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The Synthesis of L-^{14}C-Serine Ethanolamine Phosphate, L-Serine 1,2-^{14}C-Ethanolamine Phosphate, L-^{3}H-Serine 1,2-^{14}C-Ethanolamine Phosphate, and Related Compounds *

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SUMMARY

A procedure for the chemical synthesis of the following labelled phosphodiesters : L-^{14}C-serine ethanolamine phosphate, L-serine ^{14}C-ethanolamine phosphate, L-^{3}H-serine ^{14}C-ethanolamine phosphate, L-^{14}C-threonine ethanolamine phosphate, L-threonine ^{14}C-ethanolamine phosphate, L-^{14}C-serine L-^{14}C-serine phosphate and L-^{14}C-threonine L-^{14}C-threonine phosphate is reported. The variously labelled forms of these phosphodiesters have been synthesized and purified by a route and a procedure leading to unequivocal structures for the products. The labelled L-serine L-serine phosphate and L-threonine L-threonine phosphate have been obtained as by-products in the preparation of the labelled forms of L-serine ethanolamine phosphate and L-threonine ethanolamine phosphate, respectively.

All the products were satisfactorily separated, isolated, purified and crystallized from the corresponding reaction mixtures through the use of desalting procedures, suitable Ion-exchange chromatography and thin-layer techniques; L-threonine L-threonine phosphate could be purified only to some degree. Physical and chemical data and elementary analyses of the labelled phosphodiesters are presented.

INTRODUCTION.

The L-serine and ethanolamine diester of orthophosphoric acid (L-SEP) was first reported by Roberts and Lowe [1] to occur in turtle muscle, and then also observed in the tissues of fish, reptiles, birds and amphibians [2, 3]. Another compound of related structure, L-threonine ethanolamine phosphate

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(L-TEP) was successively found in fish and cyclostom tissues [3-5]. Both esters have been recently indicated to be involved in some reactions of phospholipid biosynthesis [5, 6], and to have in addition a clear evolutionary role [2, 3, 7]. Experimental evidence has been obtained indicating that the two compounds play a metabolic role probably linked to the biosynthesis of brain microsomal phospholipid [8-11].

By adaptating previous methods [5, 6, 12, 13] and by modifying older procedures [12], we have succeeded by chemical methods in synthesizing the radioactive phosphodiesters. Techniques will be now described which have allowed us to synthesize in crystalline form L-^{14}C-serine ethanolamine phosphate (L-S(^{14}C)EP), L-serine ^{14}C-ethanolamine phosphate (L-SE(^{14}C)P) and L-^{3}H-serine ^{14}C-ethanolamine phosphate (L-S(^{3}H)E(^{14}C)P), as well as the corresponding forms of L-TEP. In addition, the synthesis of the chemically related labelled compounds, L-serine L-serine phosphate (L-SSP) and L-threonine L-threonine phosphate (L-TTP), has also been achieved, as by-products in the synthesis of L-SEP and L-TEP. A preliminary report of this work has already appeared [14-16].

MATERIALS AND METHODS.

I) Radioactive materials, reagents, Ion-exchange resins, adsorbents.

1,2-^{14}C-ethanolamine hydrochloride (New England Nuclear Corp.) had a specific activity of 4.34 μC/μmole. ^{14}C-L-serine (96.3 μC/μmole) and ^{14}C-L-threonine (127 μC/μmole) were uniformly labelled products of the Radiochemical Center of Amersham. DL-3-^{3}H-serine (82.3 μC/μmole) was obtained from the New England Nuclear Corp. Carrier-ethanolamine was twice redistilled, just before using, and unlabelled L-serine and L-threonine (both from Fluka, A.G., Buchs, Switzerland) recrystallized prior to use. Reference samples of unlabelled L-SEP, L-TEP and L-SSP were prepared as described previously [5, 6], and the solids kept in a desiccator under P_2O_5 until use. Unlabelled N-carbobenzoxy-L-serine benzyl ester (Yeda, Res. and Developm., Co., Ltd., Rehovoth, Israel), with a melting point of 82° C and a nitrogen content of 4.22% (theoretical 4.25%), was used without further purification. Unlabelled N-carbobenzoxy-L-threonine benzyl ester was prepared as described previously [5] and N-carbobenzoxy-ethanolamine was synthesized according to Jones and Lipkin [12] and Rose [17].

The reagents were of analytical grade and the solvents routinely re-distilled before use. In addition, chloroform was purified by distillation from P_2O_5, N-N'-dimethylformamide dried over anhydrous calcium sulfate and fractionally distilled under normal pressure, and the anhydrous pyridine obtained by distillation of a small portion on P_2O_5, which first had been dried over NaOH pellets. The platinum oxide was obtained according to Adams *et al.* [18] and the 50% palladium on asbestos carrier was a product from Fluka,

A.G. (Buchs, Switzerland). The ninhydrin reagent was used as recommended by Jacobs [19]. Phosphorylethanolamine, phosphorylserine, phosphorylthreonine, serine, threonine and ethanolamine were commercial samples recrystallized when necessary.

The ion-exchange resins were Dowex 50 W × 4, 200-400 mesh (H^+ form) and Dowex 50 W × 4, 200-400 mesh (dry) in the NH_4^+ form. The latter was well washed exaustively with conductivity water and then regraded [20], in order to give particles of uniform size of about 15-30 μ of diameter. Both resins were re-cycled several times by standard procedures before use and finally converted into the H^+ or NH_4^+ forms, by treating with 4 N HCl or aqueous 2 N ammonia with final washings with water until the eluates were free from Cl^- or NH_4^+ ions.

Thin-layer adsorbents were cellulose MN 300 G or DEAE-cellulose MN 300 G (Macherey Nagel and Co., Düren). Sephadex G-25 (medium grade) was treated before use by the reccomended procedures.

2) General methods.

Melting points were determined into sealed capillary tubes in a thermoregulated oil-bath on samples which had first been dried at 0.1 mm Hg of pressure over P_2O_5. The physical and chemical properties, together with the elementary analyses, were compared with those of the unlabelled products obtained as described previously [5, 6]. Optical rotations were measured into small cells with the sodium D line at 23° C, and infrared spectra at the Beckman IR-4 spectrometer, equipped with sodium chloride optics and KBr disks. Acid hydrolysis of the labelled synthesized compounds was carried out as described previously [16], followed by thin-layer or column chromatography of the products and of reference compounds.

