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The incorporation of P^{32} into the inositol phosphatides of rat brain

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SUMMARY

The inositol phosphatide fraction of rat brain has been separated by chromatography on formaldehyde-treated paper into monophosphoinositide and two other inositol-containing phosphatides named phosphoinositide 1 and 2. Quantitative determination of the ether-soluble phosphatides gave the following values for rat brains of 450 to 500 mg dry weight: 14 to 21 mg phosphatidylcholine and phosphatidylethanolamine, 3.5 to 4.6 mg phosphatidylserine, 0.9 to 1.3 mg monophosphoinositide, and 0.1 to 0.2 mg each of phosphoinositide 1 and 2. All three inositol phosphatides incorporated P^{32} at a significantly higher rate than the other brain phosphatides. Of the three, monophosphoinositide always had the lowest specific activity; after 16 and 32 hours, phosphoinositide 2, which is probably identical with the triphosphoinositide of Ballou, had the highest specific activity.

It was first reported in 1954 that the rate of incorporation of labeled phosphate into the inositol phosphatides of brain was significantly higher than into other brain phosphatides. Since the only known brain inositol phosphatide was DPI,[1] first isolated by Folch (1), the higher incorporation was attributed by Dawson (2, 3) to this compound alone. For the same reason, numerous papers (4, 5, 6) dealt only with the special role of DPI. Some years later, Hokin and Hokin (7) found a highly labeled inositol phosphatide in guinea pig brain cortex and identified it as MPI on the basis of its paper chromatographic behavior. Since they could detect no DPI, they proposed that the compound investigated in previous experiments must have been MPI. At the same time, Hörhammer *et al.* (8, 9), working with the Folch fraction 1 of ox brain, isolated a second inositol-containing compound in addition to the DPI fraction by means of countercurrent distribution. According to its chromatographic behavior and degradation studies, the inositol compound was identical with MPI from soybean and liver. The DPI fraction could be separated into three components by chromatography on formaldehyde-treated paper. Thus, a method was available that appeared suitable for checking the incorporation studies and reconciling the apparent contradictions in the literature. Whereas Dawson (2, 3), Hawthorne (10), and Ballou (11) analyzed the inositol phosphates obtained by mild alkaline hydrolysis of the unfractionated inositol phosphatide fraction, the new method permitted investigation of the individual *intact* inositol phosphatides.

The present studies deal with the qualitative and quantitative content of inositol phosphatides of rat brain and their rates of P^{32} incorporation.

METHODS

P^{32} Administration and Preparation of the Brain. Adult male albino rats (250 to 290 g) were injected subcutaneously on the inside of the hind leg with 1 mc of carrier-free P^{32} as $Na_2HP^{32}O_4$ in physiological saline. Animals were killed after 2, 4, 8, 16, and 32 hours; the brains (1.8 to 2.0 g) were quickly removed, weighed, frozen in liquid air, and lyophilized.

Extraction and Separation of the Lipids. The dry brain tissue was homogenized for 3 minutes in 20 ml acetone at 40,000 r.p.m. (homogenizer type UMS, Firma Bühler, Tübingen). To remove cholesterol, neutral fat, and fatty acids, the preparation was extracted in a Soxhlet thimble with 200 ml cold acetone. Finally, the residue was extracted for 3 hours with ether in a Soxhlet apparatus. The ether extract was taken

[1] The following abbreviations are used: MPI, monophosphoinositide; DPI, diphosphoinositide; TPI, triphosphoinositide; PI-1 and PI-2, phosphoinositides 1 and 2 (two components in the fraction formerly designated "DPI" but resolved into two components in the present study).

to about 1 ml under reduced pressure, transferred to a micro tube, and made up to exactly 1 ml. The extract must not be taken to dryness, since this results in incomplete resolution in subsequent chromatography. To determine the weight of solids in the extract, 0.1 ml of the ether solution was pipetted onto a tared aluminum planchet, dried under an infrared lamp, cooled for 30 minutes over concentrated sulfuric acid in a desiccator, and weighed. An aliquot of the ether solution, corresponding to 2 mg dry extract, was used for the paper chromatographic separation.

