cm column of Dowex 2 resin (X8, 200–400 mesh, Cl^-) at 4°. The entire sample was passed, at 15 ml/min, through the column, which was then washed with H_2O (1 l.) followed by 1 l. of 0.003 *N* HCl. The fractionation of the products was achieved by elution, at the same rate, with successive 1-l. portions of 0.003 *N* HCl containing increasing concentrations of LiCl, the first portion being 0.0055 *M* in LiCl, the second 0.011 *M*, the third 0.0165 *M*, etc. One-liter fractions were collected and stored in the cold room. Total and acid-labile (10 min at 100° in 1 *N* H_2SO_4) P were determined on 0.5 ml of each fraction by the procedure of Bartlett.[9] The acid-stable P (total minus acid labile) was found in three well-separated peaks. Peak I (1.8 mg of acid-stable phosphorus) appeared in fraction 4, eluted with 0.022 *M* LiCl. It was probably a mixture of methyl phosphate and inorganic phosphate. Peak II (fractions 13–15) contained 23.7 mg of acid-stable P but very little acid-labile P. It is believed to consist primarily of P^1,P^2-dimethyl pyrophosphate. Peak III (fractions 27–31) contained approximately 60 mg of acid-stable P (59% of the starting methyl phosphate). All fractions in this peak had a ratio of total P/acid-labile P of close to 1.5. Inorganic polyphosphates were eluted shortly after this peak III.

Isolation of the Lithium Salt of MTP.—The fractions of peak III were pooled and brought to pH 7.3 with 1 *N* LiOH. The solution was concentrated at 40° in a flash evaporator to about 50 ml, then 64 ml of MeOH was added. The solution was filtered through sintered glass and the flask and the filter were rinsed with MeOH (30 ml). To the filtrate was added acetone (1880 ml). The precipitate, which formed immediately, was collected, after 48 hr in the cold, by decantation and centrifugation and was washed six times with MeOH–Me_2CO (1:20) and three times with dry ether. The product was dried overnight under vacuum at room temperature over P_2O_5 and weighed 644 mg. *Anal.* Calcd for $CH_3Li_4O_{10}P_3 \cdot H_2O$: P, 29.6. Found: P, 29.1. The ratio of total/acid-labile P was 1.48. Less than 1% of the P was present as inorganic phosphate. No Cl^- was detected. Titration between pH 8.5 and 4 gave one acid group per three P atoms.

Hydrolysis of MTP by Myosin.—A sample of myosin from rabbit skeletal muscle was kindly provided by Dr. G. Richards of the Department of Nutrition, University of California, Berkeley. It had been prepared by the method of Tonomura and coworkers[10] and preserved at −18° in 50% glycerol. The protein concentration was determined by Lowry procedure[11] with standardization by Kjeldahl. Possible changes in the color yield of the Lowry procedure were checked using albumin as a standard. A solution in 0.5 *M* KCl was obtained after precipitation of the myosin by diluting with water (10:1), centrifugation, and redissolution in and dialysis against 0.5 *M* KCl (pH 8.1 with 1 m*M* Tris-chloride buffer). Glass-distilled water was used in all procedures. The myosin solution so obtained (200 μg/ml) was kept in a plastic (cellulose nitrate) tube and in ice.

Conditions for the MTPase and ATPase assays were as follows: 0.5 *M* KCl, 5 m*M* $CaCl_2$, 0.5 m*M* substrate, pH 8.2 (Tris-chloride buffer 0.02 *M*), temperature 25°. The substrates were used as K^+ salts, obtained by passing their solutions through a column of Dowex 50, K^+. The enzymic reaction was started by adding, with magnetic stirring, 0.5 ml of the 3 m*M* substrate to 2.5 ml of a solution containing the other components, including myosin (100 μg). The reaction was stopped by adding 1 ml of a 20% trichloroacetic acid solution. After filtration, Pi was determined in 3 ml of the filtrate by the phosphomolybdate extraction method of Dreisbach,[12] 4 ml of the xylene–*i*-BuOH solvent being used for the extraction.

The enzymic activity of the myosin, obtained from the amount of Pi released after a given time and expressed as micromoles of Pi per minute per gram, was found to be 390 with ATP and 48 with MTP. Azuma and coworkers[4a] have reported that ribose triphosphate, another simple ATP analog, is hydrolyzed, also in the presence of Ca^{2+}, at one-twentieth the rate of ATP. However, we noted that the loss of activity during storage in ice was not parallel for the two substrates, the ATPase being better preserved than the MTPase. After 35 days the ATPase activity was still at 70% of the original value while the MTPase had declined to 27%. The loss of MTPase activity with time appears to be a first-order reaction with a half-life period of about 19 days in our conditions of storage of the myosin.

