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Problems Encountered in Measuring the Activity of Phosphatidate Phosphohydrolase

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The measurement of phosphate release from phosphatidate overestimates the microsomal activity of phosphatidate phosphohydrolase from rat liver, since phosphate is also produced via the glycerol phosphate that results from the deacylation of phosphatidate. The determination of phosphate production can be a reliable assay for the soluble phosphatidate phosphohydrolase in rat liver, because the glycerol phosphate formed is not hydrolysed under the conditions used.

Evidence is accumulating that phosphatidate phosphohydrolase (EC 3.1.3.4) is involved in controlling the rate of hepatic triacylglycerol synthesis (Fallon *et al.*, 1977; Pritchard *et al.*, 1977; Brindley, 1978). It is therefore important to assay this enzyme reliably and reproducibly. A variety of techniques have been developed with phosphatidate emulsions or membrane-bound phosphatidate as substrates. The latter procedure was introduced since the soluble form of the enzyme in liver and intestinal mucosa appeared to be relatively inactive towards phosphatidate emulsions (Johnston *et al.*, 1967; Smith *et al.*, 1967). This was probably caused by differences in the physical state of the phosphatidate in the two assay systems (Smith *et al.*, 1967). The presence of tightly bound Ca^{2+} in the phosphatidate used to prepare the emulsion is particularly inhibitory towards the soluble enzyme (Bowley *et al.*, 1977). Provided that Ca^{2+} -free phosphatidate is used and its physical state is carefully controlled, then it can be efficiently degraded by the soluble enzyme (Hosaka *et al.*, 1975; Bowley *et al.*, 1977; Savolainen, 1977).

When a phosphatidate emulsion is used as a substrate, phosphatidate phosphohydrolase activity is usually measured by P_i release. However, phosphatidate can also be degraded to glycerol phosphate, which in turn can be hydrolysed to P_i . The extent to which these reactions complicate the determination of the enzyme activity was therefore investigated by using the microsomal and soluble fractions obtained from rat liver.

Materials and Methods

The source or preparation of most of these have already been described (Sturton & Brindley, 1977; Whiting *et al.*, 1977).

Preparation of 1,2-diacyl-sn-[^3H]glycerol 3-phosphate and 1,2-diacyl-sn-[^3H]glycerol

These were synthesized enzymically as described by Brindley & Bowley (1976) for the preparation of membrane-bound phosphatidate except that unlabelled palmitate and 1 mM *sn*-[1,3- ^3H]glycerol 3-phosphate (500 $\mu\text{Ci}/\mu\text{mol}$) were used. The reaction was stopped after 120 min at 37°C and lipids were extracted (Hajra *et al.*, 1968). Neutral lipids and phospholipids were separated on columns of silicic acid by elution with chloroform and chloroform/methanol (4:1, v/v) respectively. 1,2-Diacyl-*sn*-[^3H]glycerol was purified from the neutral lipid fraction by t.l.c. on plates of silica gel G with *n*-hexane/diethyl ether/acetic acid (60:40:1, by vol.) for development. 1,2-Diacyl-*sn*-[^3H]glycerol 3-phosphate was purified by two different separations on t.l.c. plates of Kieselgel 60 (Merck, Darmstadt, Germany) by developing with chloroform/methanol/water/aq. 35% (w/w) NH_3 (sp.gr. 0.880) (130:42:5:3, by vol.) or chloroform/methanol/acetic acid/acetone/water (10:2:2:4:1, by vol.). Bivalent cations were removed by passing the product through Chelex resin (K^+ form) (Renkonen, 1968). The radiochemical purities of diacylglycerol and phosphatidate were >97% and they were stored under N_2 at -20°C in *n*-hexane and chloroform respectively. They were diluted for use in enzyme assays with non-radioactive substrate as indicated.

Measurement of phosphatidate breakdown

Each assay contained in 0.25 ml: 20 mM-Tris/maleate buffer, pH 6.4, 1 mM-dithiothreitol, 1.5 mM-diacyl-*sn*-[^3H]glycerol 3-phosphate (1 $\mu\text{Ci}/\mu\text{mol}$), 2 mM- MgCl_2 and 200 μg of microsomal protein or

