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Automatic Radio-chromatographic Separation and Determination of Chemically Synthesized 1,2-^{14}C-phosphorylethanolamine*

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SUMMARY

An automatic radio-chromatographic method for the purification, separation and quantitative determination of chemically synthesized ^{14}C-labelled phosphorylethanolamine is described. The chromatographic effluent from a cationic ion-exchange resin is automatically radio-determined by passage through a flow-cell filled with anthracene crystals and fitted on an automatic liquid scintillation spectrometer. The principal features of the system, and the methods used for the determination of the radioactivity content are also described.

The yield of the radioactive product has been calculated to be about 75%, the rapidity of the whole process satisfactory. The processing which has been described may be applied also for the synthesis, purification and separation of other chemically synthesized ^{14}C-labelled "phospholipid phosphoric esters".

INTRODUCTION.

Experiments on the biosynthesis of microsomal phospholipids of nervous tissues, such as phosphatidylserine, phosphatidylethanolamine and phosphatidylthreonine are being carried out in our laboratory [1, 6]. Various natural phosphomono- and phosphodi-esters, namely L-serine ethanolamine phosphodiester (SEP), L-threonine ethanolamine phosphodiester (TEP), α-glycerophosphate (GPA) and phosphorylethanolamine (PE), have been successfully assayed as *in vitro* precursors of the corresponding phospholipid moieties.

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Owing to the interest of studying the metabolic relationships of these "phospholipid phosphoric esters" with lipid biosynthesis, the chemical synthesis and purification of ^{14}C-labelled SEP and TEP was of primary importance and has already been achieved in our previous work [7, 10]. We will describe in the present paper, a micro-synthetic procedure for obtaining 1,2-^{14}C-PE. In addition, a radio-chromatographic method has been worked out, which provides a reliable means to separate and estimate the synthesized product in its chromatographic eluates. The automatic radio-chromatographic procedure can be easily applied to the separation and purification of other chemically synthesized "phospholipid phosphoric esters" of biological interest.

EXPERIMENTAL AND RESULTS.

Radioactive materials, reagents, ion-exchange resins, adsorbents.

$^{14}CH_2OH$-$^{14}CH_2NH_2$, from New England Nuclear Corporation, sealed in glass vessels, had a specific activity of 1.3 mC/mmoles. The content of the vessels was freed of ethanol by gentle evaporation on a steam bath at 40°-45° C. Carrier-ethanolamine and phosphorus oxychloride (Merck, Darmstadt, reagent grade), were twice re-distilled, just before using. Other reagents were of analytical grade, and the chromatographic solvents re-distilled before use. The ninhydrin reagent was used as described by Jacobs [11]. Unlabelled PE, from Calbiochem (California, U. S. A.), re-crystallized from ethanol, was used as reference compound.

The ion-exchange resin Zeo-Karb 225 (The Permutit Co., England), Na^+, 200-400 mesh (dry), 4.5% cross-linked, 1.8 of water regain, was well washed exhaustively with H_2O and then regraded according to Hamilton[12], in order to obtain particles of more uniform size (average diameter values of 20-25 μ). The resin was re-cycled several times by standard methods before use and finally converted in the Na^+ form. Dowex 50W $\times$ 4 resin, H^+ form, 200-400 mesh, opportunely re-cycled for use, was adopted for the desalting procedure without further purification.

Thin-layer adsorbent was cellulose MN 300 G (Macherey Nagel and Co., Düren) and thin-layer chromatography was carried out as described previously [10].

Synthesis.

The chemical synthesis of ^{14}C-labelled-PE has been scaled down to micro-levels by slightly varying a previously described technique [13]. Nearly four hours are needed for the whole procedure; high temperature (160° C) and reduced pressure (10 mm Hg) are not necessary as in previous methods.

