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Biosynthesis of Phosphatidyl Myoinositol Phosphates in Rabbit Brain*

Robin M. Saunders and Clinton E. Ballou

ABSTRACT: Rabbit brain slices were incubated separately with orthophosphate-^{32}P and myoinositol-2-^{3}H, and the incorporation of label into the phosphoinositides was determined by analysis of the intact lipids separated by DEAE-cellulose chromatography. The results are consistent with the hypothesis that the phosphatidylmyoinositol moiety remains intact during the metabolic interconversions of the phosphoinositides. Two different methods of isolation of the lipids gave similar results. Analysis of the incorporation of myoinositol-2-^{3}H by either method failed to provide any evidence for an alternative biosynthetic relationship among the phosphoinositides. Each of the different extracts obtained in the Folch procedure for isolating the phosphoinositides [Folch, J. (1949), *J. Biol. Chem. 177*, 505] contains phosphatidylmyoinositol, but only the petroleum ether extract contains the polyphosphoinositides. Mitochondria incubated with orthophosphate-^{32}P are shown to incorporate the label into all three phosphoinositides.

The incorporation of labeled precursors (orthophosphate-^{32}P, myoinositol-^{3}H, and glycerol-2-^{14}C) into the three components of the polyphosphoinositide complex by rabbit brain slices has been described (Brockerhoff and Ballou, 1962a,b). The results indicate that the glyceryl–phosphoryl–myoinositol structure is retained intact in the metabolism of the inositides, *i.e.*, phosphatidyl-L-myoinositol (I) → phosphatidyl-L-myoinositol 4-phosphate (II) → phosphatidyl-L-myoinositol 4,5-diphosphate (III). The results were obtained from analysis of the deacylated lipids. Since these observations were made, other workers (Hölzl and Wagner, 1964; Andrade and Huggins, 1964; Ellis and Hawthorne, 1962) have confirmed the data for the incorporation of radioactive orthophosphate, but conflicting results have been reported (Palmer and Rossiter, 1964) concerning the inositol incorporation. In an attempt to resolve this conflict, we have repeated and extended our studies by analyzing directly the intact lipids. The results provide further support for the biosynthetic pathway we have proposed.

Since the method of isolation of the lipids may affect the results, we have used the experimental procedure of Palmer and Rossiter (1964) and have reexamined our own technique. Similar results were obtained by both methods. It had been assumed (Brockerhoff and Ballou, 1962a) that, in the original Folch (1949) procedure, the phosphoinositides are obtained only in the petroleum ether (bp 60–70°) extract. To check this assumption, all of the fractions obtained by this multiple solvent extraction method have been analyzed for inositides. Each is shown to contain I, although the polyphosphoinositides are found only in the petroleum ether extract. Our results establish that rabbit brain mitochondria, like brain slices, are able to incorporate orthophosphate-^{32}P into all of the phosphoinositides.

Experimental Procedure

Materials and Methods. Myoinositol-^{3}H was obtained from New England Nuclear Corp. and was shown to be isotopically homogeneous by paper chromatography. Phosphorus was determined by Bartlett's method (1959) and myoinositol was assayed micro-

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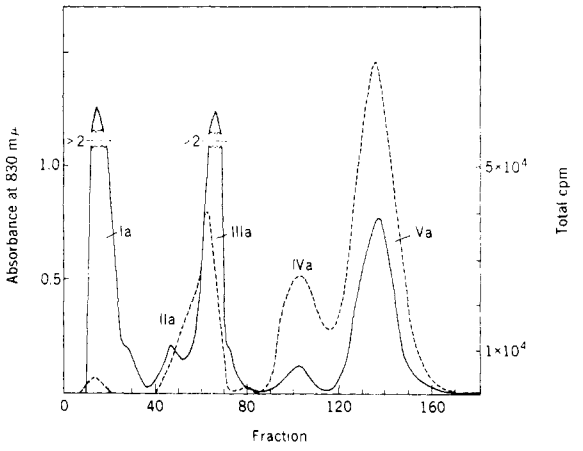

FIGURE 1: DEAE-cellulose chromatography of rabbit brain phosphoinositides after incubation with radioactive inorganic phosphate. Dashed line, radioactivity; solid line, phosphorus. Peaks Ia and IIa were not identified. IIIa contained phosphatidylinositol and phosphatidylserine, IVa contained II, and Va contained III.

