Preliminary Notes

PN 1278

Rapid labelling of diphosphoinositide in liver mitochondria

GARBUS *et al.*[1] recently described the rapid incorporation of radioactive phosphate *in vitro* into the lipid fraction of liver and kidney mitochondria. After a short incubation (5 min), most of the ^{32}P appeared in a lipid which differed from any of the known phospholipids of liver. It was suggested that the unknown lipid might be di- or triphosphoinositide. We have now identified it as diphosphoinositide (phosphatidylinositol phosphate). So far, significant amounts of di- and triphosphoinositide have only been found in nervous tissue, though DAWSON *et al.*[2] reported that traces of triphosphoinositide occur in other tissues.

Rat- or rabbit-liver mitochondria were prepared and incubated with [^{32}P]orthophosphate for 5 min as described by GARBUS *et al.*[1]. Lipid was then extracted by the method of these authors. The washed chloroform extract was concentrated *in vacuo* to a volume of 5 ml and mixed with 2 ml of 0.5 N NaOH in methanol. After 20 min, 5 ml of chloroform, 3 ml of methanol and 3 ml of water were added and the mixture was shaken well. The upper aqueous layer was then removed and the lower layer washed with fresh upper phase. The combined upper phases were diluted to 100 ml and made 5 mM with respect to sodium tetraborate as previously described[3]. The solution was applied to a Dowex-1 X10 column (formate form, 1 × 20 cm, 200–400 mesh) together with the similarly prepared hydrolysis products of the FOLCH ox brain diphosphoinositide fraction[4]. The brain inositides represented 6 mg of total P and were not radioactive. Fractions of 10 ml were collected and analysed for phosphate. Radioactivity was determined in a liquid Geiger counter. The major radioactive peak coincided in position with glycerylphosphorylinositol phosphate, the hydrolysis product of diphosphoinositide (see Fig. 1). Exact coincidence was also obtained when the peak fractions were rechromatographed on Dowex-1 X10 chloride, using 0.3 M LiCl as eluant. In the original Dowex-1-formate separation a smaller radioactive peak was seen in the position expected for glycerophosphate and a very small one was coincident with glycerylphosphorylinositol diphosphate, the hydrolysis product of triphosphoinositide.

An attempt was made to isolate diphosphoinositide from rabbit liver, but only very little was detected. Nine livers were homogenised in 10 vol. of acetone within a few minutes of death. The homogenate was centrifuged and the precipitate extracted with the same volume of chloroform–methanol (2:1, v/v), followed by chloroform–methanol–conc. HCl (800:400:3, v/v). The two chloroform–methanol extracts were combined and shaken with one-fifth their volume of 0.01 M $CaCl_2$. The lower layer was then evaporated to dryness *in vacuo* and the residue shaken with 40 ml of chloroform. After filtering, this chloroform solution of the lipids was hydrolysed as above and applied to a Dowex-1 formate column, together with a radioactive marker of glycerylphosphorylinositol phosphate prepared separately by incubating rat-liver mitochondria with ^{32}P. Elution followed the sequence shown in Fig. 1. Acid hydrolysis

of fractions from the radioactive peak eluted from the column, followed by paper chromatography[5] showed the presence of inositol and glycerol. The estimated amount of inositol was only about 20 μg per 600 g of rabbit liver. This suggests that in the incorporation experiments the mitochondrial diphosphoinositide had a high specific activity.

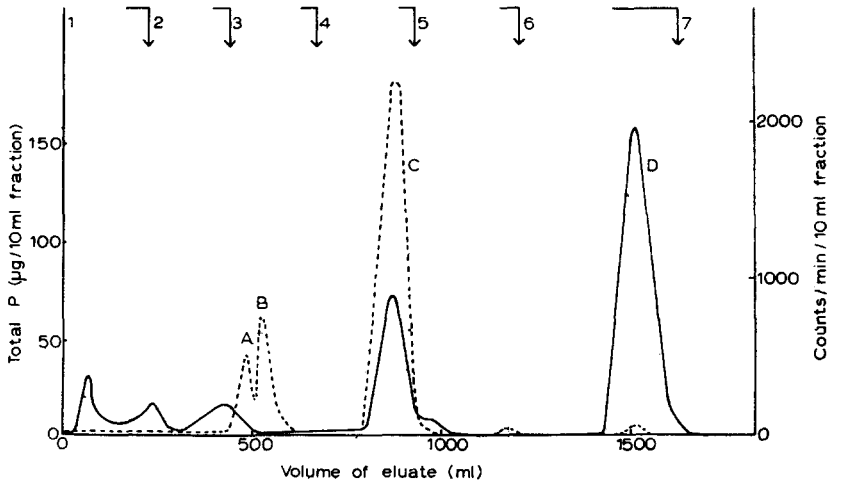

Fig. 1. Chromatography on Dowex-1 formate of hydrolysates of labelled mitochondrial lipid and the FOLCH diphosphoinositide fraction. The broken line represents radioactivity and the continuous line phosphate. Eluting solutions had the following compositions: 1, 5 mM sodium tetraborate–60 mM ammonium formate; 2, 5 mM borate–120 mM formate; 3, 5 mM borate–180 mM formate; 4, 0.2 M formate–0.1 M formic acid; 5, 0.3 M formate–0.1 M formic acid; 6, 0.5 M formate–0.1 M formic acid; 7, 1.0 M HCl. Peak identities: A, glycerophosphate; B, inorganic phosphate; C, glycerylphosphorylinositol phosphate; D, glycerylphosphorylinositol diphosphate.