1.41 ml $POCl_3$ (about 15.3 mmoles) is added dropwise to 0.55 ml of glass-distilled H_2O (about 30.7 mmoles) in a 50 ml narrow neck suction flask,

on a magnetic stirrer, at room temperature. $POCl_3$, as well as the other compounds which will be successively added, are dispensed by means of a small, graduated capillary tube fitted with a rubber valve; the liquids will thus be discharged into the flask by gentle regulation of the valve at the top of the capillary tube. The following reaction takes place :

$$2H_2O + POCl_3 \rightarrow PO(OH)_2Cl + 2HCl.$$

The HCl is transferred by aspiration into a second suction flask, connected with a water pump and containing a 40% NaOH solution kept at 0°-2° C. The HCl is thus being continuously removed during the addition of phosphorus oxychloride (50 minutes). After 30 minutes of standing, ethanolamine is added in 10 min time, first by allowing the labelled $^{14}CH_2OH$-$^{14}CH_2NH_2$ to react with the $POCl_3$ (vessels directly broken into the flask by small forceps) and then by adding the bulk of the unlabelled compound : the addition of both the labelled (0.087 mmoles, about 114 μC) and the unlabelled ethanolamine (about 2.98 mmoles) takes place at 60° C. The reaction mixture is kept at 60° C for additional 100 min on a magnetic stirrer, thus allowing the reaction :

$$CH_2NH_2\text{-}CH_2OH + PO(OH)_2Cl \rightarrow CH_2NH_2\text{-}O\text{-}PO(OH)_2 + HCl,$$

to take place. By pouring into the flask the unlabelled ethanolamine after the addition of the radioactive compound (which will therefore begin to react in an undiluted state), we have been able to obtain at the end of the synthetic process a labelled PE of higher specific activity (0.041) as compared to the expected theoretical values. The reaction mixture, while being stirred, is allowed to stand at 0° C without water pump connection, and 0.22 ml of re-distilled H_2O (about 12.2 mmoles) is added. 1 ml of N HCl is finally added, and the flask content brought up to 100° C for 50 min, in order to hydrolyze polyphosphate compounds. The reaction mixture is then cooled and concentrated under reduced pressure.

Radiochromatography.

The concentrated reaction mixture, adjusted to pH 2.20 by Na citrate buffer, 0.2 M, pH 2.20, is diluted to 4 ml, and then applied to a jacketed column of the Zeo-Karb 225 resin (1.25 × 186.5 cm). Chromatography is carried out at 23° C with Na citrate buffer 0.2 M, pH 3.10 as eluant [14, 15], in the presence of 0.05% BRIJ 35 (Watford Chemical Co., Ltd.), at a rate of about 8 ml/hr. The output of the chromatographic column is connected by means of a thin polyethylene capillary tube to a 1 ml lucite flow-cell filled by 3/5 of its capacity with a finely-divided solid fluor, i.e. with a reagent-grade recrystallized anthracene (25-50 mesh size value). A flow-cell of this type has an effective cell liquid volume of 0.4 ml, and possesses very low background values and high transmission of light. The outlet of the cell is conveniently

led to a fraction collector, and the effluent fractions may thus be automatically radio-determined and collected into 2 ml portions.

For the radio-chromatographic determinations, the flow-cell is fitted into the counting chamber of a fully automated liquid scintillation spectrometer (Nuclear Chicago mod. n. 550), equipped with a dual photomultiplier tube detector assembly (12 stage and 1 *ns* of dead time) with preamplifiers and amplifiers connected by a coincidence circuit and equipped with anti-coincidence discriminators. A three-channel monitor is used for counting; more precisely, only the data of one channel are recorded, while a second channel is used at a reduced window level, at the discriminator, in order to control occasionally the quenching effect according to the technique reported by Baillie [16]. The photomultiplier tube voltages and discriminator settings have been determined in the usual way, to give the best precision (maximal count^2/background ratio). The H.V. values were 1.000 and 1.300 V respectively for the monitor and gate phototubes, and the discriminator setting level placed between 0.5 and 9.9 V.

The radioactivity of the effluent was counted and recorded at 36 seconds intervals by a ratemeter-recorder and scaler-timer-printer combination, in order to obtain visible readings and printed data by means of a digital printer. By this way, as the radioactive effluent passes through the anthracene flow-cell, the scintillations are worked out by the photomultiplier tube detector assembly, amplified, and then recorded by the counting system. By properly correcting the recorded data, as later explained, and by plotting the corrected counts/min versus the effluent volume, an appropriate example of the separation and radio-chromatographic determination applied to the purification of labelled PE is presented (Fig. 1).

The PE peak lies between 140 and 210 ml of the effluent volume. It is well resolved, at the high load of radioactivity employed during our experiments. By the use of an appropriate splitting device, one may easily and conveniently led only a small given portion of the effluent to the counting system, thus avoiding any high radioactivity retention and raising of background in the cell. As it is shown in Figure 1, inorganic phosphate is eluted somewhat between 30 and 35 ml, whilst the unreacted labelled ethanolamine peak remains strongly adsorbed on the resin column and will be later recovered by washing the column with 2 M NH_4OH at the end of the chromatographic procedure.

