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# The Synthesis of Reagent Quantities of [2,3-$^3$H]*N*-(*n*-Propyl)hydroxylamine of High Specific Activity for Derivatizing Trace Amounts of Acyl Phosphates*

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ABSTRACT: [2,3-$^3$H]*N*-(*n*-Propyl)hydroxylamine hydrochloride was synthesized by reduction of 1-[2,3-$^3$H]-nitropropane with zinc dust in an aqueous ammonium chloride solution. The 1-[2,3-$^3$H]nitropropane was prepared by catalytic reduction of 3-nitropropene with palladium on carbon in tetrahydrofuran in the presence of approximately a stoichiometric amount of carrier-free tritium, followed by addition of carrier 1-nitropropane. The 1-[2,3-$^3$H]nitropropane was divided into a number of vials and stored in liquid nitrogen to minimize radiodecomposition, and the [2,3-$^3$H]*N*-(*n*-propyl)hydroxylamine was synthesized in batches and purified as needed. The [2,3-$^3$H]*N*-(*n*-propyl)hydroxylamine was purified by gradient elution from a Dowex 50 (Na) column followed by continuous-flow electrophoresis in a Brinkmann apparatus at two different pH values. The final product gave a single radioactive peak coinciding with the carrier propylhydroxylamine spot on paper electrophoresis and on chromatography in two solvent systems. The specific activity of the [2,3-$^3$H]*N*-(*n*-propyl)hydroxylamine prepared from different tritiated preparations ranged from 545 to 666 mc/mmole. *N*-(*n*-Propyl)-hydroxylamine was about 65% as effective as hydroxylamine in releasing inorganic phosphate from the acyl phosphate residue in a guinea pig NaK adenosine triphosphatase preparation. [2,3-$^3$H]*N*-(*n*-Propyl)hydroxylamine can be conveniently prepared in reagent quantities (0.1–0.2 mmole). It should find general use as a reagent for characterization of trace amounts of acyl derivatives.

A radioactive hydroxylamine derivative would be a valuable reagent in characterizing acyl phosphate compounds such as that found in the NaK ATPase[1] preparation incubated in the presence of [$^{32}$P]ATP, Mg, and Na (Nagano *et al.*, 1965; Hokin *et al.*, 1965; Bader *et al.*, 1966), and possibly that postulated to be an acyl phosphate intermediate in the ADP–ATP-exchange reaction in beef heart mitochondria (Colomb *et al.*, 1966).

Since hydroxylamine itself contains no atoms which can be made both radioactive and nonexchangeable with the atoms of water it was necessary to synthesize an *N*-alkylhydroxylamine containing radioactivity in stable atoms of the alkyl group. Inasmuch as some of the preparations containing acyl phosphates bound to protein are highly impure and contain very little acyl phosphate (the most active NaK ATPase preparations contain only a few hundred pmoles of acyl phosphate/mg of protein) it appeared that a hydroxylamine of high specific activity would be needed. Another problem was that the concentration of hydroxylamine required to react with acyl phosphates ranges from about 0.1 to 0.5 M. Thus it was necessary to obtain the hydroxylamine

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[1] Abbreviations used: NaK ATPase, sodium- + potassium-activated adenosine triphosphatase; PHA, *N*-(*n*-propyl)hydroxylamine; ATP, adenosine triphosphate; ADP, adenosine diphosphate.

derivative in large quantities. This paper reports on a method of preparation of [2,3-$^3$H]PHA in reagent quantities and with a specific activity ranging from 545 to 666 mc/mmole. The compound can be made 98% radiopure with three separation steps after the final step in its synthesis.