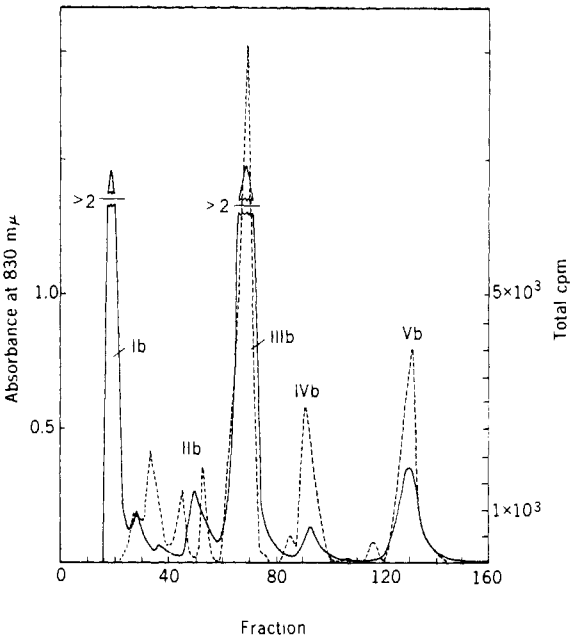

FIGURE 2: DEAE-cellulose chromatography of rabbit brain phosphoinositides after incubation with myoinositol-2-^{3}H. Dashed line, radioactivity; solid line, phosphorus. Peaks Ib and IIb were not identified. IIIb contained I plus phosphatidylserine, IVb contained II, and Vb contained III.

biologically with *Kloeckera brevis* (Snell, 1950). Paper chromatographic separations were made on Whatman No. 1 paper using solvent system A (1-propanol–ammonia, 1:1) and on formaldehyde-treated paper using solvent system B (1-butanol–acetic acid–water, 4:1:5).

Measurements of ^{32}P activity were made with a Geiger-Müller counter using aluminum planchets. Myoinositol-^{3}H measurements were made in a scintillation counter using a phosphor solution of 8% naphthalene (w/v), 0.5% 2,5-diphenyloxazole (w/v), and 0.05% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (w/v) in toluene–dioxane–ethanol (38.5:38.5:23).

Incubation. For each of the following extraction procedures, an 8-g brain from a 16-week-old rabbit was sliced approximately 1 mm thick and incubated in 75 ml of Krebs–Henseleit solution with 50 mg of glucose and 3 mcuries of orthophosphate-^{32}P (or 0.25 mcurie of myoinositol-2-^{3}H) in a 500-ml flask under oxygen–carbon dioxide (95:5) for 2 hr at 38°.

Isolation of Lipids. PROCEDURE A. The slices were collected by filtration, homogenized, and extracted by the Folch (1949) procedure. Extraction was carried out twice with acetone (75 and 50 ml), with ethanol (50 ml), and finally twice with petroleum ether (50 ml each time). A Servall Omni-Mixer was used throughout the extraction procedure and each homogenation was for 1.5 min. The combined petroleum ether extracts were evaporated to dryness, the residue was dissolved in 10 ml of chloroform–methanol–water (20:9:1), and the solution was washed through a column (10 × 1 cm) of Chelex-100 resin (Na^+ form) with an additional 50 ml of the same solvent. The combined eluent was concentrated *in vacuo* to half volume, which was diluted with 10 ml of water, and the concentration continued to remove the organic solvents. The aqueous emulsion was shaken with 0.2 g of disodium EDTA for 1 hr, during which time the pH was adjusted to 7.0 with 1 N NaOH, then it was dialyzed against several changes of water and finally lyophilized. The residue was dissolved in a little chloroform–methanol–water (20:9:1). Chromatography on DEAE-cellulose was carried out as described previously (Hendrickson and Ballou, 1964), using a 50 × 1 cm column and a gradient of 0–0.75 M ammonium acetate in the mixed solvent. Fractions of 2 ml were collected from which 0.5 ml was removed for phosphorus analysis, 0.1 ml for ^{32}P assay, or 0.2 ml for inositol-^{3}H assay. The elution patterns and radioactivities are shown in Figures 1 and 2.