Radioactivity was measured by two separate counting techniques, i.e. by a conventional gas-flow counter system with a thin-mylar window (15% efficiency on ^{14}C-labelled material) and more frequently by liquid scintillation methods, on a fully automated liquid scintillation spectrometer (Nuclear Chicago, Mod. no. 550). Two liquid scintillation mixtures (10 ml for each counting vial) were normally used, the first made with 10 gm of 2,5-diphenyloxazole and 1 gm of (4-methyl-5-phenyloxazolyl)-benzene in 1 liter of toluene, and the second with 6.5 gm of the first product, 130 mg of the second, 104 gm of naphtalene, 500 ml of toluene and 500 ml of dioxane. The finely divided silica Cab-O-Sil (Henry Cabot, Corporation), in a 4% (w/v) suspension was frequently used to improve the dispersion, especially when countings had to be made on thin-layer spots of labelled compounds [11]. For that purpose, the spot material was placed into the counting vial, 0.5 ml of distilled methanol was added with occasional shaking, and 10 ml of the 4% suspension in the scintillation mixture finally added prior to counting. Efficiency for carbon-^{14} and for tritium was normally about 80-84% and 38-40%, respectively.

Counts of ^{3}H in the double labelled samples were based on the following formula :

$$^{3}H = \frac{N_1 - aN_2}{E_1}$$

and counts of ^{14}C on the following formula :

$$^{14}C = N_2/E_2$$

in which N_1 is counts in the higher voltage channel 1; N_2 is counts in the lower voltage channel 2; E_1 and E_2 are efficiencies of the ^{3}H-counts and ^{14}C-counts, in the channel 1 and 2 respectively. These efficiencies were established by comparing the ratio of the sample counts in two appropriate windows of the channel 2 to the counts in the proper channels of the quenched double labelled standards of known efficiencies. By *a* it is indicated the ratio of counts in channel 1 and 2 of a ^{14}C-standard of the same efficiency of the ^{14}C in the double labelled sample. All counts were corrected for background counting rate, self-absorbment and decay.

Nitrogen was determined according to Jacobs [21], and phosphorus either on the chromatographic spots or on liquid samples by methods already reported [3, 7, 11]. NH_2-N was estimated by the ninhydrin reaction [3, 7, 19].

3) Ion-exchange chromatography and thin-layer procedures.

Ion-exchange chromatography and thin-layer separations were worked out as described previously [16]. Radioautography was carried out on a X-ray film (Kodirex) with a 3-10 day-exposure.

EXPERIMENTAL PART AND RESULTS.

In the following paragraphs we shall first report on the synthesis of the carbobenzoxy-compounds and their benzyl esters, and then on the synthesis of the successive products, i.e. of L-SEP and L-TEP labelled with ^{3}H or ^{14}C at different positions. Finally, we shall shortly report on the synthesis of chemically related structures, such as L-SSP and L-TTP.

1) N-carbobenzoxy-L-^{14}C-serine benzyl ester.

The original method of Baer and Maurukas [22] has been partially followed, with some modifications. In a 50 ml, 2-necked, round bottomed flask were placed 1.19 gm of magnesium oxide (Analar, B.D.H.), 1 gm of unlabelled L-serine, 200 μC (4 ml, about 225 μg, calculated on a molecular weight of 108 at the given specific activity of 96.3 μC/μmole) of uniformly labelled L-serine, 9 ml of H_2O, which were used to wash the serine-containing ampoules, 4 ml of 0.01 N NaOH to neutralize the acidic solvent of the labelled amino acid and 8 ml of diethyl ether. The solution was placed in an ice-water bath and

mechanically stirred. 2.51 gm of carbobenzoxy chloride were then added dropwise in 15 min time, and the whole mixture was kept at 0° C for about 2 hours and at room temperature for additional 30 min, always under stirring. The mixture was then freed of the magnesium oxide by centrifugation into pyrex-glass tubes, and the supernatant (about 25 ml), together with the washings (about 20 ml) of the flask and of the centrifuge tube, was placed in a small separatory funnel and twice washed with 20-25 ml portions of diethyl ether. The ether layer was removed by means of small capillary tubes. The aqueous solution (about 40 ml) was placed in a small beaker, and after the addition of the washings (20 ml) of the separatory funnel, was chilled and then brought to pH 2.5 at a pH-meter with 5 N HCl (about 2 ml). An oil separated, which then crystallized. After 3 hours of standing in an ice box, the liquid phase was decanted, while the white solid, washed at once with about 10 ml of ice-cold water, was directly dried *in vacuo* in the small beaker over sodium hydroxyde. Additional amounts of N-carbobenzoxy-L-^{14}C-serine (CBZS^{14}) were obtained by treating the aqueous phase with diethyl ether and then concentrating it under reduced pressure in a 100 ml-evaporating flask. The combined dry solids (about 2.15 gm), both collected in the 100 ml-evaporating flask, were then powdered and dissolved at 40° C as completely as possible with 40 ml of ethyl acetate with occasional stirring over a period of 45-60 minutes. The solution was therefore brought to dryness at 40° C under reduced pressure in the same 100 ml-evaporating flask, the residue dissolved in 15 ml of ethyl acetate at 65-70° C and treated with 45 ml of hot chloroform. After 1 hour of standing at room temperature, the solution was kept at about −25° C overnight. The precipitate was freed from the supernatant solution, re-dissolved and re-precipitated as above, and finally dried *in vacuo* over paraffin to constant weight on the same 100 ml-evaporating flask. The CBZS^{14} (1.77 gm, 78% of theory) melted from 118-119° C, had an optical rotation of +5.6 (*c*, 6 in glacial CH_3-COOH), a nitrogen content of 5.94% (theoretical value of 5.85%), and did not react positively with ninhydrin [3, 7, 19]. All these values were in good agreement with previous determinations [5, 6, 14, 22, 23]. Total radioactivity was 156 μC (78%) and the specific activity 0.021 (μC/μmole).

The CBZS^{14} was further converted into the N-carbobenzoxy-L-^{14}C-serine benzyl ester (CBZS^{14}BE). In the 100 ml-evaporating flask, containing 1.77 gm (7.4 mmole) of CBZS^{14}, a solution of 0.627 gm (7.45 mmole) of sodium bicarbonate in 19.4 ml of water was added in 10 min time, under vigorous stirring. To the sodium salt of the CBZS^{14}, which had been previously dried *in vacuo* by distillation at 35° C and completely essiccated at 0.1 mm Hg of pressure over sodium hydroxyde, were then added at 80° C 12.8 ml of N-N′-dimethylformamide. When solution was attained, 4.32 ml (36 mmole) of benzyl bromide were added, and the mixture was kept at 70° C for about 20 hours in the originary 100 ml-evaporating flask under water reflux. The N-N′-dimethylformamide and excess benzyl bromide were distilled off at 80° C by distillation, the last amounts being removed at 0.1 mm Hg with an