## Materials

3-Bromopropene was obtained from Fisher Scientific Co., Chicago, Ill. 1-Nitropropane was obtained from Distillation Products Industries, Rochester, N. Y. Zinc dust (90% pure) and hydroxylamine hydrochloride were obtained from Allied Chemical Corp., General Chemical Division, Morristown, N. J. Tetrahydrofuran was made peroxide free immediately before use by passage over an aluminum oxide column and was collected over calcium hydride. *N*-Methylhydroxylamine hydrochloride was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. *O*-Methylhydroxylamine hydrochloride was obtained from K & K Laboratories, Inc., Plainview, N. Y. *N*-Phenylhydroxylamine was synthesized by the method described by Vogel (1959a), mp 83°, lit. mp 81°. *N*-Benzylhydroxylamine was synthesized by the method of Neubauer (1897). The final product, recrystallized from ethyl ether–petroleum ether (bp 30–60°), had a melting point of 56.5–57°, in agreement with the literature value of 57° (Behrend and Leuchs, 1890). *N*-Cyclohexylhydroxylamine was synthesized by the method of Feuer and Vincent (1962), mp 133–136°, lit. mp 138–140°. *N*-(*n*-Propyl)-hydroxylamine oxalate was synthesized by reduction of 1-nitropropane with zinc dust by a modification of the procedure described by Beckmann (1909) for *N*-methylhydroxylamine. The *N*-propylhydroxylamine was crystallized as the oxalate salt from ethanol–water, mp 151–152.5° (uncorrected and in agreement with the literature (Ryer and Smith, 1951)). *Anal.* Calcd: C, 40.00; H, 8.23; N, 11.66. Found: C, 40.08; H, 8.30; N, 11.45. The yield was 25%.

3-Nitropropene was synthesized from 3-bromopropene (30 ml) and 20 g of $AgNO_2$ by a modification of the procedure of Askenasy and Meyer (1892). The 3-nitropropene was purified by microdistillation. It was collected at a pressure of 43–45 mm and a boiling range of 37–40°. The $n^{23.4}D$ was 1.42530. The yield from 3-bromopropene was 10%.

## Methods

*Electrophoresis on Paper.* Paper electrophoresis was performed with a Gilson high-voltage electrophorator, Model D. Samples (25 μl), previously adjusted to pH 2.0 with 2 N HCl, were applied in 2-cm bands to 5-cm-wide strips of Schleicher & Schuell 598 YD paper. The buffer was formic acid (pH 2.0, 40 ml of 85% formic acid/l.). Electrophoresis was carried out at 2° at 4000 v for varying times, as indicated.

*Radioactive Assay.* Aliquots (100 μl) of appropriately diluted samples were added to 10 ml of Bray's (1960) scintillation mixture and counted in a Packard Tri-Carb liquid scintillation spectrometer, Model 3950. Counting efficiency was determined by the internal standardization technique, using [$^3$H]toluene as a standard. For counting the paper electropherograms, the air-dried paper strips were cut into 1-cm segments parallel with and beginning at the origin. Each segment was then cut into 1-cm squares and placed at the bottom of a scintillation vial. Water (1 ml) was added, and the contents were shaken for 30 min at room temperature. Bray's (1960) solution (10 ml) was added, and the contents were mixed and counted. Care was taken that the paper squares were lying flat on the bottom of the counting vial.

*Gas–Liquid Partition Chromatography.* 1-Nitropropane was determined by gas–liquid partition chromatography under conditions similar to those described by Bethea and Adams (1961, 1962). The column was 20% Apiezon on acid- and base-washed Chromosorb W. The column temperature was 72°, and the gas flow rate was 150 cc/min.

*N-(n-Propyl)hydroxylamine Assay.* This was done by the method of Hanes (1929). A PHA standard curve was run with each assay. Carrier PHA on chromatograms was detected by staining with 0.1 M ammoniacal silver nitrate. The PHA spot gave a black spot on a white background.

*Reaction of the Protein-Bound Acyl Phosphate in NaK ATPase Preparation with Hydroxylamine and Derivatives of Hydroxylamine.* The preparation of $^{32}$P-labeled guinea pig brain NaK transport ATPase, the conditions for its reaction with hydroxylamine, and the method for determining the released [$^{32}$P]orthophosphate have been described previously (Hokin *et al.*, 1965). In the present experiments, the conditions for the reaction of $^{32}$P-labeled protein with hydroxylamine or hydroxylamine derivative were modified; the reaction time was 30 min and the final concentration of hydroxylamine or derivative was 0.1 M.