PROCEDURE B. The slices were collected by centrifugation, homogenized, and extracted by the procedure of Palmer and Rossiter (private communication) with the aid of a Servall Omni-Mixer. The tissue was homogenized with 19 volumes of chloroform–methanol (2:1) for 10 min, then 0.2 volume of methanol was added, and the suspension was centrifuged. The supernatant was decanted and the ratio of chloroform:methanol was readjusted to 2:1 before washing (extract A). The tissue residue, remaining after preparation of extract A, was extracted three times with five volumes of chloroform–methanol–concentrated hydrochloric acid (200:100:1), each for 19 min. These combined extracts were extract B.

Extracts A and B were washed by the procedure of Folch *et al.* (1957). The extracts were first shaken with

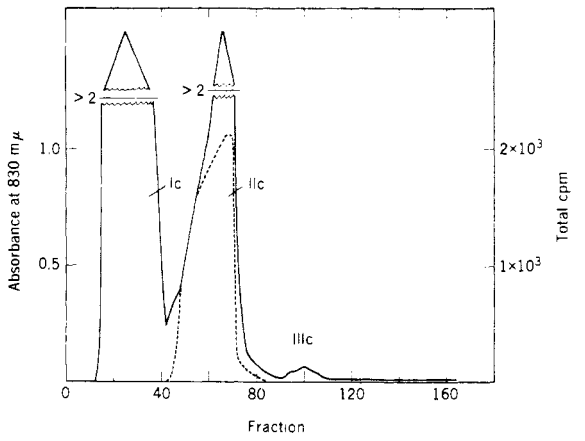

FIGURE 3: DEAE-cellulose chromatography of chloroform–methanol extract of rabbit brain slices after incubation with myoinositol-2-^{3}H. Dashed lines, radioactivity; solid lines, phosphorus (extract A). Peaks Ic and IIIc were not investigated. Peak IIc contained I and phosphatidylserine.

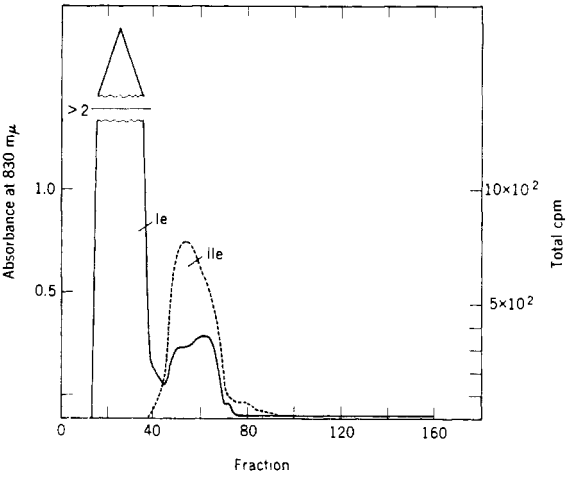

FIGURE 5: DEAE-cellulose chromatography of acetone extract of rabbit brain slices after incubation with myoinositol-2-^{3}H. Dashed line, radioactivity; solid line, phosphorus. Ie was not investigated. IIe contained I plus phosphatidylserine.

