solution and then in distilled water, air-dried, cleared in alcohol and xylene, and mounted with Histoclad (Clay Adams) synthetic mounting medium. Safranin O can be used as a counterstain if necessary. Both brush border of the villi and lymph nodes in the intestine and walls of seminal vesicles were stained. An improvement of the original 3-indolyl phosphate method has also been presented elsewhere[17] but the 5-bromo derivative yields more amorphous deposits.

(15) The histochemical experiments were performed with the assistance of Mrs. Cecilia L. Whitney. Dr. G. Evans of the Surgical Pathology Laboratory of the University of Pennsylvania Hospital generously provided some pathological tissue for this study.

(16) K. C. Tsou and H. C. F. Su, *J. Histochem. Cytochem.*, **11**, 561 (1963).

(17) R. J. Barnett, H. W. Deane, and A. M. Seligman, "Handbuch der Histochemie," VII/1, Gustav Fischer Verlag, Stuttgart, 1962.