oil pump. The residue was then dissolved in the same container with 45 ml and 7 ml portions of diethyl ether and water respectively, and the solution transferred into a 100 ml-separatory funnel by means of small capillary tubes. The aqueous layer was removed with capillary tubes, and the ether solution, first washed twice with 5 ml of a saturated sodium bicarbonate solution and then twice with 10 ml of H_2O, was finally dried with anhydrous sodium sulfate. The solvent was then evaporated under reduced pressure and the benzyl ester purified by dissolving at 40° C the residue in the same evaporation flask with 10 ml of carbon tetrachloride and by adding 4 ml of petroleum ether (40-60° C of b.p.). After the whole solution had reached room temperature, the flask was left at 20° C for 3-4 hours, and then the liquid phase decanted. The crystalline residue was recrystallized by the procedure described above, transferred into a weighed 100 ml-Erlenmeyer flask and dried *in vacuo* over sodium hydroxyde. The CBZS^{14}BE (1.70 gm, 70% of theory, over-all yield of 54% on L-serine) melted from 84° C, had an optical rotation of +5.5 (*c*, 4 in chloroform), and did not react positively with the ninhydrin reagent. Its chemical composition was found to be: N, 4.19, C, 65.59, H, 5.71 (calculated for $C_{18}H_{19}NO_5$: N, 4.25, C, 65.64, H, 5.81). All these values, together with the infra red analyses, were in good agreement with previous findings [5, 6, 12, 14, 22] obtained for the unlabelled compound. Total radioactivity at this stage was 110 μC (71% of theory from CBZS^{14}, over-all yield of 55% on L-^{14}C-serine), and the specific activity (μC/μmole) 0.021. Before using the CBZS^{14}BE for the synthesis of L-SEP, an infinite amount was chromatographed on thin-layers (the results will be referred to later).

2) N-carbobenzoxy-L-^{3}H-serine benzyl ester (CBZS^{3}BE).

CBZS^{3}BE has been obtained with the same procedure described for the synthesis of CBZS^{14}BE. Starting material was 500 μC of DL-^{3}H-3-serine (1 ml, about 640 μg, specific activity of 82.3 μC/μmole), 226.5 mg of unlabelled L-serine, 1 ml of 0.1 N NaOH to neutralize the acidic solvent of the labelled amino acid, and proportional amounts of the other reactants. The CBZS^{3}BE was 400.36 mg (56.2% of theory based on L-serine) and melted from 84° C, in good agreement with the results of the CBZS^{14}BE. Total μC were 268 (53.6% of the originary radioactivity). By considering that isotope material at the starting of the synthetic process was diluted with unlabelled L-serine, and that the concentration of the D-^{3}H-serine in mmoles of CBZS^{3}BE was consequently negligible at the end of the synthetic process, specific activity of the main chemical product, i.e. of the L-CBZS^{3}BE, was 134 μC/1.216 mmole, namely 0.110.

3) N-carbobenzoxy-^{14}C-ethanolamine (CBZE^{14}).

This compound has been synthesized as described by Rose [17], while keeping automatically the pH of the reaction mixture between 9 and 10 during

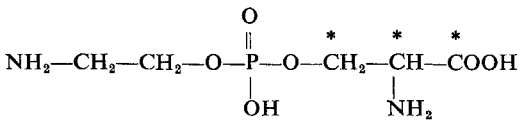
the addition of the carbobenzoxychloride [12]. A suitable small titration vessel, fitted into an automatic titration unit (Radiometer, Copenhagen), has been used in this connection. 400 μC of 1,2-^{14}C-ethanolamine hydrochloride (8.94 mg, 92.16 μmole), of specific activity 4.34 μC/μmole, were used. The content of the ampoules was freed from the ethanol by gentle evaporation on a steam bath at 40-45° C, transferred by successive portions of water (total of 2.2 ml) to the titration vessel and then diluted with 205 mg (3.36 mmole) of unlabelled freshly-redistilled ethanolamine. The specific activity after diluting was 400 μC/3.36 + 0.092 mmole, i.e. 0.116 μC/μmole. Carbobenzoxy chloride (0.596 gm) was then added dropwise to the reaction mixture over a period of 25-30 minutes at 0° C. 1 N NaOH was used to keep automatically the pH of the mixture at 9.7. The system was then processed as reported by Rose [17] with repeated crystallization of the CBZE^{14}. The final product (0.582 gm, 85% of theory on ethanolamine) had a radioactivity content of 296 μC (74% of recovery) with a specific activity of 296/2.980 mmole, i.e. of 0.099. Melting point was of 61° C, nitrogen content of 7.10% (theoretical value of 7.18%). Infrared spectra were the same than those quoted by Jones and Lipkin [12].

4) N-carbobenzoxy-L-^{14}C-threonine benzyl ester (CBZT^{14}BE).

CBZT^{14}BE has been prepared by a published procedure [24], carried out for the synthesis of the unlabelled compound. Some modifications have however been made, by precipitating the oily product of the N-carbobenzoxy--L-^{14}C-threonine with 5 N H_2SO_4 (about 2.5 ml) directly in the 100 ml-separatory funnel, in order to improve the recovery. Procedure (*b*) for the synthesis of the benzyl ester (see 24) has been used. Starting material was 300 μC (5.5 ml, about 291 μg, calculated on a molecular weight of 123 at the given specific activity of 127 μC/μmole) of uniformly labelled L-^{14}C-threonine, 1 gm of unlabelled L-threonine, 1.08 gm of magnesium oxide (Analar, B.D.H.), 3 ml of H_2O, which were used to wash the threonine-containing ampoules, 5.5 ml of 0.01 N NaOH to neutralize the acidic solvent of the labelled amino acid, and finally 6.3 ml of diethyl ether. 2.80 gm of carbobenzoxy chloride were then added and the reaction mixture processed with final crystallization of the product [24]. The CBZT^{14}BE (1.65 gm, 75% of theory, over-all yield of 57% on L-threonine), melted from 81° C, had an optical rotation of −13.1 in dry and ethanol-free chloroform (*c*, 2.7), and did not react positively with the ninhydrin reagent. Its chemical composition was found to be : N, 4.16, C, 66.71, H, 6.31 (calculated for $C_{19}H_{21}NO_5$: N, 4.08, C, 66.46, H, 6.16). All these values, together with the infrared analyses, were in good agreement with previous data [5, 6, 14, 24] of the unlabelled compound. Total radioactivity at this stage was 170 μC (76% of theory from carbobenzoxy-L-^{14}C-threonine, over-all yield of 57% on L-^{14}C-threonine), and the specific activity 0.035 μC/μmole. Before using the CBZT^{14}BE for the synthesis of L-TEP, an infinite amount was chromatographed on thin-layers (the results will be referred to later).

5) Synthesis of L-S(^{14}C)EP.