Estimation of the radioactive product.

The amounts of the radioactive compound which has circulated through the flow-cell during the whole procedure may be determined from the recorded radioactivity values, R, expressed as counts/minutes. If these values are higher than 500,000 cpm, they need to be corrected, because of the dead time, T, of the detector, so that during a given counting period, t, the $cpm_{(t)}$ will be :

$$cpm_{(t)} = \frac{R}{1 - (RT)}.$$

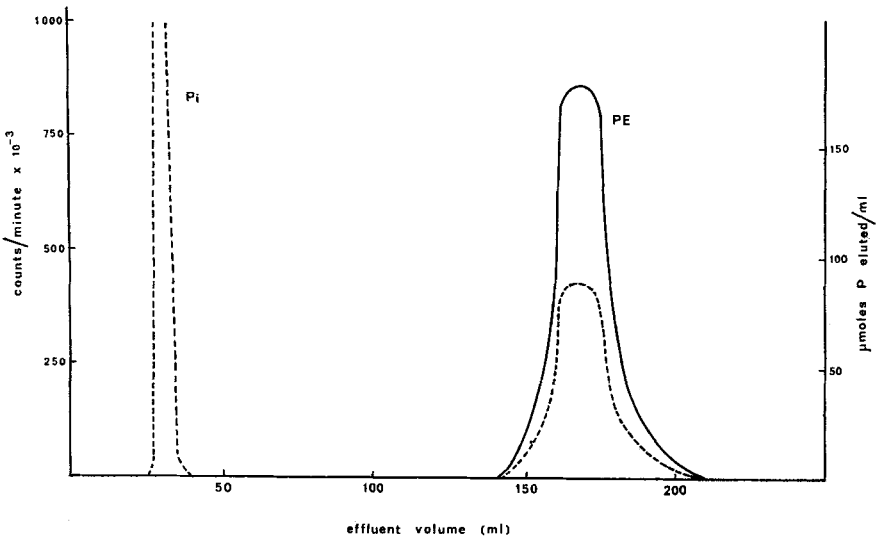

FIG. 1. The radio-chromatographic separation of the chemically synthesized ^{14}C-labelled phosphorylethanolamine.

P_i, inorganic phosphate; PE, labelled phosphorylethanolamine. Dotted line, phosphorus values; full line, radioactivity levels. For experimental conditions, see text.

Owing to the fact that each recorded value is referred to a single successive fraction of the effluent, it is necessary, at first, to calculate the integral of all the $cpm_{(t)}$ of the chromatographic peak, in order to obtain the radioactivity data of the total amounts of the chromatographic peak separated on the resin column. The integral :

$$cpm = \int_{t_0}^{t_n} cpm_{(t)}\, dt$$

may be graphically determined by a planimetric determination of the area of the peak recorded with an analogic means. More simply, we add all the $cpm_{(t)}$ values, obtained at 1 min interval, by means of a digital recorder, or more precisely

$$cpm = \frac{cpm_{(t0)} + cpm_{(tn)}}{2} + \sum_{i=1}^{i=n-1} cpm_{(t_i)}$$

The simplest way to correct the background value is to calculate it on the total data obtained as described previously. It is not possible with the flow-counting system to estimate the background values, B, during the chromatographic run. Conversely, it is easy to determine B, before, B_0, and after, B_n, the separation of the compound(s). On the other hand, during the chromatography of highly labelled compounds, the background levels may vary by a

noticeable degree during the whole experimental procedure, owing to the fact that the flow-cell has a radioactivity retention value of about 5 counts per 10^5 sample counts. We suggest the following formula for correction of the background values :

$$B = \frac{B_0 + B_n}{2} (t_n - t_0)$$

More precisely, we subtract a trapezoidal portion at the basis of the peak $(cpm)_{(t)} = f(t)$.

The effective total cpm of the separated compound, c/min, will therefore be :

$$c/min = \int_{t_{(0)}}^{t_{(n)}} cpm_{(t)} dt - B$$

The values of Figure 1 are expressed in c/min, and are thus corrected according to the given formulas.