## Procedure and Results

*Comparison of the Effectiveness of Substituted Hydroxylamines in Reacting with Protein-Bound Acyl Phosphate in Guinea Pig Brain NaK ATPase.* Before attempting the synthesis of a radioactive derivative of hydroxylamine, the reactivity of several derivatives toward the protein-bound acyl phosphate of guinea pig NaK ATPase was investigated (Table I). *O*-Methylhydroxylamine and *N*-phenylhydroxylamine were unreactive. The other *N*-alkylhydroxylamines tested showed comparable reactivities although all were less reactive than hydroxylamine. *N*-(*n*-Propyl)hydroxylamine was chosen as the derivative to be made radioactive.

*Tritiation of 3-Nitropropene.* To determine whether the double bond or the nitro group of 3-nitropropene was more susceptible to catalytic reduction, 1 mmole of 3-nitropropene and 1 ml of tetrahydrofuran were shaken with 200 mg of 10% palladium on carbon (Mozings, 1946) in the presence of exactly 1 mmole of hydrogen. Examination of the products by gas–liquid

TABLE I: Comparison of the Effectiveness of Substituted Hydroxylamines in Reaction with the Acyl Phosphate Residue of Guinea Pig Brain NaK ATPase Preparation.[a]

| Compound | % of Acyl Phosphate Reacted |
|---|---|
| Control | 13 |
| Hydroxylamine | 100 |
| *O*-Methylhydroxylamine | 21 |
| *N*-Methylhydroxylamine | 70 |
| *N*-(*n*-Propyl)hydroxylamine | 63 |
| *N*-Cyclohexylhydroxylamine[b] | 53 |
| *N*-Phenylhydroxylamine | 13 |
| *N*-Benzylhydroxylamine | 63 |

[a] $^{32}$P-labeled guinea pig brain NaK ATPase was incubated with 0.1 M solutions of hydroxylamine or derivatives in 0.1 M acetate buffer (pH 5.4) for 30 min at room temperature. The control was incubated under the same conditions in the absence of any hydroxylamine. Release of [$^{32}$P]orthophosphate was determined as described previously (Hokin *et al.*, 1965). Hydroxylamine released 76% of the label corresponding to 100% of the acyl phosphate present. [b] *N*-Cyclohexylhydroxylamine was used as a suspension since it was not soluble at a concentration of 0.1 M.

partition chromatography showed that approximately 75% of the 3-nitropropene had been reduced to 1-nitropropane. Reduction of 3-nitropropene with limiting amounts of tritium, therefore, seemed a feasible procedure for producing 1-[2,3-$^3$H]nitropropane.

After establishing conditions for hydrogenation in our laboratory ampoules containing freshly distilled 3-nitropropene, 10% palladium on carbon (hydrogenated before use; Mozings, 1946) in tetrahydrofuran, and 10% nitropropane in tetrahydrofuran were sent to New England Nuclear Corp., Boston, Mass., with exact instructions for carrying out the tritiation. 3-Nitropropene (1 mmole, 0.085 ml) was added to 2 ml of tetrahydrofuran containing 200 mg of 10% palladium on carbon (hydrogenated before use; Mozings, 1946). Tritium gas (25 c) was introduced into the reaction flask, and the reaction mixture was stirred magnetically for 1 hr at room temperature. The unreacted tritium was removed and the product was centrifuged to remove the catalyst. The reaction flask and the centrifuge tube were washed sequentially three times with 3 ml of 10% nitropropane in tetrahydrofuran. The reaction product and washings were pooled and divided into ten 1-ml fractions containing on the average about 2 c each and sealed in glass ampoules. Upon receipt in our laboratory, the ampoules were stored in liquid nitrogen until used.