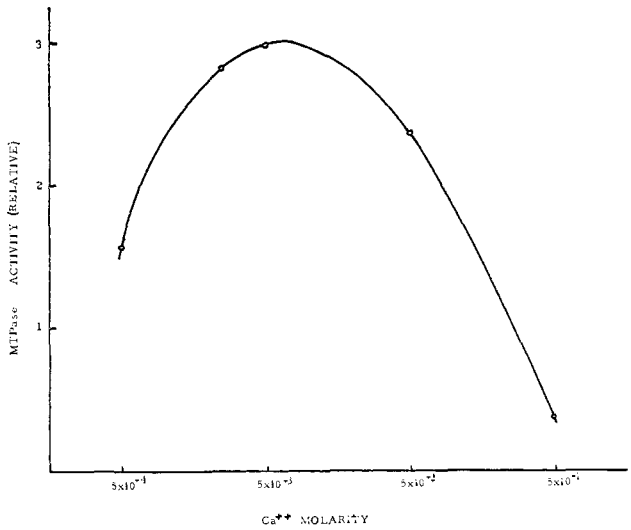

Figure 1.—Ca^{2+} dependence of myosin methy triphosphatase Experimental conditions: 0.5 *M* KCl, 0.5 m*M* MTP, pH 8.2, 25°

The dependence of MTPase activity on Ca^{2+} concentration is shown in Figure 1. Maximum activity was obtained when Ca^{2+} was about 5 m*M*. No activity was observed in the absence of Ca^{2+}. Green and Mommaerts,[13] working in somewhat different conditions (0.15 *M* KCl, pH 9.0), have reported an optimal Ca^{2+} concentration of approximately 1 m*M* in the case of ATP.

Acknowledgment.—I am indebted to Dr. J. Eiler and to Dr. M. Morales who sponsored this work and to Dr. G. Richards for gifts of myosin and much helpful advice.

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2-Hydroxy-3-naphthoic Acid Anilide Phosphate as a Fluorescent Histochemical Substrate for Phosphatase[1]

K. C. TSOU AND SADAO MATSUKAWA

Harrison Department of Surgical Research, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

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The development of fluorogenic substrates for histochemistry of normal and cancer cells is of importance because of the inherent sensitivity of fluorescence measurement over that of the absorbance measurement. Several years ago, Rutenberg, *et al.*, reported[2] the use of 2-hydroxy-3-naphthoic acid anilide phosphate for histochemical demonstration of phosphatase. Because of the presence of a small amount of naphthol-AS, a simultaneous coupling procedure was recommended and fluorescent study was not possible. A more detailed study was therefore undertaken in order

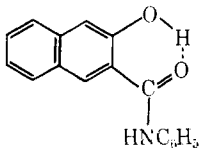
(1) This work was supported by Research Grant AT-30-1-3784 from the U. S. Atomic Energy Commission and U. S. Public Health Grant CA 07339.

(2) A. M. Rutenberg, R. J. Barrnett, K. C. Tsou, B. Monis, and R. Teague, *J. Histochem. Cytochem.*, **6**, 90 (1958).

to improve the synthesis and purification of the final substrate.

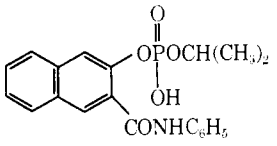
Initially a comparison of fluorescence intensity was made on sixteen naphthol-AS derivatives. Only 2-hydroxy-3-naphthoic acid anilide itself has a relatively highest fluorescence at 520 mμ when excited at 300 mμ. Thus there is no advantage in choosing any other derivative for this purpose.