Paper Chromatography of the Phosphatides. The inositol phosphatides were chromatographed on paper impregnated with formaldehyde as previously described (12) by using the upper phase of a butanol—acetic acid—water mixture 4:1:5 as the solvent. The running time was 15 hours for 40 to 45 cm at 20°. Chromatograms were dried at room temperature, treated with Nile blue (0.1% solution), and washed for 30 minutes with water to remove excess dye. For autoradiography, the film (Perutz-Per-X film) was exposed to the chromatogram for 20 days.

Measurement of Radioactivity. Liquid samples (*e.g.* the acetone or ether extract) were dried on aluminum planchets under an infrared lamp. Chromatogram spots were cut out and placed on aluminum planchets. Radioactivity was measured with a Telefunken MS Str. 610/1 scaler and an MS MD 632/1 windowless methane flow counter. All values were corrected to the activity at the start of the experiment with the aid of a standard preparation.

Phosphate Analysis. Total and inorganic phosphate in tissues and extracts were determined by the method of Ernster *et al.* (13). The organic phosphate in the phosphatides separated on the chromatograms was determined by a modification of the method of Lowry (14). The excised chromatogram spots, containing 0.1 to 5.0 μg P on up to 90 mg of paper, were weighed, cut into small pieces, and placed in Fiolax test tubes (18 x 180 mm) with 0.4 ml 72% perchloric acid (analytical grade) with a trace of ammonium molybdate added as catalyst. For ashing, the tubes were placed at an angle in holes (19 x 25 mm) in an electrically heated aluminum block, brought up to 180° in 2 hours and kept at this temperature for 1 hour. The resulting metaphosphates were converted to orthophosphate by adding 2 ml water to the cooled samples and placing them in a boiling water bath for 10 minutes. After cooling, 0.3 ml of 2.5% ammonium molybdate solution, followed by 0.3 ml of 10% ascorbic acid solution (freshly prepared) were added, mixed immediately, and incubated at 38° for 2 hours. The extinction was measured at 820 mμ in a Zeiss spectrophotometer PMQ II. In each series of estimations, parallel determinations were made of (1) the blank values of the reagent solution and the chromatogram paper, and of (2) a standard preparation.

Identification of the Phosphatides. The following test materials were available for the identification of the individual chromatogram spots: MPI prepared from ox heart after Faure and Morelec-Coulon (15); MPI and "DPI" from ox brain, isolated by countercurrent separation (8, 9); and ox brain "DPI" generously given by Professor J. Folch. The origin of the other substances has already been reported (12).

Detection of Inositol in Phosphoinositide 1 and 2. The spots of phosphoinositides 1 and 2 were eluted with chloroform—methanol 1:1 and hydrolyzed (16), and the water-soluble products were separated by two-dimensional chromatography using isopropanol—acetic acid—water 3:1:1 and butanol— acetic acid—water 4:1:5. Both spots yielded inositol, detected with silver nitrate and identified by its R_f values (0.35 in the former solvent, 0.09 in the latter).

RESULTS

Qualitative Composition of the Inositol Phosphatide Fraction of Rat Brain. In the incorporation studies with radioactive material, only small amounts of tissue can be processed. There is, therefore, some difficulty in detecting the inositol phosphatides which, in rat brain, are present in low concentrations. Fractionation according to Folch (1), column purification (17), and paper chromatography of the total lipid extract (18) all gave unsatisfactory results. The tissue was therefore pre-extracted with cold acetone to remove undesirable material. This removed 10% of the brain dry substance. The ether extract of the acetone-extracted residue constituted 8% to 10% of the brain dry weight. It contained the majority of the phosphatides and was suitable for the paper chromatographic separation and identification of the inositol phosphatides.