To determine the amount of 1-[2,3-$^3$H]nitropropane present in the reaction product 1.8 mc of material plus 1 mmole of carrier 1-nitropropane were hydrogenated in the presence of 200 mg of 10% palladium on carbon (Mozings, 1946) in a closed system (Vogel, 1959b). Hydrogen consumption was followed until completion. The 1-[2,3-$^3$H]propylamine present in the preparation before and after hydrogenation was determined by adding carrier 1-propylamine, electrophoresing duplicate aliquots at 4000 v for 40 min, cutting out paper segments, and counting, as described under Materials and Methods. One of the duplicate electropherograms was not cut into segments but was sprayed with ninhydrin to locate the 1-propylamine. The preparation received from New England Nuclear Corp. contained 5% of its radioactivity as 1-propylamine; this increased to 21% after hydrogenation. Thus, 16% of the radioactivity in the tritiated reaction product was [2,3-$^3$H]-PHA potential material; *i.e.*, 1-[2,3-$^3$H]nitropropane.

*Reduction of 1-[2,3-$^3$H]Nitropropane to 1-[2,3-$^3$H]N-(n-Propyl)hydroxylamine.* All manipulations were carried out in an efficient fume hood. A tritium gas monitor was placed just outside the hood. No radioactivity was registered throughout the operation. The contents of two vials of 1-[2,3-$^3$H]nitropropane (containing 2 mmoles of 1-nitropropane and on the average 4 c of radioactivity) were allowed to thaw and were then immediately transferred (with two 0.5-ml rinsings of tetrahydrofuran) to an ice-cold 50-ml erlenmeyer flask containing a magnetic stirring bar and 4 ml of 0.6 M ammonium chloride solution. Between additions the reaction flask was stoppered to prevent losses of volatile 1-[2,3-$^3$H]-nitropropane. The reaction flask was fitted with a Liebig condensor surmounted by a coil condensor, through which ice-cold water was continuously pumped. The contents of the reaction flask were stirred with a magnetic stirrer at 60°. Zinc dust (600 mg) was added *via* the Liebig condensor in about seven equal portions over a 20-min period. At the end of this time traces of zinc dust in the lower condensor were washed into the reaction flask with 0.5 ml of tetrahydrofuran, and the reduction was continued for 10 min. The reduction mixture was then centrifuged. The supernatant fluid and three warm water washings of the zinc oxide in the reaction flask and in the centrifuge tube were made up to 16–20 ml, adjusted to pH 6.5 with 0.1 N HCl, and frozen overnight.

*Purification of [2,3-$^3$H]N-(n-Propyl)hydroxylamine.* The above reaction product was applied to a 1 × 20 cm Dowex 50-X8 (Na) column (100–200 mesh), which had been previously equilibrated with 0.005 M sodium phosphate buffer (pH 6.5). The sample was rinsed onto the column with an additional 50 ml of phosphate buffer, and a linear gradient of 0–2 M KCl in 0.005 M sodium phosphate buffer was then allowed to develop. The distribution of radioactivity eluted from the column can be seen in Figure 1. The recovery of radioactivity was 70–80%. When standard PHA was chromato graphed in an identical manner it eluted in exactly the same position as the second peak shown in Figure 1; 93% of the standard PHA was recovered.

The fractions in the PHA peak were pooled, adjusted to pH 1 with 2 N HCl, and dried in a flask evaporator at 40°. The [2,3-$^3$H]PHA hydrochloride was extracted