To synthesize L-S(^{14}C)EP, which has the following formula :



1.70 gm (5.15 mmole) of CBZS^{14}BE (see paragraph 1) were dissolved into the originary 100 ml-Erlenmeyer flask with 5 ml of chloroform and 0.72 ml of quinoline, and cooled in an ice-bath. To the solution were added, during the period of 1 hr, 1.13 gm (5.15 mmole) of phenyl phosphorodichloridate (Aldrich) in 10 ml of chloroform. After removing the ice-bath and stirring for another hour, the flask was brought to 20° C in a water-bath and 2.10 ml of dry pyridine quickly added. 1.007 gm (5.15 mmole) of unlabelled CBZE dissolved in 5 ml of chloroform was then added at 20° C over the course of one hour, and the whole mixture stirred overnight at 20° C.

25 ml of cold 6 N sulfuric acid was then added and the mixture transferred into a 100 ml-separatory funnel together with 3 ml of chloroform which was used in 1 ml portions to wash the flask. The chloroform layer was then successively washed with two 25 ml portions of ice-cold 6 N sulfuric acid, two 25 ml portions of H_2O, with 0.5 N $NaHCO_3$ and finally with other three 25 ml portions of H_2O, the aqueous phases being removed with small capillary tubes. The chloroform layer was then shaked in the separatory funnel with a mixture of acid-washed charcoal (Norit A)/anhydrous sodium sulfate (1/10), and directly evaporated to dryness in the hydrogenolysis vessel (50-60 ml of maximal volume). The Norit A/sodium sulfate material was exhaustively washed with chloroform, the washings transferred into the hydrogenolysis vessel and also evaporated. An oily yellow residue (3.26 gm, 95%), which constitutes the mixture of the neutral labelled phosphate esters, resulted at the end of the process. An optical rotation of $-4.2°$ at 30° C (*c*, 4.8 in ethyl alcohol) was observed at this stage.

The oily residue was dissolved in 23 ml of absolute ethanol, and 1.1 ml of 10 N perchloric acid and 0.6 gm of 50% palladium on asbestos were added. The mixture was then subjected to hydrogenolysis at the atmospheric pressure in the apparatus described in Figure 1.

Hydrogenolysis with palladium, carried out as described by Jones and Lipkin [12], led to the uptake of 0.0133 mole of hydrogen (89% of theory) in about 12 hr. The palladium catalyst was removed by filtration and washed with 2 ml of H_2O, 3 ml of absolute ethanol and three 3-ml portions of water. The filtrate and washings were collected in a larger hydrogenation vessel (110-120 ml of maximal volume), supplemented with 0.3 gm of platinum oxide and subjected to the second hydrogenolysis. 0.049 mole of hydrogen (72% of theory) was consumed.

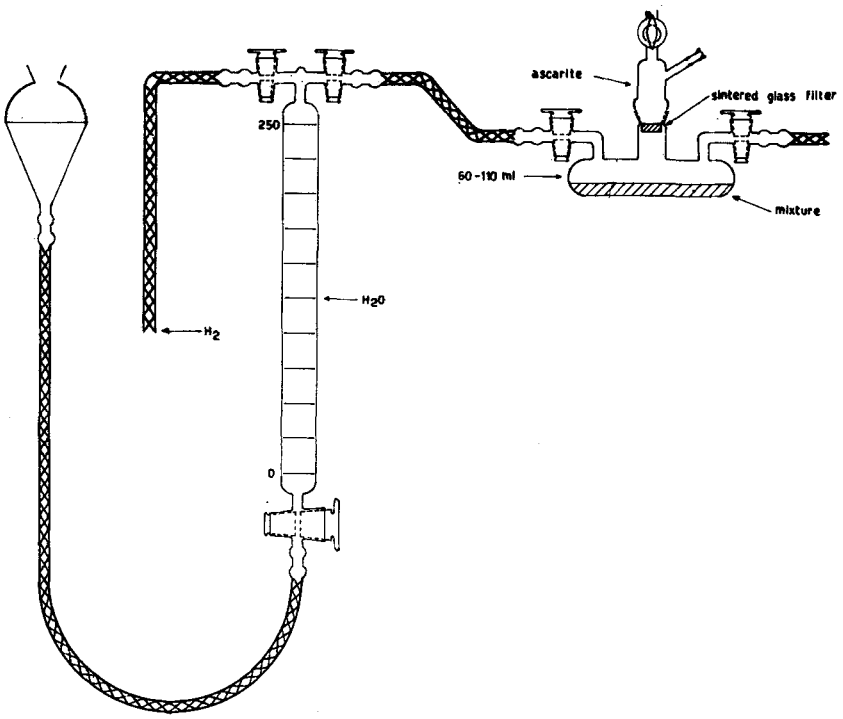


FIG. 1. A diagram of the hydrogenolysis apparatus used in the present work.

At this stage, the mixture of the neutral esters, which was shown by thin-layer chromatography and radioautography to contain several ninhydrin-reacting and -unreacting compounds, was subjected to purification as follows :

(*a*) *Step 1.* The reaction mixture was brought to a pH of 1.8-2.0 by the addition of 10% NaOH and then concentrated *in vacuo* to few ml directly into the hydrogenolysis vessel. The reaction mixture was filtered directly on the top of a column (2.05 cm × 9.2 cm, i.e. 30 ml) of Dowex 50 W × 4, H^+, 200-400 mesh. Very small aliquots of the filtrate were analyzed by thin-layer chromatography and radioautography at this phase.

(*b*) *Step 2.* The column was washed with H_2O until conductivity of the eluate had almost disappeared. The absorbed material was then eluted with 2 N aqueous ammonia, which was removed by evaporation in a small rotating evaporator. Small amounts of the concentrated eluate were again tested at this phase as above. Desalification with Sephadex G-25 column (1.20 × 65 cm) was also introduced as an alternate way in place of the Dowex 50 W treatment. 0.1 N acetic acid was used as eluant.

(*c*) *Step 3.* Only one fractionation of the components with a Dowex 50 W

resin, NH_4^+, was found necessary at this phase of the work. The eluate was in fact transferred to a column (2.25 cm × 123 cm) of finely graded Dowex 50 W ion-exchange resin, NH_4^+ form (489 ml), and allowed to pass through at a rate of about 30 ml/hr, with water as eluant [13]. The effluent was collected in 5 ml fractions, and a rapid screening of alternate fractions carried out by thin-layer chromatography. Complete separation of all the components of the system was achieved at this phase [16, 25], with L-SEP clearly separated from the L-SSP, which is a by-product of the synthesis.