As it is known, cpm values are to be divided by the efficiency, E, of the counting system, formed by the flow-cell and the spectrometer, in order to have dpm. The amount of the radioactive compound, expressed as μc, is, in fact, μc = dpm/2,220,000. In the case of a flow-counting system, a last correction factor must be necessarily applied, owing to the fact that every arbitrary fraction ε of radioactive compound remains in the flow-cell and is counted for the entire time, V/*v*, expressed as minutes, and which is necessary for the flow-cell to be passed by the effluent. Taking V as the cell volume, expressed as ml, and *v* the rate of the effluent, expressed as ml/min,

$$dpm = \frac{c/min}{E} \frac{v}{V}$$

We have estimated E by injecting 7 ml of a ^{14}C-labelled leucine standard aqueous solution (96 *nc*, 213,120 dpm) at a rate of 0.18 ml/min into the 0.4 ml flow-cell, and the effective total c/min found to be 127,410. Thus :

$$E = \frac{127{,}410 \times 0.18}{213{,}120 \times 0.4} = 0.269.$$

The amounts of the radioactive PE which has passed through the anthracene suspension has been calculated therefore as follows :

$$\mu c = \frac{156{,}620{,}000 \times 0.1(3)}{0.269 \times 0.4} \cdot \frac{1}{2{,}220{,}000} = 87.4.$$

Methods of controlling the content of the chromatographic effluent.

Phosphorus and nitrogen were estimated on the radioactive fractions, by the methods of Strickland *et al* [17] and Jacobs [18], respectively. The radioactivity of the single fractions of the PE peak has been determined by conven-

tional liquid scintillation method in 1 ml of absolute ethanol and 10 ml of 10 °/₀₀ PPO and 1 °/₀₀ dimethyl-POPOP in purified toluene.

In the whole range of the PE peak, the measurements of radioactivity of the single fractions agreed well with the values reported in Figure 1; moreover, phosphorus/nitrogen ratio was close to 1 in all the fractions. The phosphorus values have been compared with the radioactivity levels, as shown in Figure 1, and a constant specific activity of 0.041 μC/μmoles has been found throughout the whole peak.

Samples of the reaction mixture (see *synthesis*) and of each chromatographic fraction have been assayed by thin-layer chromatography, either on scintillation layers [19] or on cellulose plates. Free-NH_2 groups and phosphate were located on the same plate, by allowing the ninhydrin staining and the molibdate reaction procedures to take place successively on the scintillation layer.

The chromatographic fractions, displaced at an effluent volume between 140 and 210 ml, were shown to contain on the scintillation plates only the radioactive spot of PE, which also stained positively with ninhydrin and with the molibdate reagent. Its R_f value strictly corresponded to marker PE, as shown by thin-layer chromatography. The quantitative estimation of phosphorus and nitrogen on the PE spots scraped off from the cellulose layers agreed well with the expected values and with the theoretical P/N ratio.

Figure 2*a* shows a thin-layer chromatogram spotted from left to right with non-radioactive control of PE and of a mixture of PE and ethanolamine, with small aliquots of the initial reaction mixtures, before radiochromatography (applied in high and small amounts respectively), and finally (right) with aliquots of some fractions of the ion-exchange chromatographic eluates, taken out before, during and after the emergence of the PE peak. The plate was developed with the solvent system, methyl-ethyl-ketone, methyl-cellosolve, acetic acid, H_2O (15, 5, 2, 8, by volume), and then sprayed with the ninhydrin reagent. It may be seen from Figure 2*a* that the reaction mixture is clearly purified by means of a single ion-exchange chromatographic run, and that the PE spot appears well separated and without chromatographic impurities. The corresponding autoradiography of Figure 2*b* shows the same results. The spot of labelled ethanolamine is practically absent, and may be eluted from the Zeo-Karb ion-exchange resin column only by the 2 N aqueous ammonia (see Radiochromatography).

It is worth mentioning that at an effluent volume of the chromatographic eluates of about 30-35 ml, the thin-layer plate showed the presence of a spot of inorganic orthophosphate.

Control procedure of the desalted PE.