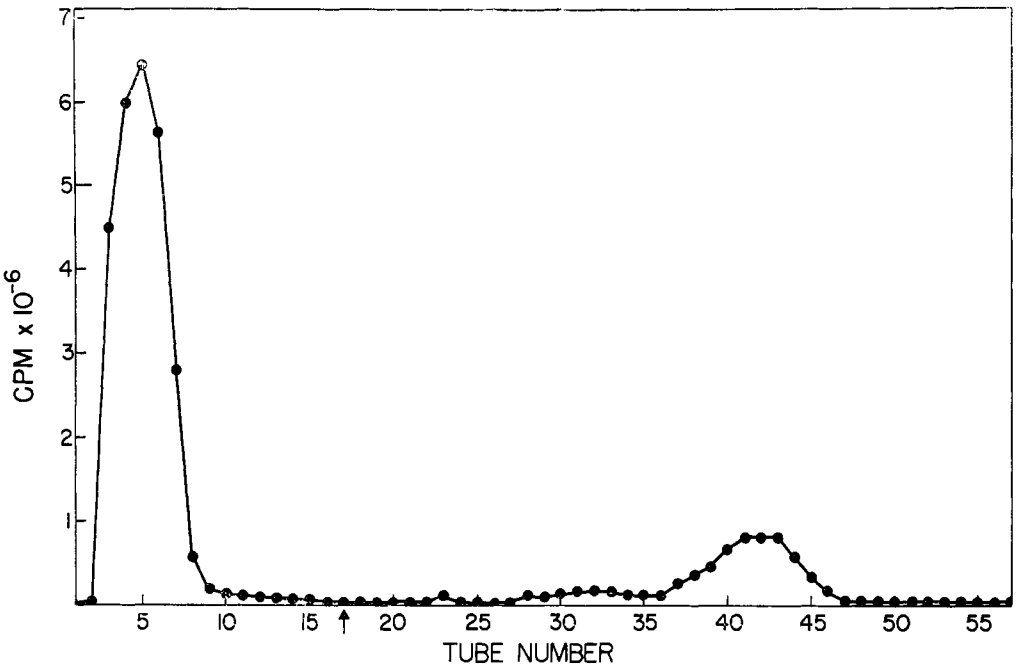


FIGURE 1: Chromatography of zinc reduction mixture on Dowex 50-X8 (Na) column. Fractions of 5 ml were collected by means of a drop counter. The gradient was started at fraction 17 (arrow) and continued until 200 ml was collected. The column was operated at room temperature and had a flow rate of 57 ml/hr. A 25-$\mu$l aliquot of each fraction was diluted with water (dilution factor = $11.2 \times 10^6$), and 100 $\mu$l was counted in 10 ml of Bray's (1960) solution. The recovery of radioactivity from the column was 75%. Fractions 36–47 inclusive were pooled.

from the residue with two 20-ml portions of hot absolute ethanol. The supernatant fluids were pooled and stored at −20°. The recovery of radioactivity during the extraction procedure was 90–100%. Electrophoresis of the PHA peak at this point, as described under Methods, revealed that only 54% of the radioactivity was [2,3-$^3$H]PHA, the remaining radioactivity being divided between two contaminant peaks positioned on either side of the PHA peak (Figure 2).

The [2,3-$^3$H]PHA obtained from the Dowex 50 column was dried in a flash evaporator at 40°, dissolved in 5 ml of water, and then subjected to continuous-flow electrophoresis (Brinkmann Instruments, N. Y., Model F.F.) in formic acid (pH 2.2) (Figure 3). By this procedure separation of the slower migrating contaminant was achieved. The fractions of the peak containing the PHA were pooled, 20 drops of 2 N HCl was added (this changes the PHA from the volatile formate to the nonvolatile hydrochloride salt on flash evaporation), and the material was dried in a flash evaporator at 40°. The residue was dissolved in 5 ml of 0.0075 M sodium citrate buffer (pH 5.34) and subjected to continuous-flow electrophoresis in this same buffer. A sharp separation of the [2,3-$^3$H]PHA from the faster migrating radioactive contaminant was achieved (Figure 4). The fractions in the PHA peak were pooled, acidified to pH 1–2 with 2 N HCl, and dried in a flash evaporator at 40°. The residue was suspended in 5 ml of absolute ethanol and adjusted to pH 4 with 2 N NaOH. Sodium citrate was removed by centrifugation. The sodium citrate residue was extracted once with 1 ml of absolute ethanol, and the supernatant fluids were pooled. The Brinkmann electrophoresis apparatus was decontaminated by washing with Radiacwash (Atomic Products Corp., Center Moriches, Long Island, N. Y.).