(d) Step 4. The fractions containing L-SEP (between about 75-90) were combined, quickly evaporated at 35° C nearly to dryness under reduced pressure, and the residue was crystallized from aqueous methanol to yield colorless, hygroscopic microcrystals. The compound was three-fold re-crystallized and finally washed with dry methanol.

Two-dimensional thin-layer chromatography of the labelled L-S(^{14}C)EP revealed the presence of only one ninhydrin-reacting spot, which was superimposed on the radioautograph. The R_f values were 0.12 and 0.30 in the methylethylketone, methylcellosolve, 20% acetic acid (45, 15, 30, v, v, v), solvent and in the water-saturated phenol, respectively. The crystalline L-S(^{14}C)EP showed to possess the same physical and chemical properties of the unlabelled corresponding product synthesized previously [5, 6, 7, 12]. Part of these results is shown in Table I.

TABLE I. The analytical results and physical properties of chemically synthesized L-S(^{14}C)EP

	Labelled material [a]	Unlabelled reference compound [b]
Melting point (corr.)	144° C	145° C
α_D^{23} (in water)	—15.2 (*c*, 0.81)	—15.8 (*c*, 0.84)
Infrared absorption maxima (cm^{-1})	781, 832, 896, 912, 995, 1037, 1412, 1531, 2080	780, 832, 896, 912, 996, 1036, 1413, 1531, 2080
Compounds released on acid hydrolysis (6 N HCl, 5 hr, 100° C)	phosphorylserine, phosphorylethanolamine, serine, ethanolamine, inorganic phosphate	phosphorylserine, phosphorylethanolamine, serine, ethanolamine, inorganic phosphate
Analyses (%) : C	27.21	27.71
H	6.42	6.30
P	12.32	12.11
N	10.96	10.82

[a] Crystallized from aqueous methanol, two-fold re-crystallized, and dried over P_2O_5.
[b] The product has been obtained as described previously [5, 6]. Theoretical values for $C_6H_{13}N_2O_6P(CH_3OH)$: C, 27.70; H, 6.59; P, 11.91; N, 10.78. Molecular weight : 260.2.

The total amount of the L-S(^{14}C)EP was 0.180 gm (13.4% of theory, based on L-CBZS^{14}BE, and over-all yield of 7.3%, based on L-serine), and the total radioactivity 14.5 μC (13.2% from CBZS^{14}BE, and over-all yield of 7.25%, based on L-^{14}C-serine). Specific activity was 0.021 μC/μmole.

6) Synthesis of L-SE(^{14}C)P.

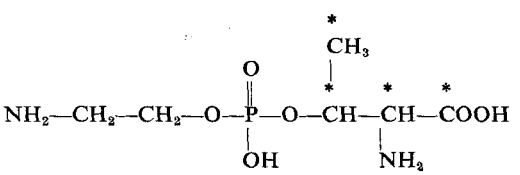
This compound was prepared by the same procedure used for the synthesis of L-S(^{14}C)EP (see paragraph 5). Starting material was 0.582 gm (2.98 mmole) of CBZE^{14}, with a specific activity of 0.099 μC/μmole (see paragraph 3), and 0.984 gm (2.98 mmole) of unlabelled CBZSBE. The synthesized crystalline phosphodiester possessed the same physical and chemical properties of the L-S(^{14}C)EP (see paragraph 5). The total amount of L-SE(^{14}C)P was 0.147 gm (0.566 mmole, 19% of theory on CBZE and over-all yield of 16.4% based on the ethanolamine). Total radioactivity was 56 μC (about 19% based on CBZE^{14} and over-all yield of 14% based on 1,2-^{14}C-ethanolamine). Specific activity was 0.1 μC/μmole.

7) Synthesis of L-^{3}H-serine ^{14}C-ethanolamine phosphate (L-S(^{3}H)E(^{14}C)P).

This compound was obtained by the same procedure described above. Starting material was 0.400 gm (1.21 mmole, 134 μC, specific activity of 0.110) of CBZS^{3}BE (see paragraph 2) and 0.236 gm (1.21 mmole, 120 μC, specific activity of 0.099) of CBZE^{14} (see paragraph 3). 0.266 gm of phenyl phosphorodichloridate was used as the coupling agent. At the end of the process the phosphodiester showed the same physical and chemical properties of L-S(^{14}C)EP (see paragraph 5 and Table I). Total amount of L-S(^{3}H)E(^{14}C)P was 0.047 gm (0.183 mmole, 15.1% of theory, based on the CBZ-compounds). Specific activity of the tritium in the phosphodiester was 0.116, and that of the ^{14}C 0.09.

8) Synthesis of L-T(^{14}C)EP.

L-T(^{14}C)EP, which has the following formula



was prepared by the procedure described above for the various labelled forms of L-SEP (see paragraphs 5, 6 and 7). 1.65 gm (4.80 mmole) of CBZT^{14}BE (see paragraph 4), of specific activity 0.035 μC/μmole, were mixed together with 0.936 gm (4.80 mmole) of unlabelled CBZE and 1.06 gm of phenyl

phosphorodichloridate. Hydrogenolysis was carried out as described previously as well as the following purification steps. Chromatography on column of the Dowex 50 W resin, NH_4^+ form, was also introduced in this case, only one fractionation being necessary to obtain clear separation of the labelled L-TEP from other contaminants [25]. The L-T(^{14})EP-containing fractions (between about 68-76), well separated from L-TTP (Fig. 2), which represents a by-product of the synthesis of L-TEP, identified by means of reference compound, were combined, quickly evaporated nearly to dryness and directly treated with 30 volumes of methanol/acetone (1/4, v/v) in about 15 min at room temperature. The precipitate was dissolved in minimal amounts of water and reprecipitated as above. The micro-crystalline powder was washed repetitively with anhydrous acetone and finally dried *in vacuo* over P_2O_5 and liquid paraffin.

The labelled L-TEP was chromatographed by two-dimensional thin-layer chromatography : only one ninhydrin-reacting spot was observed, which was superimposed on the radioautograph. The R_f values were 0.13 and 0.43 in the methylethylketone, methylcellosolve, 20% acetic acid solvent (45, 15, 30, v, v, v) and in the water-saturated phenol, respectively. L-T(^{14}C)EP showed

TABLE II. The analytical results and physical properties of chemically synthesized L-T(^{14}C)EP.