0.2 volume of 0.05% calcium chloride, the mixture was centrifuged, and the lower chloroform phase was retained. This was washed six times with prepared "upper phase" solution (chloroform–methanol–water, 3:48:47), which contained 0.02% calcium chloride. The first four "upper phase" washes also contained 0.1 N hydrochloric acid to reduce protein contamination (Dittmer and Dawson, 1961). The washed chloroform layer was evaporated to dryness and dissolved in a small volume of chloroform–methanol–water (20:9:1). Each extract was then treated exactly as described for the petroleum ether extract in procedure A and chromatographed on DEAE-cellulose using a 50 × 1 cm column and a 0–0.75 M ammonium acetate gradient. Fractions of 2.2 ml were collected, from which 0.5 ml was removed for phosphorus analysis and 0.2 ml for inositol-^{3}H

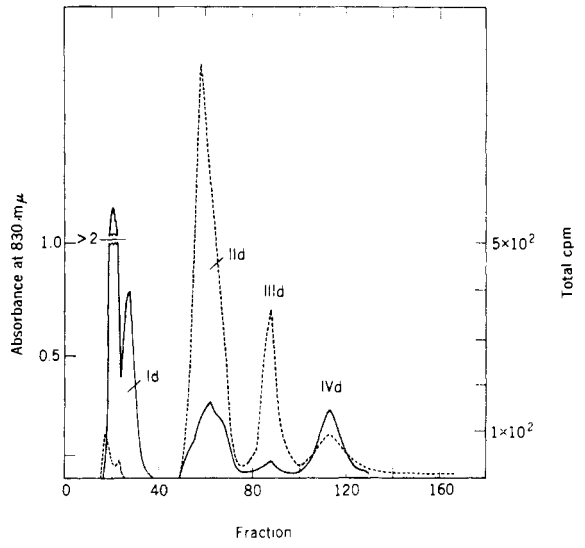

FIGURE 4: DEAE-cellulose chromatography of chloroform–methanol–hydrochloric acid extract of rabbit brain slices after incubation with myoinositol-2-^{3}H. Dashed line, radioactivity; solid line, phosphorus (extract B). Peak Id was not investigated. IId contained I, IIId contained II, and IVd contained III.

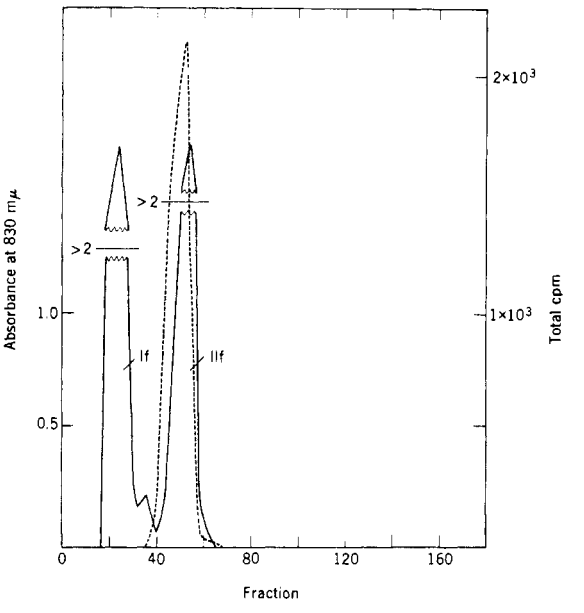

FIGURE 6: DEAE-cellulose chromatography of ethanol extract of rabbit brain slices after incubation with myoinositol-2-^{3}H. Dashed line, radioactivity; solid line, phosphorus. If was not identified. IIf contained I plus phosphatidylserine.