2-Hydroxy-3-naphthoic acid anilide (naphthol-AS) (I) was found to exhibit a distinct 3050-cm^{-1} band which was attributed to its hydrogen-bonded formula Ia. This intrahydrogen bonding does not change with



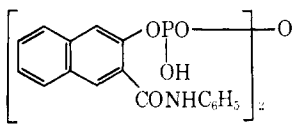
Ia

concentration and contributes to the low solubility and substantivity of the naphthol to tissue. In treating this compound with $POCl_3$ or PCl_5 for the preparation of the corresponding phosphate, it is thus necessary to overcome the hydrogen bonding in order to facilitate the reaction. Favorable conditions for the preparation of this substrate can be found in the experimental part. Two of the by-products of this reaction are identified. A diester of isopropyl 2-naphthyl-3-carboxyanilide phosphate (III) was isolated, when isopropyl alcohol



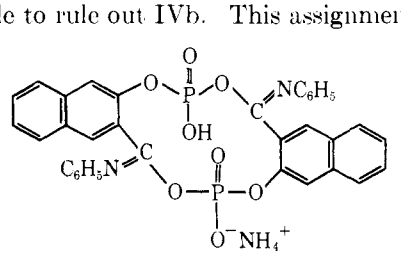
III

was used as a component of the chromatographic solvent system. This compound was not hydrolyzed by intestinal alkaline phosphatase, but could be hydrolyzed by liver and kidney enzymes in tissue experiments. Also isolated was the corresponding pyrophosphate (IV) which was not unanticipated since a



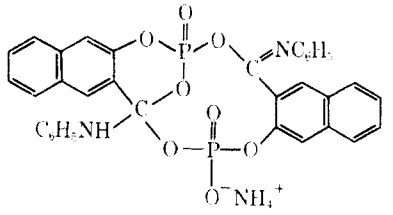
IV

similar compound was isolated as a by-product of 3-indolyl phosphate.[3] What was different in this work was that the compound IV could not have the structure as anticipated, based on ir and uv spectra, but did have the cyclic structure IVa, even though it was not possible to rule out IVb. This assignment is based



IVa

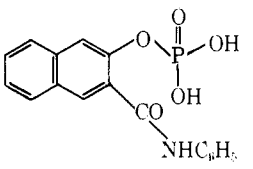
(3) K. C. Tsou, H. C. F. Su, D. J. Rabiger, H. Heymann, and A. M. Seligman, *J. Med. Chem.*, **10**, 662 (1967).



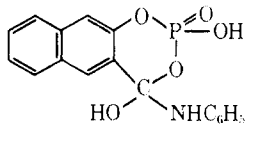
IVb

on the extreme stability of this compound to alkali and its resistance to alkaline phosphatase as well as on titration and ir measurements, which did not show the 1660- and 1540-cm^{-1} band as a similar compound with structure IV. While the pyrophosphate IV can be a competitive inhibitor for alkaline phosphatase as we noted in the past, there has been no evidence that this novel cyclic phosphate competes in any way with the enzymatic hydrolysis of V.

The other interesting structure problem of naphthol-AS phosphate (V) which has histochemical and biochemical implications is that this substrate can exist in either its open-chain form V as expected or in a tautomeric form Va. This assignment is based on the



V



Va

relative intensity of the 1660-cm^{-1} band. In Va, the 1660-cm^{-1} band decreases sharply and a new C–O–P band at 1375 cm^{-1} appears. Structure Va will, as expected, be hydrolyzed by phosphodiesterase, and examples of this reaction will be reported elsewhere, together with more detailed fluorescence studies.

Histochemically, 2-hydroxy-3-naphthoic acid anilide phosphate was tested as an alkaline phosphatase substrate first with a simultaneous coupling method[1] in the presence of diazotized 5-amino-2-benzoylamino-1,4-diethoxybenzene (fast blue BBN) to a level that one could not see the color of the dye under a light microscope. Fluorescence microscopy was then applied to the section incubated with the substrate only. It was found that as little as 0.1 mg of substrate was needed to observe the activity, for instance, along the villi of the intestine. Thus, the fluorescence method is at least as much as 30 times more sensitive than the colorimetric method for histochemical purposes.

Experimental Section

All 2-hydroxy-3-naphthoic acid anilide (naphthol-AS) and derivatives are purified from commercial samples (Pfister Chemical Co.) by recrystallization from $MeCONMe_2$ and MeOH.

Preparation of 2-Hydroxy-3-naphthoic Acid Anilide Phosphate. A. $POCl_3$.—2-Hydroxy-3-naphthoic acid anilide (naphthol-AS) (5.26 g, 0.01 mole) was suspended in anhydrous THF (130 ml) at 60°. $POCl_3$ (3.68 g, slight excess) was added with stirring. After 20 min, pyridine (1.9 g) was added and the mixture was warmed for 15 min. After the reaction was allowed to stand at room temperature overnight the pyridine hydrochloride (2–3 g) was removed by filtration. This precipitate contained a small amount of naphthol-AS. The solvent was removed under reduced pressure with an efficient rotary evaporator, and an oily yellow solid remained. This solid was dissolved in a small amount of acetone and separated by decantation from the naphthol-AS, which is sparingly soluble in acetone. The acetone solution was

then concentrated to give a crude product which showed three spots upon hydrolysis and paper chromatography: R_f 0.78 (AS), R_f 0.90 (cyclic dimer), R_f 0.22 (AS − PO_4). Thus, V could be isolated in a very low yield by paper chromatography, as seen later.

