THE SITE OF DIPHOSPHOINOSITIDE SYNTHESIS IN RAT LIVER

Robert H. Michell* and J.N. Hawthorne
Department of Medical Biochemistry and Pharmacology
The University, Birmingham 15, England.

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Garbus et al., (1963) showed that short incubations of liver mitochondria with $^{32}P_i$ yield a labelled phospholipid with the properties of a phosphoinositide. This was identified as diphosphoinositide (DPI) (Galliard and Hawthorne, 1963; Morgan, 1964). The labelling was inhibited by most inhibitors of mitochondrial electron transport and oxidative phosphorylation. Oligomycin was originally reported not to inhibit the reaction (Garbus et al., 1963), but was later shown to be a very effective inhibitor (Michell et al., 1964; Hajra et al., 1965; Galliard et al., 1965), suggesting that ATP, and not a high-energy intermediate of oxidative phosphorylation, is the phosphate donor. All studies with liver so far published have been made with mitochondria and attempts have been made to relate DPI synthesis to some specifically mitochondrial process, possibly ion-accumulation (Hawthorne, 1964; Hajra et al., 1965). However, in brain the phosphorylation of phosphatidylinositol (PI) to DPI has been shown in a microsomal fraction (Colodzin and Kennedy, 1964) as well as in other subcellular fractions (Dawson, 1965; Kai and Hawthorne, unpublished). Optimum conditions for the phosphorylation of PI to DPI with γ-^{32}P-ATP in liver cell fractions have now been established. This communication shows that the synthesis of DPI is extra-mitochondrial and evidence is presented which suggests that the reaction is primarily localised in the plasma membrane.

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* Medical Research Council Student.

MATERIALS AND METHODS

Protein estimation and the preparation of γ-^{32}P-ATP and purified lipids are described elsewhere (Galliard et al., 1965). Subcellular fractionation of rat liver was performed as described by Sedgwick and Hübscher (1965), with two modifications: 1) The homogenate was filtered through a fine mesh nylon cloth before fractionation; 2) the medium was 0.3M sucrose, not 0.3M sucrose/ 0.002M EDTA.

The basic incubation medium for incorporation studies was: 100 mM KCl; 40 mM $MgCl_2$ (15 mM for the mitochondrial fraction); 30 mM sucrose; 20 mM Tris/HCl buffer, pH 7.4; 5 mM ATP containing from 10^5 to 10^6 counts/100 sec/ µmole of ^{32}P. Incubations were for 2 to 5 min, in total volume of 1 ml, at 37°, with a protein content of 1 to 4 mg. The incorporations were stopped, lipids extracted, and the radioactivity of DPI determined, all as described elsewhere (Galliard et al., 1965). Liver homogenate synthesised 0.5 to 2 µmoles DPI per mg protein per min under these conditions. Other enzyme assays were also done as described by Galliard et al. (1965) except 5'-nucleotidase. This was assayed as follows. Cell fractions (less than 1 mg protein) were incubated for 15 min at 37° in 2 ml of a medium containing: 100 mM KCl; 10 mM $MgCl_2$; 50 mM Tris/HCl buffer, pH 7.4; 5 mM AMP; 10 mM sodium potassium tartrate. The reaction was stopped with 1 ml of 25% (w/v) TCA and P_i was assayed in 2 ml of the supernatant, by the method of King (1932). The inclusion of tartrate in the assay medium as an inhibitor of acid phosphatase was suggested by El-Aaser and Reid (1965).

RESULTS AND DISCUSSION

$^{32}P_i$-DEPENDENT LABELLING OF DPI

It has been shown that DPI labelling from $^{32}P_i$ is an energy-dependent process, for which mitochondria are necessary (Galliard et al., 1965). Absence of mitochondria, or blockage of ATP synthesis by inhibition of

respiration or oxidative phosphorylation, abolishes DPI labelling. Atractyloside, which inhibits transport into and out of the tightly-bound nucleotide pool of the mitochondrion (Heldt et al., 1965; Brierly and Green, 1965; Klingenberg and Pfaff, 1965) also blocks $^{32}P_i$-dependent labelling of DPI. This led Hajra et al. (1965) to postulate that the phosphorylation of PI occurs on the exterior of the mitochondrial membrane.

ATP-DEPENDENT LABELLING OF DPI

After incubation of a liver mitochondrial fraction with γ-^{32}P-ATP a labelled lipid was detected and identified as DPI by methods similar to those of Galliard et al. (1965). Hajra et al. (1965) observed the same phosphorylation. This labelling has also now been shown in other subcellular fractions from rat liver. The incorporation showed an absolute requirement for divalent cations. At optimum concentration (15 mM for mitochondrial and 40 mM for other fractions) Mg^{2+} was the most efficient activator, but at low concentrations (1 mM) Mn^{2+} was more efficient than Mg^{2+}. Ca^{2+} was ineffective. The reaction was stimulated (up to 30-fold in the nuclear fraction) by PI, with maximum synthesis at concentrations of 5 mM and above. Other phospholipids (phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid) inhibited the reaction. At 30° incorporation proceeded linearly with time for only 3-4 min, approaching a constant level after that time.