In the ether extract, the inositol phosphatides are three times more concentrated than in a chloroform—methanol 2:1 extract, which contains 25% to 30% of the brain dry weight for the same quantity of inositol phosphatides. Correspondingly, phosphate analyses show that the ether extract contains a higher proportion of organic phosphate.

Cholesterol and neutral fats (R_f, 0.90), phosphatidylcholine and phosphatidylethanolamine (R_f, 0.72 and 0.68), phosphatidylserine (R_f, 0.55), MPI (R_f, 0.47), and two other inositol-containing phosphatides, which we have named phosphoinositide 1 and 2 (R_f, 0.41 and 0.36), can be clearly recognized on the chromatogram (see Fig. 1). The compounds were identified by co-

chromatography with authentic samples. Both phosphoinositide 1 and 2 contained inositol.

Thus, as recently reported (19), rat brain, like ox brain, contains both MPI and DPI. The ox-brain "DPI" provided by Professor J. Folch and prepared by ourselves could be separated into three components (9), but the corresponding fraction from rat brain only yielded two inositol-containing substances. Although the ratio of inositol to phosphate in these compounds has not yet been determined, it is fairly certain that one of the two components is a true DPI. In recent work, Dittmer and Dawson (20) and Ballou *et al.* (11, 21, 22) obtained inositol mono-, di-, and triphosphate by careful degradation of the separated products. The other component of the "DPI" fraction may therefore be a TPI whose existence was first proposed by Dittmer and Dawson, and Ballou.

Quantitative Estimation of the Ether-Soluble Inositol Phosphatides of Rat Brain. After the paper chromatographic separation of the lipid extract, the individual phosphatide spots were cut out and ashed. This procedure prevents the losses that cannot be avoided if the phosphatides are first eluted from the formaldehyde-treated paper (18). According to Hokin (24), only 65% to 85% of the phosphatides can be eluted from silicic acid-impregnated paper (23). The recovery for one inositol phosphatide was as low as 35%. The amount of material was calculated by using the determined phosphate values and the following molecular weights: phosphatidylcholine, 800; phosphatidylethanolamine, 763; phosphatidylserine, 790; MPI, 863; "DPI", 850.[2]

Table 1 gives the levels of inositol phosphatides in 5 rat brains. In the ether extract of rat brain, MPI constitutes 2.2% to 3.0%, and phosphoinositides 1 and 2 each 0.3% to 0.6% of the total solids.

The in vivo *Incorporation of P^{32} into the Inositol Phosphatides of Rat Brain.* From Table 2, it is seen that inorganic phosphate is incorporated rapidly into all three inositol phosphatides of rat brain. Two hours after the subcutaneous P^{32} injection, the inositol phosphatides are highly labeled in comparison to phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine. These last three phosphatides have little or no activity after two hours; after 16 and 32 hours, they reach the 2-hour activity of the inositol phosphatides. Of the inositol phosphatides, MPI has the lowest incorporation rate, while the labeling of phosphoinositide 2 is the highest of the three after 16 and 32 hours.

Thirty-two hours after P^{32} injection, the ratio of specific activities is approximately as follows: phosphatidylserine–phosphatidylcholine–MPI–phosphoinositide 1–phosphoinositide 2 = 1:2:4:6:8.

[2] Calculated by Folch.