PE is desalted after radiochromatography through a procedure already described elsewhere [14, 15, 20], on a Dowex 50W resin, by following the

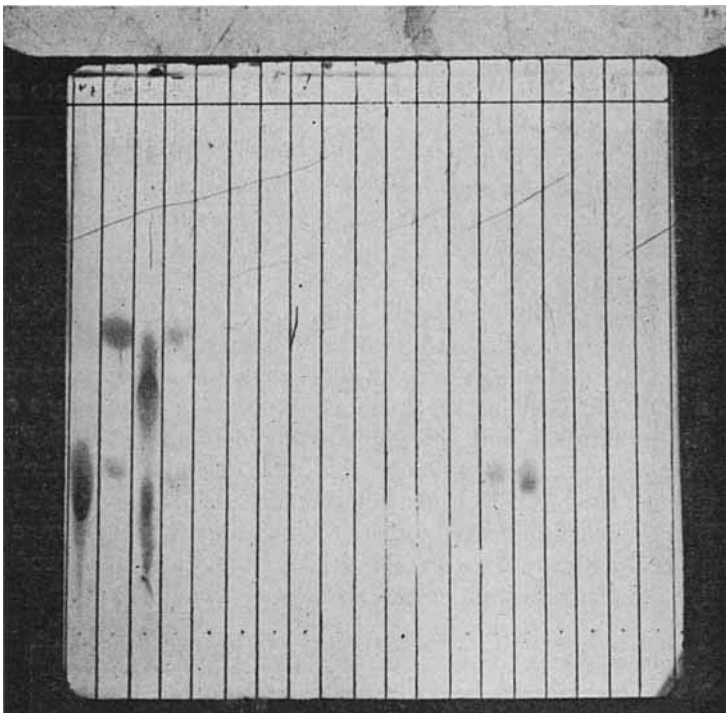

FIG. 2*a*. Thin-layer chromatogram of the reaction mixture (left) and of the ion-exchange chromatography fractions (right). For experimental conditions and for the description of the chromatogram, see text.

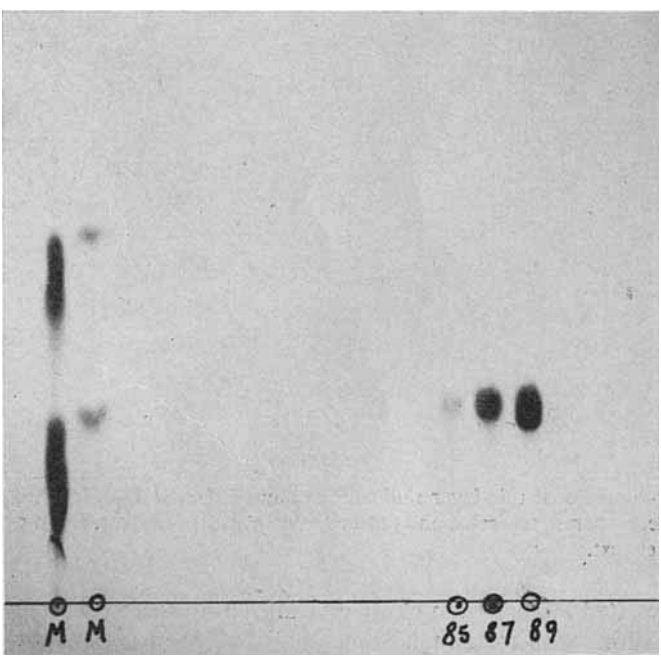

FIG. 2*b*. Thin-layer radioautography of the reaction mixture (left) and of the ion-exchange chromatography fractions (right). For the experimental conditions and for the description of the radioautography, see text.

desalting through a conductivity test of the effluent. Aqueous ammonia, which was used as a displacing agent, is easily removed by successive evaporations under reduced pressure into a small rotatory evaporation flask.

The concentrated desalted PE has been analysed by two-dimensional thin-layer chromatography with the following solvent systems : (1), methyl-ethyl-ketone, methyl-cellosolve, acetic acid, H_2O (15, 5, 2, 8, by volume), and (2), water-saturated-phenol, and the corresponding radioautography is shown in Figure 3. Only one spot is present, that reacted in the expected way with ninhydrin and with a molibdate-staining reaction. The estimations of radio-activity and of nitrogen and phosphorus content of the desalted PE agreed with the data of the radio-chromatographic analyses and of the determinations of the effluent : total μC, 87.4, and total mmoles 2.131. The μC and mmoles of the PE in the reaction mixture before radiochromatography were estimated and found to be 87.8 and 2.14 respectively. Thus, no loss of product does practically take place during the ion-exchange chromatography.