B. With PCl_5.—Naphthol-AS (1.3 g, 0.005 mole) was suspended in 50 ml of dioxane and PCl_5 (2.6 g, 0.0125 mole) was added with stirring. The reaction was exothermic and a clear yellow solution was obtained after continuous stirring. Pyridine (0.09 g, 0.0125 mole) was then added after 10 min, and the mixture was allowed to stand overnight. Pyridine hydrochloride (0.4 g) was removed by filtration. From the precipitate, 33 mg of naphthol-AS was recovered. The dioxane solution was poured into ice water with stirring. A light yellow crystalline precipitate was obtained which was collected and treated with 1 *N* Na_2CO_3. Naphthol-AS (0.7 g) was again recovered as an insoluble product. The alkaline solution was now treated with Amberlite-IR-120 and lyophilized to obtain 225 mg of the crude naphthol-AS phosphate. An additional 339 mg (yield 35%) of the AS phosphate was recovered from the dioxane solution. This crude acid is adequate for most histochemical purposes, but is not pure enough for fluorescence study as its paper chromatogram shows three spots, R_f 0.21 (AS phosphate), R_f 0.80 (AS), and R_f 0.95 (cyclic dimer), even though the latter two spots are weak.

Purification of Naphthol-AS Phosphate by Paper Chromatography.—Since the analytical sample and the ammonium salt are obtained from extensive purification and a commercial sample[4] of this and related phosphates is now available from various sources, it is necessary for us to include the purification of this compound here.

Ascending paper chromatography was used in testing. When BuOH was used, the R_f values were very close. Only *i*-PrOH–NH_4OH–H_2O (7:1:2) was found to be useful for paper strip separation (R_f 0.20 (AS phosphate), R_f 0.78 (AS), R_f 0.90 (cyclic dimer)). Preparative paper chromatography employed 20 mg of compound dissolved in 0.5 ml of Me_2CO, spotted on four sheets of Whatman No. 1 paper of 23-cm width, and chromatographed, using the *i*-PrOH–NH_3–H_2O system. The low R_f and high R_f value strips were cut out and extracted with warm H_2O or Me_2CO. A low R_f compound (14 mg) was analyzed and found to be the isopropyl ester of III. *Anal.* $C_{20}H_{20}NO_5P + NH_3 + 9H_2O$: C, 42.55; H, 7.26; N, 4.96; P, 5.50. Found: C, 42.76; H, 7.31; N, 5.05; P, 5.40. After extensive drying on P_2O_5, a trihydrate could be obtained (P: calcd, 6.80; found, 6.80). Because of the low P content, this compound cannot be a triphosphate. Titration with 0.1 *N* NaOH on the free acid (prepared from Amberlite IR-120) shows basic monoacid and has the same equivalent as expected. Ir spectroscopy shows an *i*-Pr band at 7.60 μ (1370 cm^{-1}).

The high R_f compound agrees with the expected IVa as a monoammonium salt and as a dihydrate. *Anal.* $C_{34}H_{26}N_2O_9P_2 + NH_3 + 2H_2O$: C, 56.69; H, 4.57; N, 5.82. Found: C, 55.83; H, 4.08; N, 5.82. The ir spectrum supports the cyclic structure based on the presence of a band at 1660 cm^{-1} (–C=N–), the absence of a band at 1650 cm^{-1} (amide II band), and a strong band at 1375 cm^{-1} in the –P–O–C deformation region.

Ascending Paper Column Preparatory Chromatography.—The paper column was packed with Whatman cellulose powder CF-11 in a tapered column to about 12 cm (15-mm diameter). Five of these were used for 100 mg of the sample. After the compound was applied to the bottom of the tube, chromatography was done with the usual *i*-PrOH–NH_3–H_2O (7:1:2) in a large graduated cylinder. Solvent may be added if necessary to maintain the reservoir at the bottom of the graduated cylinder. It took 1–1.5 hr to complete this development and the column was pushed out while wet and cut out under light into five portions and extracted (H_2O). From the top, 28 mg was recovered as the pyrophosphate. The next layer contained a violet fraction, R_f 0.23 (0.8 mg), which is AS phosphate; the third fraction contained 69 mg, R_f 0.22. The ir spectrum showed that they are the same monoammonium salt. This salt was converted into the free acid by treating with IR-120 and then lyophilized. The resulting acid showed a weak 1660-cm^{-1} band, sintered at 110–120°, and melted at 240–243°. *Anal.* $C_{17}H_{14}NO_5P \cdot 2H_2O$: C, 53.78; H, 4.74; N, 3.70; P, 8.17. Found: C, 54.10; H, 4.41; N, 3.99; P, 8.34.