500 μ g of soluble protein. Duplicate assays were performed in parallel and these were incubated at 37°C for 20 min with the soluble protein and for 15 min with microsomal protein. The procedures for stopping the reactions were as follows. (a) The release of P_i was measured in assays that were stopped with 0.25 ml of 0.61 M-trichloroacetic acid (Bowley *et al.*, 1977). (b) The formation of lysophosphatidate was determined in incubations that were stopped with 0.5 ml of butan-1-ol (Bjerve *et al.*, 1974). Lipid from the butanol phase was chromatographed on t.l.c. plates of Kieselgel 60 with chloroform/methanol/acetic acid/acetone/water (10:2:2:4:1, by vol.) for development. (c) The products of the reaction were extracted by the method of Bligh & Dyer (1959). The formation of diacyl[3H]glycerol was determined on samples of the bottom phase that were chromatographed on t.l.c. plates of silica gel G with n-hexane/diethyl ether/acetic acid (60:40:1, by vol.) for development. [3H]Glycerol phosphate and [3H]glycerol in the top phase of the extract were separated by shaking twice with Dowex resin (Cl^- form). The difference between the 3H in the top phase before and after Dowex treatment was taken as a measurement of glycerol phosphate formation. The identity of the glycerol and of the glycerol phosphate was confirmed by separation on t.l.c. plates of microgranular cellulose after developing twice with di-isopropyl ether/butan-1-ol/90% (w/v) formic acid (3:3:2, by vol.).

Measurement of the degradation of 1,2-diacyl-*sn*-glycerol

The assay system and conditions were the same as those described for the degradation of phosphatidate except that the substrate was a mixed emulsion containing 360 nmol of unlabelled phosphatidate and 15 nmol of 1,2-diacyl-*sn*-[3H]glycerol (2.5 μ Ci/ μ mol). The reactions were stopped and extracted by the method of Bligh & Dyer (1959) and the appearance of monoacylglycerol and glycerol was measured as described above.

Determination of protein and radioactivity

Protein was determined by a biuret method and radioactivity was measured by liquid-scintillation counting (Whiting *et al.*, 1977).

Results and Discussion

Table 1 shows the extent of phosphatidate degradation by microsomal and soluble fractions under conditions that were previously used to measure phosphatidate phosphohydrolase activity (Pritchard *et al.*, 1977; Whiting *et al.*, 1977). The rates of formation of the products shown were proportional to the time of incubation and to the concentration of cell fraction added.

The formation of P_i and of diacylglycerol after incubation of phosphatidate with the soluble fraction was very similar (Table 1). Phosphatidate was degraded to glycerol phosphate, but less than 5% of the water-soluble products was isolated as glycerol, thus indicating that there was little deacylation of diacylglycerol. This was confirmed by adding 1,2-diacyl-*sn*-[3H]glycerol to the incubations and by finding that only $4.0 \pm 0.7\%$ (mean $\pm$ s.d. from three independent experiments) was converted into glycerol. Lysophosphatidate and monoacylglycerol were not detected in any of the experiments. The addition of 1 mM-phosphatidylcholine as a mixed emulsion with the phosphatidate produced an optimum stimulation of about 5-fold in diacylglycerol production (Table 2). A similar stimulation was observed in the synthesis of P_i in other independent experiments (not shown). P_i was not released from phosphatidylcholine in the absence of phosphatidate. A smaller stimulation by phosphatidylcholine in the rate of P_i release from phosphatidate has already been reported by Hosaka *et al.* (1975). The addition of phosphatidylcholine also approximately doubled the quantity of glycerol phosphate formed from phosphatidate, but this only amounted to less than 10% of the phosphatidate degraded (Table 1).

The results in Table 1 show that the activity of the soluble enzyme can be measured by the formation

Table 1. Degradation of 1,2-diacyl-*sn*-[3H]glycerol 3-phosphate by the microsomal and soluble fractions of rat liver
1,2-Diacyl-*sn*-[3H]glycerol 3-phosphate was incubated with the soluble and microsomal fractions of rat liver as described in the Materials and Methods section. The results are given as means $\pm$ s.d. from six and nine independent experiments with soluble and microsomal fractions respectively.

Fraction	Product formed (nmol)			
	P_i	Diacylglycerol	Glycerol phosphate	Glycerol
Soluble	14.5 ± 2.5	15.2 ± 3.1	4.3 ± 1.5	Not detected
Microsomal	15.0 ± 1.0	10.3 ± 3.4	2.6 ± 1.2	4.4 ± 2.2

Table 2. *Effect of phosphatidylcholine on the degradation of 1,2-diacyl-sn-[³H]glycerol 3-phosphate by the microsomal and soluble fractions of rat liver*

1,2-Diacyl-sn-[³H]glycerol 3-phosphate was incubated with the soluble and microsomal fractions of rat liver as described in the Materials and Methods section. Phosphatidylcholine (1 mM) was added in three independent experiments as indicated.