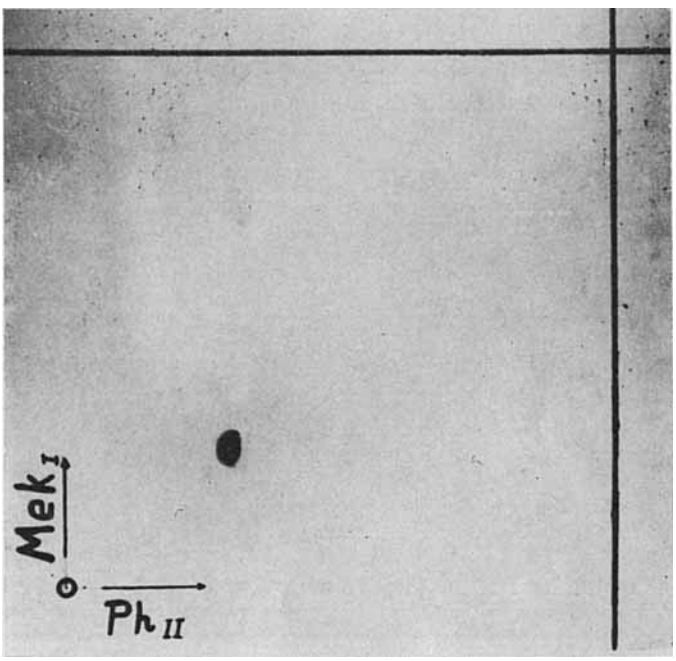

FIG. 3. Two-dimensional thin-layer radioautography of the desalted, labelled phosphoryl-ethanolamine, prepared, separated and purified as described in the text. For the experimental conditions, see text.

The desalted PE has been easily crystallized by successive treatments and recrystallizations with methanol. Some analytical properties of the crystalline PE are reported in Table 1 and compared with the properties of the desalted

uncrystallized compound. It may be seen from Table 1 that melting point and phosphorus and nitrogen content of the crystalline product are close to the theoretical values, and that there is no difference between the properties of the desalted un-crystallized PE and those of the crystalline compound.

TABLE I. Analytical properties of the radio-chromatographed ^{14}C-labelled phosphoryl-ethanolamine.

	Column eluate (desalted product)	Crystalline product
Amount examined	1 ml	10 mg
μmoles P	199	70.5
μmoles N	202 [a]	69.4
% of N (theoretical)	—	9.93
% of N (found)	—	9.72
% of P (theoretical)	—	21.93
% of P (found)	—	21.84
P/N ratio	0.99	1.02
Melting point (corr.)	—	242° C
Specific activity (μC/μmol P)	0.041	0.041

[a] This value has been obtained after calculation of the amount of ammonia (trace amounts estimated by Nessler's reagent).

DISCUSSION.

The chief advantage of the synthetic processing here described is the possibility of working on very small volumes of reactants (in the range of a milliliter), without the use of reduced pressure or high temperature. Only four hours are necessary for the chemical synthesis and about forty hours for the complete processing. The yield in mmoles is satisfactory (69%), and that in μC even more (77%).

The main advantages of the automatic radiochromatography, as compared to purification procedures by repeated crystallizations of the product, are : (1), the yield is almost quantitative, (2), the process is completely automatic and can be worked out without control and overnight, (3), the procedure is quick, lasting about 35 hours, (4), the radio-chromatographed product, after desalting, is already pure to be used in the *in vitro* incorporation experiments, (5), the position of the radioactivity peak represents a tool for the identification of the substance, (6), the radioactivity values automatically recorded are easily employed for the quantitative estimation of the product and for the yield of the synthetic processing.

The type of the flow-cell used in these experiments has the following features : (1), anthracene does not give any chemical quenching, (2), anthracene

does not dissolve in the aqueous streams, (3), the position of the fluor inside the cell and its fine subdivision make the contact very intimate on a large surface, and the efficiency very high, (4), the continuous flow system is among the easiest ones to be managed, (5), the system does not modify the standard flow conditions of a chromatographic column, although a slight pressure has been applied to counterbalance the cell resistance, (6), the cell packing procedure and the flow system make it possible to achieve high reproducibilities of results and good standardizations for quantitative determinations.

Methods of column chromatography separation for the "phospholipid phosphoric esters" have been described [14, 15, 20]; they will be used for the automatic radio-chromatographic separation here described, in order to prepare and purify the esters, of primary importance for the studies on the biosynthesis of phospholipids in nervous tissues.

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