SUBCELLULAR DISTRIBUTION OF THE ATP-DEPENDENT SYSTEM

DPI labelling was measured in each of the isolated cell fractions, the incubation time being 2 min. A set of markers was assayed on the same preparations. These were: Nuclear DNA; mitochondrial, succinate:INT* reductase (succinate: INT oxidoreductase, E.C. 1.2.99.1); lysosomal, β-glucuronidase (β-D-glucuronide glucuronohydrolase, E.C. 3.2.1.31); endoplasmic reticulum, glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, E.C. 3.1.3.9.); and soluble, 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate NADP oxidoreductase (decarboxylating), E.C.1.1.1.44.).

* 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride.

5'-nucleotidase (5'-ribonucleotide phosphohydrolase, E.C. 3.1.3.5.), an enzyme which has been shown to occur in liver plasma membranes both histochemically (Novikoff et al., 1962) and by isolation of the purified membranes (Emmelot et al., 1964), was also assayed. The results are given in Figure 1.

Relative specific activities:

i.e. $\frac{\text{Units of enzyme activity, or } \mu g \text{ DNA, per mg protein of fraction}}{\text{Units of enzyme activity, or } \mu g \text{ DNA, per mg protein of homogenate}}$

are plotted on the ordinates. Protein contents (as percentages of the total protein of the filtered homogenate) are plotted on the abscissae. The fractions are designated: D (debris - this was the material filtered out of the homogenate before fractionation, and its activity is not included in the recoveries); N (nuclear); Mt (mitochondrial); L (lysosomal): Mc (microsomal); S (supernatant). The recoveries (R) are given as percentages of the activity of the filtered homogenate. From the distribution of the marker enzymes and DNA in the individual fractions it can be seen that a reasonable fractionation of the subcellular structures was achieved. The distribution of 5'-nucleotidase was significantly different from that of the other markers, but similar to that reported by El-Aaser and Reid (1965). The distribution of the enzyme catalysing the labelling of DPI closely paralleled that of 5'-nucleotidase, suggesting that phosphorylation of PI is also a plasma membrane phenomenon. Thus the results that Hajra et al., (1965) obtained with atractyloside would now indicate, not labelling on the exterior of the mitochondrion, but on contaminating membrane fragments.

Another site of DPI synthesis is the erythrocyte ghost. Here the activity is about one-tenth of that in the liver homogenate (Hokin and Hokin, 1964).

Triphosphoinositide (TPI) labelling from ^{32}P-ATP in brain occurs mainly in a fraction rich in myelin (Dawson, 1965), which is derived from the Schwann cell plasma membrane. TPI labelling also occurs in the erythrocyte ghost, to about the same extent as that of DPI (Hokin and Hokin, 1964). Labelling of

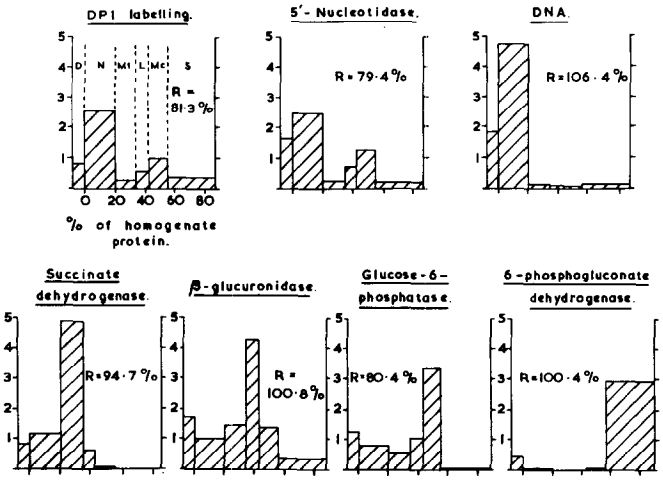

Fig. 1. Subcellular distribution of DPI labelling and markers.

both DPI and TPI is virtually confined to their monoesterified phosphate groups. It seems that DPI and TPI may be characteristic constituents of plasma membranes of various types, including those of the liver cell, erythrocytes and myelin. A negligible proportion of rat liver DPI synthesis occurs in the mitochondrial fraction. Further studies along these lines may lead to a clearer understanding of the function of these lipids.

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