	Labelled material [a]	Unlabelled reference compound [b]
Melting point (corr.)	176° C	176° C
α_D^{23} (in water)	—37.5 (*c*, 0.91)	—37.1 (*c*, 0.78)
Infrared absorption maxima (microns)	12.80; 10.87; 10.30; 9.65; 9.26; 8.13; 7.40; 7.11; 6.55; 6.08; 3.35; 2.92.	12.80; 10.86; 10.30; 9.65; 9.27; 8.13; 7.41; 7.12; 6.55; 6.08; 3.35; 2.92.
Compounds released on acid hydrolysis (6 N HCl, 5 hr, 100° C)	phosphorylthreonine, phosphorylethanolamine, threonine, ethanolamine, inorganic-phosphate	phosphorylthreonine, phosphorylethanolamine, threonine, ethanolamine, inorganic phosphate
Analyses (%) : C	30.2	30.7
H	5.8	6.0
P	13.4	12.9
N	11.2	11.3

[a] Precipitated from methanol-acetone (1-4, v-v), two-fold washed with dry acetone, and dried over P_2O_5.

[b] The product has been obtained as described previously [5, 6]. Theoretical values for $C_6H_{15}N_2O_6P$: C, 29.8; H, 6.2; P, 12.8; N, 11.6. Molecular weight : 242.

to possess at the final stage of the purification the same physical and chemical properties of the unlabelled corresponding product synthesized previously [5, 6, 7]. Part of the results is shown in Table II.

The total amount of the L-T(^{14}C)EP was 0.120 gm (0.496 mmole, 10.3% of theory, based on L-CBZT^{14}BE, and over-all yield of 6%, based on L-threonine), and the total radioactivity 17 μC (10% from CBZT^{14}BE, and over-all yield of 5.7%, based on L-^{14}C-threonine). Specific activity was 0.034 μC/μmole).

9) Synthesis of L-TE(^{14}C)P.

The procedures used for the synthesis of this compound were similar to those already described. Starting material was 0.582 gm (2.98 mmole) of CBZE^{14}, with a specific activity of 0.099 μC/μmole (see paragraph 3), and 1.02 gm (2.98 mmole) of unlabelled CBZTBE. The total amount of the L-TE(^{14}C)P was 0.092 gm (0.480 mmole), and the yield 16.1% of theory, based on CBZE, and 13.9%, based on the ethanolamine. Total radioactivity was 47 μC (about 16%, based on CBZE^{14}, and over-all yield of 11.7%, based on 1,2-^{14}C-ethanolamine (see paragraph 3). Specific activity was 0.098 μC/μmole.

Discussion.

The method here described for the chemical synthesis of labelled L-SEP and L-TEP modifies and simplifies more tedious procedures [12, 13], worked out for the synthesis of the unlabelled forms of the phosphodiesters, and yields in addition pure crystalline material. Modifications have been worked out in order to handle much smaller amounts of starting material and to achieve satisfactory recoveries. The chromatographic separation on the cellulose column [12, 13] has been omitted, and thin-layer chromatography techniques have been adopted in order to obtain rapid and reliable screenings at any stage of the synthetic processing.

The complete process of synthesis, separation, purification and crystallization of the variously labelled forms of L-SEP or L-TEP was achieved within 20 days, with clear-cut results of their structure. Moreover, suitable ion-exchange chromatography procedures have yielded pure crystalline labelled compounds at the end of the chemical process.

A rapid and satisfactory screening of the labelled phosphodiesters, together with their breakdown products formed during the hydrogenolysis step, has always been carried out after each main step of synthesis and purification, and a complete separation and isolation of the various components of the phosphate ester mixture has been achieved through the ion-exchange fractionation (paragraph 5, step 3). The latter brings to a complete separation of the various labelled and unlabelled compounds after the hydrogenolysis and desalification [16, 25], as shown in Figure 2.

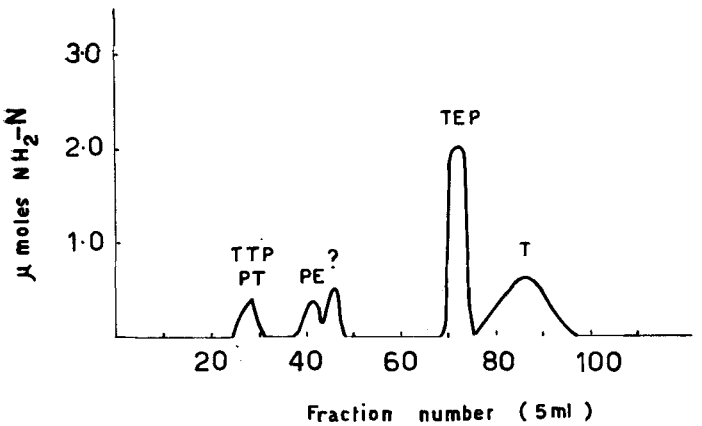


FIG. 2. Synthesis of labelled L-TEP. Ion-exchange chromatogram on a Dowex 50 W resin column, NH_4^+ form, of the reaction mixture after desalting (see, for analogy, paragraph 5, step 3, for the purification of L-SEP).

TTP = L-threonine L-threonine phosphate; PT = phosphorylthreonine; TEP = L-threonine ethanolamine phosphate; PE = phosphorylethanolamine; T = threonine; ? = unidentified.

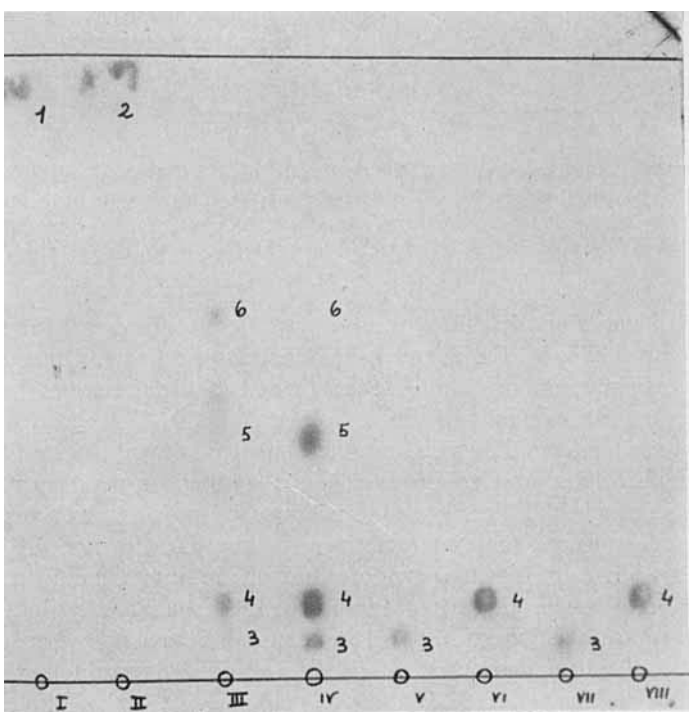


FIG. 3. Thin-layer autoradiograph of the synthesis of L-^{14}C-serine-labelled L-SEP and L-SSP.