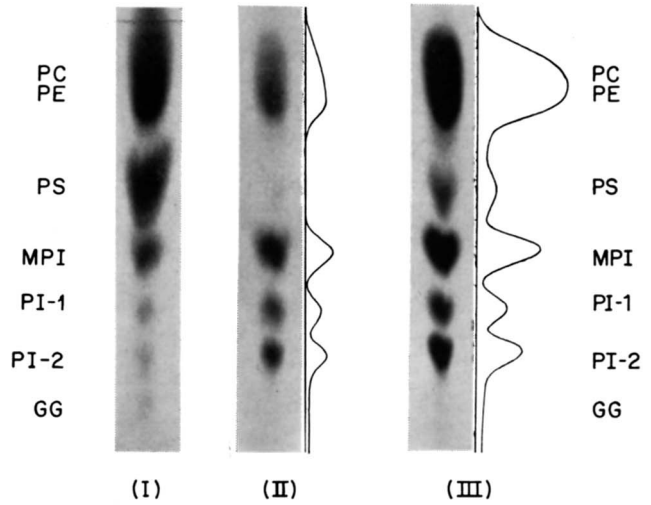

FIG. 1. Chromatograms and autoradiograph showing the distribution of P^{32} among the phosphatides 2 and 16 hours after injection of P^{32}. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; MPI, monophosphoinositide; PI-1, phosphoinositide 1; PI-2, phosphoinositide 2; GG, ganglioside. (I) Paper chromatogram of the ether-soluble lipids from rat brain. Formaldehyde paper, butanol-acetic acid-water 4:1:5, descending at 20°, stained with Nile blue (0.1%). (II) Autoradiogram of the rat-brain phosphatides 2 hours after P^{32} injection. Perutz-Per-X film; exposure time 20 days; extinction curve made with an Elphor Integraph (Bender u. Hobein, München); slit 1 × 18 mm; surface values: PC and PE = 4.1, PS = 0, MPI = 2.4, PI-1 = 1.3, PI-2 = 1.5. (III) Same as II, but 16 hours after the P^{32} injection. Slit 1 × 21 mm; surface values: PC and PE = 17.7, PS = 1.7, MPI = 5, PI-1 = 1.9, PI-2 = 3.4.

Figure 1 gives the autoradiograms with their corresponding densitometer-extinction curves and shows the distribution of P^{32} in the different phosphatides of the ether extract of rat brain. After two hours of P^{32} incorporation, 56.0% of the radioactivity of the ether-soluble phosphatides is present in the inositol phosphatides, as seen from the areas. Areas were obtained by densitometer readings of the autoradiogram and are a measure of relative activity. Such values can only

TABLE 1. THE INOSITOL PHOSPHATIDE CONTENT OF THE ETHER EXTRACTS OF 5 RAT BRAINS (ALL WEIGHTS IN MG)

Brain Number	1	2	3	4	5
Phosphatidylcholine and phosphatidylethanolamine			20.7	13.9	
Phosphatidylserine			4.6	3.5	
MPI	1.0	0.9	1.2	0.9	1.3
PI-1	0.15	0.10	0.20	0.17	0.24
PI-2	0.19	0.19	0.18	0.16	0.24
Ether extract	35.7	34.7	53.3	36.2	43.5

TABLE 2. THE SPECIFIC ACTIVITY OF RAT BRAIN PHOSPHATIDES 2, 4, 8, 16, AND 32 HOURS AFTER THE *in vivo* ADMINISTRATION OF P^{32}

Compound	Specific Activity (cpm/μg P)* Time of P^{32} Incorporation (hours) 2	4	8	16	32
Phosphatidylcholine and phosphatidylethanolamine	6	18	37	61	128
Phosphatidylserine		6	26	22	59
MPI	84	150	179	207	268
PI-1	159	213	283	265	380 ± 10
PI-2	89	248	247	334	455 ± 50

* Mean values of 5 determinations.

be compared if they are produced under identical conditions (the same width and length of slit).

DISCUSSION

The results of the experiments reported here show that all three inositol phosphatides of rat brain incorporate labeled phosphate at a significantly higher rate than the other brain phosphatides and that the two component phosphatides of the "DPI" fraction (PI-1 and PI-2) show a higher rate of phosphate uptake than MPI.[3]

There are two possible explanations for the findings of Hokin and Hokin (4), who attributed the high incorporation rate to MPI alone. First, the brain cortex of the guinea pig may contain only MPI. This would agree with the findings of Hörhammer *et al.* (9), who found only MPI in ox-grey matter but both MPI and DPI fractions in the white matter. Second, the brain cortex of the guinea pig may contain a DPI substance which remained on the origin of the chromatogram in Hokin's method and was not recognized.