Fraction	Phosphatidylcholine	Expt. ...	Product formed (nmol)											
			P _i			Diacylglycerol			Glycerol phosphate			Glycerol		
			I	II	III	I	II	III	I	II	III	I	II	III
Soluble	Absent					21	16	11	6.2	3.0	3.0	Not detected		
Soluble	Present					77	75	79	8.2	7.2	7.1	Not detected		
Microsomal	Absent		9	22	18	9	12	12	3.9	2.7	1.0	2.9	9.1	5.6
Microsomal	Present		14	32	26	11	26	22	2.6	0.1	0.1	3.3	6.1	3.7

of either P_i or diacylglycerol. However, care should be taken if 1,2-diacyl-sn-glycerol 3-[³²P]-phosphate is used as a substrate and the appearance of water-soluble products is monitored. It is possible for sn-glycerol 3-[³²P]phosphate to increase the water-soluble radioactivity by as much as 30% (Table 1). This problem can be alleviated by including phosphatidylcholine in the incubations (Table 2).

The measurement of phosphatidate phosphohydrolase activity in the microsomal fraction is more complex. The production of diacylglycerol was only about 68% of the P_i formed (Table 1). This discrepancy can be accounted for by the deacylation of phosphatidate leading to the production of glycerol phosphate and glycerol (Table 1), and of unesterified fatty acids (Tzur & Shapiro, 1976). Glycerol could also have been formed from 1,2-diacyl-sn-[³H]-glycerol, but when this was added only 6.0±0.5% (mean±s.d. from three independent experiments) was converted into [³H]glycerol.

Attempts were made to decrease the hydrolysis of glycerol phosphate by inhibiting alkaline phosphatase (EC 3.1.3.1) and acid phosphatase (EC 3.1.3.2). Omitting Mg²⁺ ions from the incubations or adding F⁻ ions decreased the rate of glycerol production, but these procedures also inhibited phosphatidate phosphohydrolase activity. It was also possible that adding phosphatidylcholine to the incubations might decrease the deacylation of phosphatidate and diacylglycerol by providing an alternative substrate for lipase activity. The rate of production of glycerol phosphate was decreased in all three experiments, whereas that of diacylglycerol and P_i was increased (Table 2). Shapiro & Tzur (1968) also showed that the microsomal phosphatidate phosphohydrolase activity is stimulated by phosphatidylcholine.

The rate of production of diacylglycerol in the presence of phosphatidylcholine was 79–85% that of P_i (Table 2), whereas in the absence of phosphatidylcholine this rate had a mean value of about

69% (Tables 1 and 2). The measurement of phosphatidate phosphohydrolase activity can probably be made more reliable by carefully adjusting the concentration of phosphatidylcholine in different experimental conditions. The best measurement of this activity is made by determining the formation of diacylglycerol. The more convenient measurement of P_i release should only be used if the deacylation of phosphatidate and the hydrolysis of glycerol phosphate do not contribute significantly to P_i production. Measuring the rate of release of water-soluble ³²P from 1,2-diacyl-sn-glycerol 3-[³²P]-phosphate is even more subject to error since glycerol phosphate is likely to contribute to this activity (Tables 1 and 2).

The extent to which the deacylation of phosphatidate and of diacylglycerol complicate the measurement of phosphatidate phosphohydrolase activity depends upon the experimental conditions chosen. Some authors have recognized these problems and have claimed that deacylation (Coleman & Hübscher, 1962) or dephosphorylation of glycerol phosphate (Rosenthal & Pousada, 1966) did not significantly affect the results obtained by measuring P_i release. These conclusions can also be made by using the soluble fraction of rat liver, but the measurement of the rate of P_i release is not a reliable assay for the microsomal enzyme (Tables 1 and 2). In the work of Tzur & Shapiro (1976) the rate of diacylglycerol formation was only 23–34% that of P_i release. It is therefore important to check the products of phosphatidate degradation under the conditions to be used before deciding how to assay phosphatidate phosphohydrolase. This is particularly important when studying the subcellular distribution of the enzyme since the deacylation of phosphatidate and of diacylglycerol may vary markedly in different fractions. The interpretation of these results is further complicated by there being more than one phosphatidate phosphohydrolase activity in mitochondrial and microsomal

fractions (Jamdar & Fallon, 1973; Lamb & Fallon, 1974; Kako & Patterson, 1975; Caras & Shapiro, 1976).

The apparent enzyme activity that is normally detected in the microsomal fraction far exceeds the ability of this fraction to degrade the phosphatidate that it synthesizes from glycerol phosphate (Johnston *et al.*, 1967; Smith *et al.*, 1967; Whiting *et al.*, 1977; Pritchard *et al.*, 1977). This discrepancy probably results from both the overestimation of activity caused by measuring the rate of P_i release and the inclusion of activities that are not directly concerned with glycerolipid synthesis.

The present paper is intended as a cautionary note. It lists some of the problems that should be considered when interpreting published work on phosphatidate phosphohydrolase and in planning future studies.

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