*Radiochemical Purity.* Samples of the [2,3-$^3$H]PHA

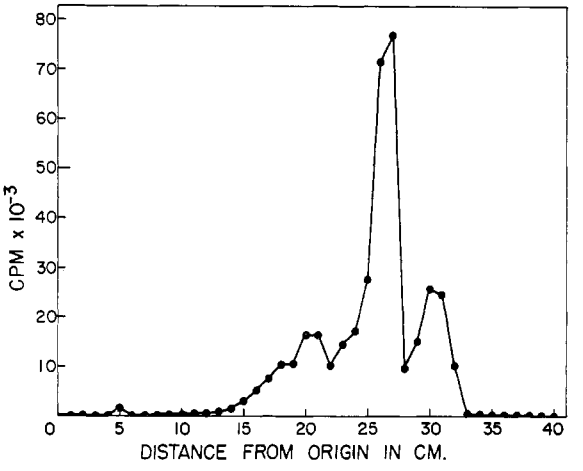


FIGURE 2: Electropherogram of [2,3-$^3$H]PHA peak from Dowex 50 column. The buffer was formic acid (pH 2). The distribution of radioactivity in cationic compounds is shown. Recovery was 90%. The center peak corresponded to stained carrier PHA run in parallel.

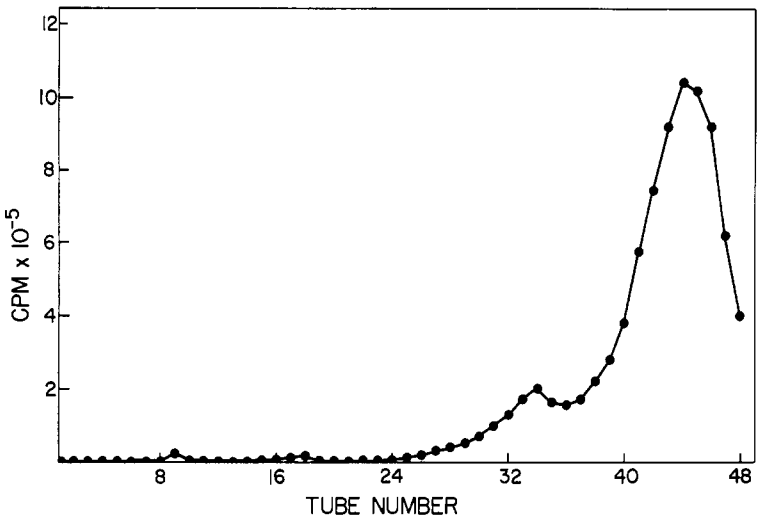


FIGURE 3: Spectrum of radioactivity in cationic material obtained by continuous-flow electrophoresis at pH 2.2 of [2,3-$^3$H]PHA peak from Dowex 50 column. Conditions of the run were: electrode buffer was 2.62 N formic acid, $\mu$ = 0.0232, pH 1.68; separating buffer was 0.235 N formic acid, $\mu$ = 0.00625, pH 2.2. The instrument was operated at 13°, 2000 v, and 275 ma. The sample (5 ml) was applied at a rate of 2.1 ml/hr with the background buffer flow at 210 ml/hr, the retention time between the plates being 40 min. The counting procedure was similar to that described for the Dowex column. The recovery of radioactivity was 97%. Fractions 40–48 inclusive were pooled.