I = carbobenzoxy-L-serine; II = carbobenzoxy-L-serine benzyl ester; III = reaction mixture at step 1 (see paragraph 5 of the text); IV = compounds at step 2; V = L-SSP after step 3; VI = L-SEP after step 3; VII = L-SSP after step 4; VIII = L-SEP after step 4.

For the spots : 1 = labelled carbobenzoxy-L-serine; 2 = labelled carbobenzoxy-L-serine benzyl ester; 3 = L-SSP; 4 = L-SEP; 5 = serine; 6 = unidentified.

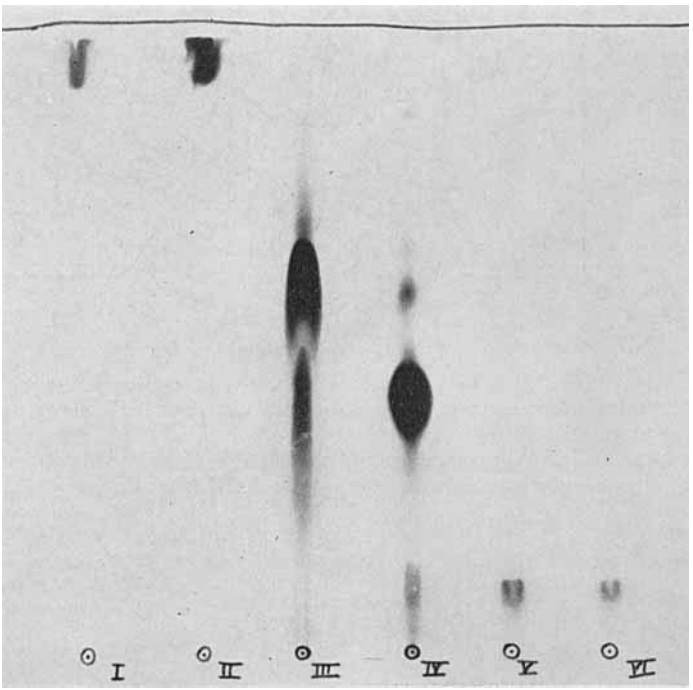


FIG. 4. Thin-layer autoradiograph of the synthesis of L-^{14}C-threonine-labelled L-TEP. I = carbobenzoxy-L-threonine; II = carbobenzoxy-L-threonine benzyl ester; III = reaction mixture at step 1 (see, for analogy, paragraph 5 of the text); IV = reaction mixture at step 2; V = L-TEP after step 3; VI = L-TEP after step 4.

Figure 3 and Figure 4 show thin-layer radioautographs of the synthetic process of labelled L-S(^{14}C)EP and L-T(^{14}C)EP, respectively. Other examples of chromatographic separation of the labelled phosphodiesters have been reported by us in previous work [16, 25].

As it appears from Figure 3, another labelled phosphodiester has been separated and isolated as a by-product of the synthetic processing of L-SEP. The product, which had already been examined by previous workers [12], has been isolated, purified and characterized as the L-^{14}C-serine L-^{14}C-serine phosphate (L-SSP). By repeated evaporations of the homogeneous chromatographic fractions 24-30 (step 3, paragraph 5), the product was obtained as a chromatographically pure white powdery material, which possessed the same physical and chemical characteristics of the unlabelled product synthesized previously [12, 15].

The compound was chromatographed on thin-layer plates by two dimensional procedures, and only one ninhydrin-reactive spot was observed, which was superimposed on the radioautograph. The R_f values were 0.06 and 0.24 for the methylethylketone, methylcellosolve, 20% acetic acid solvent (45, 15,

30, v, v, v) and for the water-saturated phenol, respectively, and were similar to the values found for the unlabelled chemically synthesized L-SSP.

The total amount of the labelled L-SSP was 0.070 gm (0.257 mmole, on the basis of a molecular weight of 272). The over-all yield, based on the L-serine (see paragraph 1) was 5.4%. Total radioactivity was 8 μC (4% over-all yield, based on the L-^{14}C-serine), and specific activity 0.031 μC/μmole.

A new phosphodiester-like compound has been detected also during the synthesis of the labelled forms of L-TEP, presumably representing a by-product of that synthesis. The compound, which was easily separated from the L-TEP in the fractions 25-31 by the chromatography with the Dowex 50 W resin, NH_4^+ (step 3, paragraph 5), was however still contaminated with traces of phosphorylthreonine, as shown in Figure 2. The product has been chromatographed on thin-layer plates by two-dimensional procedure : a broad ninhydrin-reactive spot, probably contaminated with phosphorylthreonine and which was superimposed on the radioautograph was observed. The R_f values were 0.04 and 0.37 for the methylethylketone, methylcellosolve, 20% acetic acid solvent (45, 15, 30, v, v, v) and for the water-saturated phenol, respectively. On the basis of its hydrolysis products, P/N ratio and analogy with L-SSP, the product was identified as the L-^{14}C-threonine L-^{14}C-threonine phosphate (L-TTP).

The total amount of the threonine-labelled L-TTP was 0.050 gm (3.8% over-all yield, based on L-threonine). Total radioactivity was 7 μC (2.3% over-all yield, based on ^{14}C-L-threonine), and specific activity 0.045 μC/μmole. Owing to the presence of traces of phosphorylthreonine in the L-TTP fraction, these last results cannot be conclusive.

In the Tables III and IV the comprehensive results of the synthetic processing of L-SEP and L-TEP, together with values of the synthesis of L-SSP and L-TTP, are shown. Table III describes the synthesis of the serine-labelled-L-SEP (see paragraph 5), and Table IV that of the threonine-labelled-L-TEP (see paragraph 8). It can be seen that the specific radioactivities of the crystalline L-SEP and L-TEP are 0.021 and 0.034 respectively, in good agreement with the levels of 0.022 and 0.037 of the ^{14}C-L-serine and ^{14}C-L-threonine in their starting reaction mixtures. The specific radioactivity of L-SSP (Table III) is 0.031 (1.5 times that of L-SEP), and that of L-TTP (Table IV) 0.045 (1.3 times that of L-TEP).

Over-all yields of the crystalline L-SEP and L-TEP, in their various labelled forms, are in the range of 10%. These values are not very different from those obtained by us and by previous workers for the synthesis of unlabelled phosphodiesters [5, 6, 12, 13]. In addition, the chemical process gives the possibility to obtain a by-product from the synthetic process of L-SEP, namely L-SSP, in a perfectly homogeneous form, with a recovery of 5-6% as based on L-serine. L-TTP, which is a by-product of the synthesis of L-TEP, has also been obtained during our studies, with an over-all yield of 4% as

TABLE III. The chemical synthesis of L-^{14}C-serine ethanolamine phosphate and of L-^{14}C--serine L-^{14}C-serine phosphate, through the carbobenzoxycompounds.