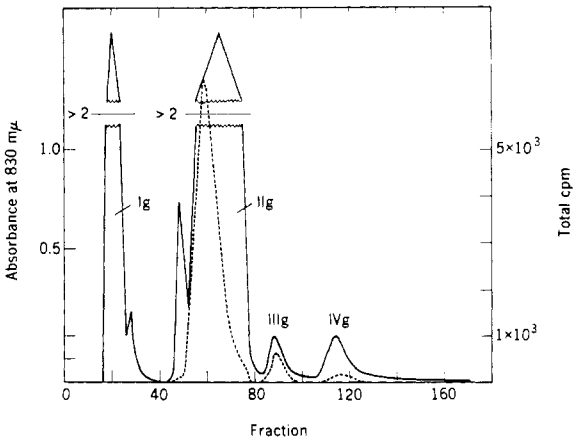

FIGURE 7: DEAE-cellulose chromatography of petroleum ether extract of rabbit brain slices after incubation with myoinositol-2-^{3}H. Dashed line, radioactivity; solid line, phosphorus. Ig was not investigated. IIg contained I plus phosphatidylserine, IIIg contained II, and IVg contained III.

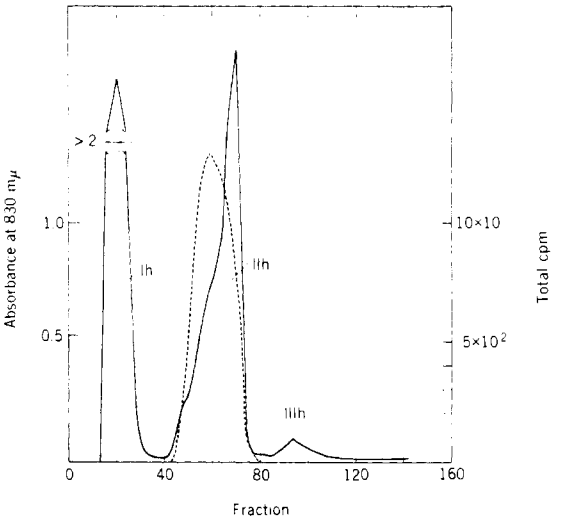

FIGURE 8: DEAE-cellulose chromatography of chloroform–methanol–water extract of rabbit brain slices after incubation with myoinositol-2-^{3}H. Dashed line, radioactivity; solid line, phosphorus. Peaks Ih and IIIh were not identified. IIh contained I plus phosphatidylserine.

assay. The elution patterns and radioactivities are shown in Figures 3 and 4. The results were quantitatively similar to those of the other extraction procedures, indicating that inositides were not selectively lost during the extraction.

PROCEDURE C. The slices were collected by centrifugation, homogenized, and extracted. Extraction was carried out twice with acetone (70 and 50 ml), with ethanol (50 ml), twice with petroleum ether (50 ml each time), and finally with 50 ml of chloroform–methanol–water (20:9:1). A Servall Omni-Mixer was used and each extraction was for 1.5-min duration, except the last, which was for 5 min. Each extract was treated exactly as described for the petroleum ether extract in procedure A and chromatographed under identical conditions, analyzing for phosphorus and radioactivity. The elution patterns and radioactivities are shown in Figures 5–8.

Incubation of Mitochondria with Orthophosphate-^{32}P. A whole rabbit brain was homogenized in ice-cold 0.32 M sucrose using a Potter-Elvehjem homogenizer to give a final concentration of 10% (w/v). The mitochondrial fraction was isolated by differential centrifugation following the method of Eichberg *et al.* (1964). The crude mitochondria were washed once with 0.32 M sucrose and incubated in 35 ml of Krebs–Henseleit solution with 2 mcuries of orthophosphate-^{32}P and 25 mg of glucose in a 100-ml flask under oxygen–carbon dioxide (95:5) for 1 hr at 38°, in order to duplicate the conditions for the slices.

The mitochondria were collected by centrifugation and extracted as in procedure A. The petroleum ether extract was treated exactly as described for the whole brain preparation. Fractions of 2.2 ml were collected from which 0.2 ml was removed for ^{32}P assay. The elution pattern is shown in Figure 9.