TABLE I

EFFECT OF INHIBITORS ON LABELLING OF MITOCHONDRIAL DIPHOSPHOINOSITIDE

Mitochondria from 2 g liver were incubated for 5 min at 30° with 40 μmoles $MgCl_2$, 60 μmoles potassium glutamate, 80 μmoles Tris–HCl buffer (pH 7.4), 500 μmoles sucrose and 4.8 μmoles [^{32}P]orthophosphate (approx. 90 μC) in a total volume of 4.8 ml.

Conditions	*Glycerylphosphorylinositol phosphate peak (counts/min)*
No additions	8220
10^{-3} M sodium cyanide	40
$5 \cdot 10^{-5}$ M dinitrophenol	150
Under nitrogen	380
Boiled mitochondria	0

Samples from the mitochondrial glycerylphosphorylinositol phosphate peak were incubated at 37° with alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) in 0.1 M ethanolamine–HCl buffer (pH 9.5) containing 5 mM $MgCl_2$. Inorganic phosphate was then removed from the digest by the extraction method of MARTIN AND DOTY[6]. After 2 h of incubation all the radioactivity of the

original glycerylphosphorylinositol phosphate was found in the inorganic phosphate fraction. In a control experiment with boiled enzyme no radioactive inorganic phosphate was liberated. This indicates that Reaction 1 was taking place in the mitochondria.

$$\text{Phosphatidylinositol} + \text{ATP} \rightarrow \text{diphosphoinositide} + \text{ADP} \qquad (1)$$

Experiments in which oxidative phosphorylation was inhibited indicated that the labelling of diphosphoinositide was dependent upon ATP production. Results are given in Table I.

It seems unlikely that diphosphoinositide could be an intermediate in oxidative phosphorylation[1]. Moreover, its structure contains no high-energy phosphate bonds. However, the rapid and transient labelling may be explained by assuming that the lipid is acting as a carrier in cation transport. During the early stages of incubation there would be a greater need for active transport, *e.g.* to restore mitochondrial potassium lost during the preparation of the mitochondria.

T.G. holds a Department of Scientific and Industrial Research Scholarship for training in research.

Department of Medical Biochemistry and Pharmacology, Medical School, Birmingham (Great Britain)

T. GALLIARD
J. N. HAWTHORNE

[1] J. GARBUS, H. F. DELUCA, M. E. LOOMANS AND F. M. STRONG, *J. Biol. Chem.*, 238 (1963) 59.
[2] R. M. C. DAWSON, N. HEMINGTON AND J. B. DAVENPORT, *Biochem. J.*, 84 (1962) 497.
[3] G. HÜBSCHER, J. N. HAWTHORNE AND P. KEMP, *J. Lipid Res.*, 1 (1960) 433.
[4] J. FOLCH, *J. Biol. Chem.*, 177 (1949) 505.
[5] J. N. HAWTHORNE AND G. HÜBSCHER, *Biochem. J.*, 71 (1959) 195.
[6] J. B. MARTIN AND D. M. DOTY, *Anal. Chem.*, 21 (1949) 965.

Received April 29th, 1963

Biochim. Biophys. Acta, 70 (1963) 479–481

PN 1277

Tightly-bound proteolipid phospholipid in bovine brain white matter

In 1961 WEBSTER AND FOLCH-PI[1] demonstrated that aqueous tripotassium citrate when added to chloroform–methanol solutions of crude proteolipid rendered 80% of it insoluble. These authors suggested that tripotassium citrate split lipid–protein bonds, liberating lipid and precipitating protein. The present communication, as well as confirming the above results, will show that there is still tightly-bound phospholipid associated with the insoluble protein residue after citrate splitting.

Crude proteolipid isolated from bovine brain white matter was purified[2] and dissolved in chloroform–methanol (2:1) containing 5% water. This was treated with tripotassium citrate as above and the insoluble material, collecting at the interface, was removed, washed with chloroform–methanol (1:1) and extracted 3 times with chloroform–methanol (1:1) containing 0.5% conc. HCl. These acid chloroform–methanol extracts were pooled and the acid removed by equilibration with water[3]. Table I shows the distribution of phosphorus among the various fractions of tripotassium citrate-treated proteolipid.