Dilute NH_4OH was used now to convert this free acid to the monoammonium salt which is readily soluble in H_2O and is useful for fluorescence study.

Fluorescence Assay.—Fluorescence measurements were carried out on an Aminco fluorometer and recorded with an X-Y recorder. Naphthol-AS was dissolved in 0.1 *N* NaOH and was found to be excited at 300 mμ to give fluorescence maxima at 520 mμ, whereas the phosphate has no 520-mμ emission. Enzymatic hydrolysis of the phosphate was therefore carried out in glycine buffer and its product could be detected as low as 0.01 μg/ml.

Histochemical Study.—Fresh frozen sections of tissue were cut on a Pearse cryostat and incubated immediately for alkaline phosphatase with the following substrate solution, in a small coupling jar: naphthol-AS phosphate ammonium salt (0.1 mg), 3 ml of glycine buffer (pH 9.8), 3 ml of 2 *M* NaCl, and 1 ml of 1% $MgCl_2$. After 30 min, the sections were rinsed quickly in distilled H_2O and mounted on glycerol for examination. They can also be dehydrated in the usual manner and mounted in Histoclad. The bluish green fluorescence of the dye can be seen vividly along the villi (Figure 1). In the case of the intestine, leucocytes and macrophages are also stained.

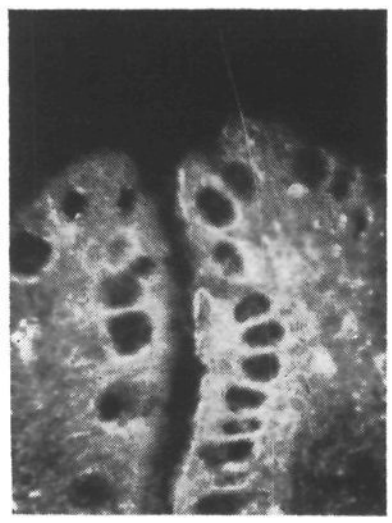


Figure 1.—Alkaline phosphatase in rat small intestine as demonstrated by the fluorescence method.

For acid phosphatase, calcium–formalin-fixed sections were used and citrate buffer (pH 5.0) was used in place of the glycine buffer. There was conspicuously more staining in the peribiliary region of liver when compared with the 5-iodoindoxyl method.[5]

At least part of this activity is attributed to phosphodiesterase activity. An application of this method to glioma showed brilliant yellowish fluorescent tumor cells. In Leydig cell tumor of the rat (Figure 2) this method reveals differential stain around tumor cells and some clearly in the nucleus.

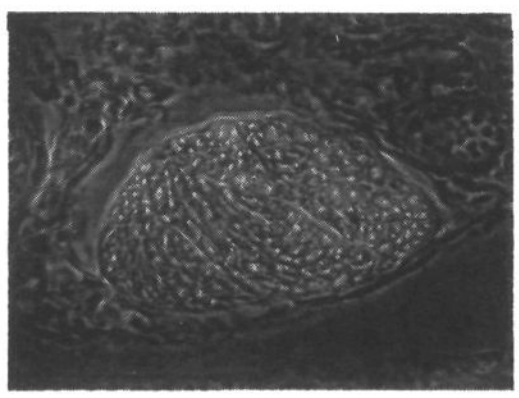


Figure 2.—Acid phosphatase in rat Leydig cell tumor with fluorescence phase contrast.

Acknowledgment.—We wish to acknowledge the generous cooperation of Dr. Gerald Peskin through the use of his Aminco spectrophotometer in part of this work, and Miss Diana Weiss and Miss Patty Lu Kaliher for the fluorometric study.

(4) A commercial "naphthol-AS free" naphthol-AS phosphate was analyzed to contain 66% naphthol-AS.

(5) G. Evans, C. Whinney, and K. C. Tsou, *J. Histochem. Cytochem.*, **14**, 171 (1966).