Results

^{32}P Incorporation in Experiment A. The peak tubes were pooled and the isolated fractions were identified as described earlier (Hendrickson and Ballou, 1964) using paper chromatographic technique A for deacylated lipids and system B for intact lipids. The results are given in Figure 1. Peak IIIa gave two spots in system B corresponding to phosphatidylserine and I,

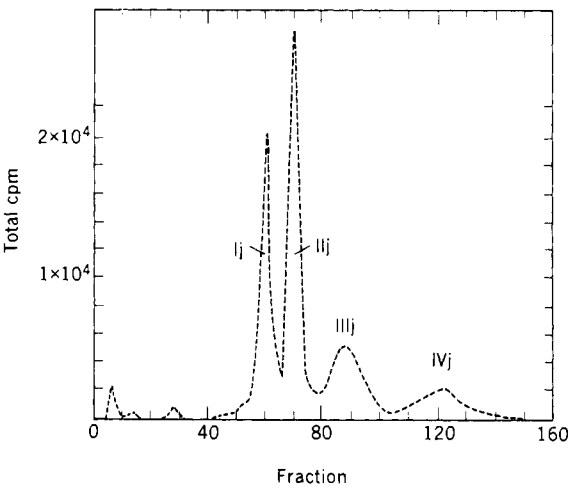

FIGURE 9: DEAE-cellulose chromatography of rabbit brain mitochondrial phosphoinositides after incubation with radioactive inorganic phosphate. Dashed line, radioactivity. Ij contained phosphatidylserine, IIj contained I, IIIj contained II, and IVj contained III.

TABLE I: Specific Radioactivities in Phosphoinositides of Rabbit Brain Slices after Incubation with Radioactive Inorganic Phosphate and with Myoinositol-2-^{3}H.[a]

Inositide	Concn of Inositide in Brain (μmoles/8 g)	^{32}P Expt[b] (counts/min per μmole) Total Activity	Activity in Position 1	Activity in Monoesterified Phosphate Groups	Myoinositol-^{3}H Expt[b] Total Activity (counts/min per μmole)
I	5.7	38,550	38,550	None	18,360
II	0.4	286,000	29,100	256,900	3,250
III	1.74	293,000	2,050	290,950	346

[a] Isolation by procedure of Folch (1949). [b] The difference in the relative specific activities of the three components in these two experiments reflect variables in the technique or the metabolic capabilities of the different tissue samples.

which accounts for the failures of the curves for total phosphate and radioactivity to correspond. These components could not be separated completely by the reported procedures (Hanahan and Olley, 1958; Rouser *et al.*, 1961). The specific activity of I was determined by removing the spot corresponding to I, digesting it with 70% perchloric acid, and assaying for phosphorus and radioactivity. Table I gives the specific activities of the inositides and the distribution of radioactivity in the different positions of the inositol ring, determined as described previously (Brockerhoff and Ballou, 1962b).

Myoinositol-2-^{3}H Incorporation in Experiment A. The peaks were isolated and identified as above. Figure 2 shows the results. In a similar manner, peak IIIb was shown to be a mixture of phosphatidylserine and I. A scan of a paper chromatogram of the peak indicated only one radioactive spot corresponding to I. The inositol content of IIIb was determined directly on the mixture. Table I gives the specific activities of the inositides. On microbiological assay, no inositol was found in fractions 23–39, 41–50, or 51–56. Each of these tritium-labeled peaks appeared to contain carbohydrate on the basis of the phenol–sulfuric acid test. In another myoinositol-2-^{3}H incorporation experiment described below, these radioactive peaks were not detected.

Myoinositol-2-^{3}H Incorporation in Experiment B. The peaks were isolated and identified as described above. Figure 3 shows the results. The specific activities of the inositides are given in Table II. Palmer and Rossiter (1964) and Dittmer and Dawson (1961) have reported that all of I is extracted from brain tissue with neutral chloroform–methanol. We find that this is not so (see Figure 4), although, as reported, no polyphosphoinositide is extracted with this solvent.