The different incorporation rates for phosphoinositides 1 and 2 in the present study could best be explained by the presence of TPI, which was recently discovered by Dittmer and Dawson (20) and Ballou *et al.* (11, 21, 22). If DPI and TPI are produced from MPI by stepwise phosphorylation, the three inositol phosphatides would be expected to have different specific activities. TPI, synthesized from MPI and two molecules of P^{32}, would be expected to have the highest activity, followed by DPI, synthesized from MPI and one molecule of P^{32}. The lowest activity would be found in the MPI, because of its relatively slow *de novo* synthesis. If phosphoinositide 2 in our experiments is identical with the TPI of Ballou, the difference in incorporation rates would be in best agreement with this hypothesis of a stepwise phosphorylation. If one assumes that the results with rat brain are applicable to other species, some contradictions in the literature in this field have thus been explained.

[3] Since this paper was prepared, J. N. Hawthorne, at the 5th International Congress of Biochemistry in Moscow, reported that, after 6 hours of P^{32} incorporation, the highest specific activity was found in a "DPI."

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REFERENCES

1. Folch, J. *J. Biol. Chem.* **146**: 35, 1942.
2. Dawson, R. M. C. *Biochim. et Biophys. Acta* **14**: 374, 1954.
3. Dawson, R. M. C. *Biochem. J.* **57**: 237, 1954.
4. Hokin, L. E., and M. R. Hokin. *Biochim. et Biophys. Acta* **18**: 102, 1955.
5. McMurray, W. C., K. P. Strickland, J. F. Berry, and R. J. Rossiter. *Biochem. J.* **66**: 634, 1957.
6. Magee, W. L., J. F. Berry, and R. J. Rossiter. *Biochim. et Biophys. Acta* **21**: 408, 1956.
7. Hokin, L. E., and M. R. Hokin. *J. Biol. Chem.* **233**: 818, 1959.
8. Hörhammer, L., H. Wagner, and J. Hölzl. *Biochem. Z.* **330**: 591, 1958.
9. Hörhammer, L., H. Wagner, and J. Hölzl. *Biochem. Z.* **332**: 269, 1960.
10. Ellis, R. B., and J. N. Hawthorne. *Biochim. et Biophys. Acta* **51**: 385, 1961.
11. Grado, C., and C. E. Ballou. *J. Biol. Chem.* **235**: PC 23, 1960; *J. Biol. Chem.* **236**: 54, 1961.
12. Hörhammer, L., H. Wagner, and G. Richter. *Biochem. Z.* **331**: 155, 1959.
13. Ernster, L., R. Zetterström, and O. Lindberg. *Acta Chem. Scand.* **4**: 942, 1950.
14. Lowry, O. H., N. R. Roberts, K. Y. Leiner, Mei-Ling Wu, and A. L. Farr. *J. Biol. Chem.* **207**: 1, 1954.
15. Faure, M., and M. J. Morelec-Coulon. *Ann. inst. Pasteur* **91**: 537, 1956.
16. McKibbin, J. M. In *Methods of Biochemical Analysis*, edited by D. Glick, New York, Interscience Publishers, Inc., Vol. VII, p. 111, 1959.
17. Hanahan, D. J., J. C. Dittmer, and E. Warashina. *J. Biol Chem.* **228**: 685, 1957.
18. Lissau, Ä. Dissertation, München, 1961.
19. Hörhammer, L., J. Hölzl, and H. Wagner. *Naturwissenschaften* **48**: 103, 1961.
20. Dittmer, J. C., and R. M. C. Dawson. *Biochim. et. Biophys. Acta* **40**: 379, 1960.
21. Tomlinson, R. V., and C. E. Ballou. *J. Biol. Chem.* **236**: 1902, 1961.
22. Brockerhoff, H., and C. E. Ballou. *J. Biol. Chem.* **236**: 1907, 1961.
23. Lea, C. H., D. N. Rhodes, and R. D. Stoll. *Bicchem. J.* **60**: 353, 1955.
24. Hokin, L. E., and M. R. Hokin. *J. Biol. Chem.* **233**: 805. 1958.