Compound [a]	Amounts [b]	μC [b]	S. A. (μC/μmole)
Serine	1.00	200	0.022
CBZS^{14}	1.77 (78%)	156 (78%)	0.021
CBZS^{14}BE	1.70 (54%)	110 (55%)	0.021
L-S(^{14}C)EP (step 1)	0.45 (18%)	37 (18%)	0.022
L-S(^{14}C)EP (step 3)	0.33 (13%)	27 (13%)	0.022
L-S(^{14}C)EP [c]	0.18 (7.3%)	14.5 (7.2%)	0.021
L-SSP (step 3)	0.12 (9%)	14 (7%)	0.030
L-SSP [d]	0.07 (5.4%)	8 (4%)	0.031

[a] For abbreviations, see the text.
[b] Between brackets are shown the over-all yields based on the originary serine or radioactivity.
[c] Three-fold crystallized compound.
[d] The product has been obtained as a powdery dried compound by various evaporations of the corresponding homogeneous chromatographic fractions obtained through the Dowex 50 W resin, NH_4^+ form (see the text), and finally dried over phosphorus pentoxide.

TABLE IV. The chemical synthesis of L-^{14}C-threonine ethanolamine phosphate and L-^{14}C--threonine L-^{14}C-threonine phosphate, through the carbobenzoxy-compounds.

Compound [a]	Amounts [b]	μC [b]	S. A. [b]
Threonine	1.00	300	0.037
CBZT^{14}	1.62 (75%)	224 (75%)	0.036
CBZT^{14}BE	1.65 (57%)	170 (57%)	0.035
L-T(^{14}C)EP (step 1)	0.46 (23%)	64 (21%)	0.034
L-T(^{14}C)EP (step 3)	0.22 (11%)	31 (10%)	0.034
L-T(^{14}C)EP [c]	0.12 (6%)	17 (5.7%)	0.034
L-TTP (step 2)	0.11 (8.3%)	16 (5%)	0.045
L-TTP [d]	0.05 (3.8%)	7 (2.3%)	0.045

[a] For abbreviations, see the text.
[b] Between brackets are shown the over-all yield based on the originary threonine or radioactivity. S. A. in μC/μmole.
[c] Micro-crystalline powder, three-fold re-crystallized.
[d] Step 3 of the synthesis of that compound (see the text). The product is not entirely homogeneous at the chromatographic analysis and is contaminated by traces of phosphoryl-threonine. Data are only indicative.

based on L-threonine, but further work is necessary to produce this ester in a satisfactory pure form.

CONCLUSIONS.

The synthetic processing which has been reported in this work gives a reliable way to prepare in a pure crystalline state L-SEP and L-TEP, labelled in either the L-serine and L-threonine respectively or in the ethanolamine moiety. In addition, L-SEP labelled contemporarily with ^{3}H-L-serine and ^{14}C-ethanolamine has also been synthesized. The procedures given in this paper provide a simple and quick way for the separation, identification and purification from their reaction mixtures of the crude labelled forms of L-SEP, L-TEP, L-SSP and L-TTP, after chemical synthesis had taken place.

The method could prove useful in the chemical preparation of these compounds, and may find some application, because L-SEP and L-TEP are phosphates of noticeable biological interest [6-11] and L-SSP and L-TTP may have some significance in the metabolism of phospholipids.

The recovery rates are satisfactory and the rapidity of the whole procedure promising for other future applications.

REFERENCES

1. ROBERTS, E. and LOWE, I. P. — *J. Biol. Chem.*, **211** : 1 (1954).
2. ROSENBERG, H. and ENNOR, A. H. — *J. Biochem. (Tokyo)*, **50** : 81 (1961).
3. PORCELLATI, G. and CURTI, B. — *Boll. Soc. It. Biol. Sper.*, **38** : 1867, 1872, (1962).
4. ROSENBERG, H., ENNOR, A. H., HAGERMAN, D. D. and SUGAI, S. — *Biochem. J.*, **84** : 536 (1962).
5. PORCELLATI, G. — *Boll. Soc. It. Biol. Sper.*, **39** : 1870 (1963).
6. PORCELLATI, G. — *Biochim. Biophys. Acta*, **90** : 183 (1964).
7. PORCELLATI, G., FLORIDI, A. and CIAMMARUGHI, A. — *Comp. Biochem. Physiol.*, **14** : 413 (1965).
8. DI JESO, F., MALCOVATI, M. and PORCELLATI, G. — *IXth International Conference on the Biochemistry of Lipids*. Noordwijk am Zee, Commun. no. 55.
9. PORCELLATI, G. — *2nd Meeting of the Federation of European Biochemical Societies*. Vienna, 1965, commun. no. A 74.
10. PORCELLATI, G., DI JESO, F. and MALCOVATI, M. — *Life Sci.*, **5** : 769 (1966).
11. PORCELLATI, G., DI JESO, F., MALCOVATI, M. and BIASION, M. G. — *Life Sci.*, **5** : 1791 (1966).
12. JONES, E. E. and LIPKIN, D. — *J. Amer. Chem. Soc.*, **78** : 2408 (1956).
13. BEATTY, I. M. and MAGRATH, D. I. — *J. Amer. Chem. Soc.*, **82** : 4983 (1960).
14. MONTANINI, I., SIMONCINI, I. and PORCELLATI, G. — *Boll. Soc. It. Biol. Sper.*, **40** : 1221 (1964).
15. SIMONCINI, I. and PORCELLATI, G. — *Boll. Soc. It. Biol. Sper.*, **40** : 1967 (1964).
16. PORCELLATI, G. — *3rd Symposium on Chromatography*, Bruxelles, 14-15 September 1964, Société Belge des Sciences Pharmaceutiques, p. 123.
17. ROSE, W. G. — *J. Amer. Chem. Soc.*, **60** : 1384 (1947).
18. ADAMS, R., VOORHEES, V. and SHRINER, R. L. — In «Organic Syntheses», Vol. I, John Wiley and Sons, New York, N.Y., 1944, p. 463.
19. JACOBS, S. — *The Analyst*, **81** : 413 (1956).
20. HAMILTON, P. B. — *Anal. Chem.*, **30** : 914 (1958).
21. JACOBS, S. — *The Analyst*, **85** : 257 (1960); **87** : 53 (1962).
22. BAER, E. and MAURUKAS, J. — *J. Biol. Chem.*, **212** : 25 (1955).
23. FRUTON, J. S. — *J. Biol. Chem.*, **146** : 463 (1942).
24. BAER, E. and ECKSTEIN, F. — *J. Biol. Chem.*, **237** : 1449 (1962).
25. PORCELLATI, G. — *J. Chromat.*, **18** : 168 (1965).