Myoinositol-2-^{3}H Incorporation in Experiment C. Figures 5–8 show the identification of the peaks determined as described above. Each extract contains I, although only the petroleum ether extract yields polyphosphoinositides. Specific activities of the inositides and the concentration of I in each extract are given in Table III. The concentration of I was calculated from inositol assay of a known aliquot.

TABLE II: Specific Radioactivities in Phosphoinositides of Rabbit Brain Slices after Incubation with Myoinositol-2-^{3}H.[a]

Inositide	Concn of Inositide in Brain (μmoles)[b]	Activity (counts/min per μmole)
Extract A		
I	8.0	15,800
Extract B		
I	0.6	13,500
II	0.2	11,400
III	0.45	1,260

[a] Isolation by procedure of Rossiter and Palmer (personal communication). [b] Calculated from inositol assays on the purified components. The concentration of I is increased, and that of III decreased with respect to the values given in Table I. This may be due to enzymic hydrolysis of III during extraction by the procedure of Rossiter and Palmer.

Concentration of Inositide in Brain. Table I gives values for concentrations of inositides of individual brains averaged over six experiments. The figures quoted were determined after incubation of the tissue for 2 hr. The amount of III decreases considerably during incubation. For II and III the results were calculated from the total phosphorus content of each peak and from inositol assay. The figure for I was determined from the inositol assay and by using paper chromatographic system B on a known aliquot of the

TABLE III: Specific Radioactivities in Phosphoinositides of Rabbit Brain Slices after Incubation with Myoinositol-2-^{3}H and Extraction with Various Solvents.

Extract	Inositide	Concn of I (μmoles/ extract)	Activity (counts/ min per μmole)
Acetone	I	0.62	19,300
Ethanol	I	1.23	19,000
Pentane	I	3.18	19,050
	II		11,200
	III		1,590
Chloroform–methanol–water	I	1.36	20,000

elution peak, in a manner analogous to that employed in assaying its ^{32}P specific activity.

^{32}P Labeling of Inositides in Mitochondria. The peaks were isolated as described above and identified using paper chromatographic technique B. Table IV shows the identification and specific activities. In this experiment, separation of I and phosphatidylserine was achieved. In all experiments, peaks not fully characterized did not contain inositol.

Discussion

In our previous studies on the metabolism of the phosphoinositides of rabbit brain, analyses were made on the glycerol myoinositol phosphates obtained by deacylation of the lipid extracts. In the work described herein, analysis of the intact lipids has provided substantiating evidence that the phosphatidylmyoinositol structure is retained intact in the metabolism of the three inositides. The specific activity of the myoinositol moiety decreases in the same progressive manner as that of the phosphatidyl phosphate group. This labeling pattern closely parallels that already reported (Brockerhoff and Ballou, 1962a,b) and provides more evidence for the proposed biosynthetic pathway.

Recently, Palmer and Rossiter (1964) have published findings on the *in vitro* incorporation of myoinositol-^{3}H into the phosphoinositides of cat brain slices in which the specific activities of the inositides were in the order I < II $\gg$ III (*cf.* I > II $\gg$ III). From this they concluded that II may not be synthesized by direct phosphorylation of I. Contrary to this proposal, Colodzin and Kennedy (1964) have shown that brain microsomes synthesize II from I in the presence of adenosine triphosphate (ATP), an observation we have confirmed (unpublished work). In an attempt to resolve the conflicting reports, we have repeated our studies with rabbit brain using the procedure employed by Palmer and Rossiter. The results (Table II) show that the myoinositol-2-^{3}H incorporation pattern, although somewhat different from that obtained by the Folch extraction procedure, agrees with our previous work since the specific activity of I > II > III. The specific activity of I is slightly higher in the neutral extract than in the acidified extract, the reason for which is not known.