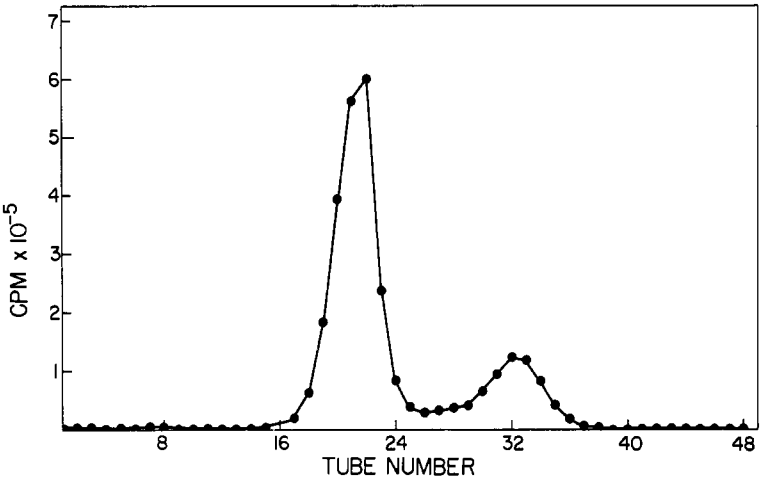


FIGURE 4: Spectrum of radioactivity in cationic material obtained by continuous-flow electrophoresis at pH 5.34 of [2,3-$^3$H]PHA purified by electrophoresis at pH 2.2. Conditions of the run were: electrode buffer 0.0075 M Na citrate, $\mu$ = 0.1095; separating citrate buffer was 0.0125 M, $\mu$ = 0.0365, pH 5.34. The instrument was operated at 2000 v, 198 ma, 7°. The sample (5 ml) was applied at a rate of 2.1 ml/hr, with the background buffer flow at 210 ml/hr, the retention time between the plates being 40 min. The recovery of radioactivity was 82%. Fractions 18–24 inclusive were pooled.

purified as described above were diluted with carrier PHA oxalate (2 mg/ml) acidified to pH 2 with 2 N HCl and the radiopurity was tested by chromatography on Whatman No. 1 paper in (1) *t*-butyl alcohol–2 N HCl–water (75:10:15, v/v), (2) methanol–ether–water–HCl (30:50:15:4, v/v), and (3) by electrophoresis on paper as described above. All systems gave a single [2,3-$^3$H]PHA peak with the $R_F$ values for 1 and 2 being 0.68 and 0.97, respectively. The radiopurity was 98%. It is worth pointing out that a single Brinkmann electrophoresis run in formic acid at pH 2.2 with a slower buffer flow rate than above could give [2,3-$^3$H]-PHA of 95% radiopurity.

*The specific activity* of the [2,3-$^3$H]PHA was determined by counting and assaying for PHA on appropriate aliquots as described under Methods. The

specific activity of the [2,3-$^3$H]PHA prepared from two different tritiated preparations was 545 and 666 mc/mmole.

*The yield* of [2,3-$^3$H]PHA, based on the amount of 1-[2,3-$^3$H]nitropropane present in the tritiated material received from New England Nuclear Corp., ranged from 20 to 50%. Based on the total amount of radioactivity present in the tritiated material, the yield ranged from 3 to 8%.

## Discussion

The preparation of radiopure [2,3-$^3$H]PHA of high specific activity in amounts sufficient to use as a reagent for reacting with possible acyl phosphate intermediates in enzymes has been achieved. From the amount of crude 1-[2,3-$^3$H]nitropropane obtained after catalytic reduction of 3-nitropropene with 25 c of tritium it should be possible to obtain a total of 1 mmole of 98% radiopure [2,3-$^3$H]PHA with a specific activity as high as 666 mc/mmole. This enables one to react appreciable volumes of biological material containing trace amounts of acyl phosphate with [2,3-$^3$H]PHA. It should be possible to recover much of the [2,3-$^3$H]PHA after reaction with the biological material although we have not investigated this in detail.

The use of [2,3-$^3$H]PHA may find fairly wide application in biochemistry. In addition to its use in characterizing acyl phosphates, [2,3-$^3$H]PHA should also prove useful in characterizing ester bonds (Harding, 1965), carboxylic acid anhydrides, and possibly other types of bonds, when these bonds are present in amounts too small to characterize by nonradioactive methods.

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