The specific activity of I, relative to II and III, is lower when extraction is done by procedure B (as compared with procedure A). It was considered possible that there might be two metabolically different pools of I, as has been reported by Hokin and Hokin (1963) for phosphatidic acid in avian salt gland, and that petroleum ether extracted only I of high activity. To test this hypothesis, the first experiment using myoinositol-2-^{3}H was repeated and the specific activities of I were determined in each extract. Although each extract contains I, the specific activities are the same within experimental error, thus providing no evidence that two different pools of phosphatidylmyoinositol exist. The low yield of II and III obtained in the extraction by procedure B is probably a result of their concomitant enzymatic degradation to I.

Since these experiments were carried out, Rossiter and Palmer (1965) have reported that *in vivo* incorporation of myoinositol-^{3}H into cat brain phosphoinositides gives results which are consistent with those reported from this laboratory (Brockerhoff and Ballou, 1962b). Thus, only the *in vitro* study with cat brain slices gives anomalous results. This may be an experimental artifact, but it warrants further investigation.

In preliminary experiments on the incorporation of orthophosphate-^{32}P into phospholipids by mitochondria, all three phosphoinositides have been found to be present and to be labeled. Further purification of the mitochondria by gradient density centrifugation (Eichberg *et al.*, 1964), or by washing, results in loss of activity, and no label could be detected in II or III, although I still became labeled. Garbus *et al.* (1963) have described the incorporation of ^{32}P into a lipid in liver and kidney mitochondria which they suggested to be either II or III. Galliard and Hawthorne (1963) and Michell *et al.* (1964) have demonstrated the phosphorylation of I to yield II in the same systems. The latter workers found a trace of radioactive material having the properties of III. Our results suggest that the

TABLE IV: Identification of Peak Components in Figure 9 and Specific Radioactivities in Phosphoinositides of Rabbit Brain Mitochondria after Incubation with Radioactive Inorganic Phosphate.

Peak	Component	Activity (counts/min per μmole)
Ij	Phosphatidylserine	7,040
IIj	I	41,600
IIIj	II	142,500
IVj	III	21,800

major activity for the synthesis of III resides in the microsomal fraction which is separated from the mitochondria during their purification.

Acknowledgment

The authors are indebted to Dr. R. J. Rossiter for details of his experimental procedure prior to publication.

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Stimulation of Protein Synthesis *in Vitro* by Partially Degraded Ribosomal Ribonucleic Acid and Transfer Ribonucleic Acid*

John J. Holland, Clayton A. Buck,† and B. J. McCarthy

ABSTRACT: An *in vitro* protein-synthesizing system from *Escherichia coli* is capable of using purified ribosomal ribonucleic acid (RNA) as a template for amino acid incorporation under certain conditions. This template activity is optimal when neomycin is added and when the secondary structure of ribosomal RNA is destroyed by heating to cause a number of phosphodiester bond cleavages in each molecule. After more extensive heating to destroy its secondary structure, soluble RNA also acts as a template for amino acid incorporation.

Nirenberg and Matthaei (1961) showed in their original study of cell-free protein synthesis that ribonucleic acid (RNA) extracted from washed *Escherichia coli* ribosomes was capable of slight stimulation of protein synthesis. However, the specific activity of ribosomal RNA was less than 5% of that of tobacco mosaic virus (TMV) RNA. Furthermore, sucrose gradient sedimentation studies indicated that only a fraction of the RNA from ribosomes was active and that the soluble RNA fraction was inactive as a template. Subsequently, other investigators have used this system to measure messenger RNA (m-RNA) activity in total RNA extracted from bacterial and mammalian cells. This assay is made on the assumption that ribosomal and transfer RNA (t-RNA) contribute little to the template activity.

It is shown below that both ribosomal and soluble

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