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Inhibition of Glucose 6-Phosphatase by Pure and Impure C-Type Phospholipases

REACTIVATION BY PHOSPHOLIPID DISPERSIONS AND PROTECTION BY SERUM ALBUMIN

By B. R. CATER,* PAMILLA TRIVEDI and T. HALLINAN
Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, 8 Hunter Street, London WC1 1BP, U.K.

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1. Pure or impure C-type phospholipases hydrolysed rat liver microsomal phosphatides *in situ* at 5° or 37°C. At 5°C mean hydrolysis of total phospholipids was 90% by *Bacillus cereus* and 75% by *Clostridium perfringens* (*Clostridium welchii*) C-type phospholipases. 2. Four degrees of inhibition of glucose 6-phosphatase (D-glucose 6-phosphate phosphohydrolase; EC 3.1.3.9) resulted. (*a*) At 37°C inhibition was virtually complete and apparently irreversible. (*b*) At 5°C phospholipase C inhibited 50–87% of the activity expressed by intact control microsomal fractions. (*c*) Bovine serum albumin present during delipidation alleviated most of this inhibition: at 5°C phospholipase C plus bovine serum albumin inhibited by 0–35% (mean 18%): simultaneous stimulation by the destruction of its latency seems to offset glucose 6-phosphatase inhibition, sometimes completely. (*d*) If latency was first destroyed, phospholipase C plus bovine serum albumin inhibited 30–50% of total glucose 6-phosphatase activity at 5°C. Only this inhibition is likely largely to reflect the lower availability of phospholipids, essential for maximal enzyme activity, as it is virtually completely reversed by added phospholipid dispersions. Co-dispersions of phosphatidylserine plus phosphatidylcholine (1:1, w/w) were especially effective but Triton X-100 was unable effectively to restore activity. 3. Considerable glucose 6-phosphatase activity survived 240min of treatment with phospholipase C at 5°C, but in the absence of substrate or at physiological glucose 6-phosphate concentrations the delipidated enzyme was completely inactivated within 10min at 37°C. However, 80mM-glucose 6-phosphate stabilized it and phospholipid dispersions substantially restored thermal stability. 4. It is concluded that glucose 6-phosphatase is at least partly phospholipid-dependent, and complete dependence is not excluded. For reasons discussed it is impossible yet to be certain which phospholipid class(es) the enzyme requires for activity.

In liver, glucose 6-phosphatase (EC 3.1.3.9) appears to be an integral enzyme (Singer & Nicholson, 1972) of the endoplasmic-reticular membranes. It has been frequently reported as being phospholipid-dependent (Beaufay & de Duve, 1954*b*; Feuer & Goldberg, 1967; Duttera *et al.*, 1968; Lumper *et al.*, 1969; Garland & Cori, 1972), but doubt has recently been cast on this. Instead it has been proposed that a small fraction of the enzyme can participate in a stimulatory detergent-like interaction with phospholipids when these are restored to delipidated microsomal fractions (Snoke & Nordlie, 1972) or even that glucose 6-phosphatase is phospholipid-constrained (Zakim, 1970).

* Present address: BIBRA, Woodmansterne Road, Carshalton, Surrey, U.K.

Beaufay & de Duve (1954*a*) first provided evidence that glucose 6-phosphatase required phospholipids. Treatment of microsomal fractions with *Clostridium perfringens* phospholipase C elicited two distinct responses from glucose 6-phosphatase: (i) substantial inhibition, (ii) sensitization to further inactivation when microsomal fractions were post-incubated at 25–37°C (after the phospholipase C action had been stopped with EDTA). Duttera *et al.* (1968) confirmed the inhibitory effect of delipidation by phospholipase C at 20°C and, observing that glucose 6-phosphatase activity could be restored with phospholipid dispersions, concluded that the enzyme was phospholipid-dependent. Similar conclusions were drawn for glucose 6-phosphatase from deoxycholate-dispersed microsomal fractions which had been delipidated chromatographically (Garland &

Cori, 1972). Zakim (1970) also observed inhibition of glucose 6-phosphatase by phospholipase C, but, finding that serum albumin could restore the pyrophosphate–glucose phosphotransferase activity of the enzyme to greater than control values after treatment with phospholipase C or phospholipase A2, and that added asolectin (soya-bean phospholipids) slightly inhibited the enzyme in control microsomal fractions, he concluded that phospholipids did not support glucose 6-phosphatase, but played the diametrically opposite role of constraining the enzyme's maximal activity.

Since the reports cited above arrived at opposite conclusions, it was decided in this study to investigate further the effects of phospholipid depletion on glucose 6-phosphatase. The ability of phospholipid dispersions to reverse the effects of delipidation on the enzyme was also examined.

Pure and impure phospholipase C from *Bacillus cereus* and a crude preparation from *Cl. perfringens* were used to deplete microsomal fractions of phospholipids. Since preliminary studies confirmed the profound destabilizing effect of *Cl. perfringens* phospholipase C on glucose 6-phosphatase (Cater *et al.*, 1970), delipidation has been mainly done at 5°C. To strengthen interpretation, the hydrolysis of phosphatidylcholine, the major endoplasmic-reticular phosphatide, or of other phospholipids has been measured and is reported for every experiment.

Materials and Methods

Animals and radioactive labelling

Most experiments were performed on 150–300g male Ash–Wistar rats killed by cervical fracture. Animals were starved for 16–24h and were usually killed 30–40min after the intraperitoneal injection of one of the radioactive phospholipid bases, choline, ethanolamine, serine or inositol, or 60min after injection of radioactive palmitate. In single-label experiments, radiochemical dosage was typically 10–20μCi of ^{3}H or 5–25μCi of ^{14}C/100g. To eliminate possible difficulties due to variable ^{14}C spillover during radioactive counting, dosage was arranged so the incorporation of ^{3}H was 10–20 times that of the ^{14}C precursor in double-label experiments.

Preparation of subcellular fractions

Microsomal fractions were sedimented in 60min at 104000 or 134000$g_{av.}$. All operations were performed at 0–5°C, and fresh microsomal preparations prepared the same day were used in almost all experiments. In a few experiments 1:1 (w/v) sucrose homogenates were made and the 10000$g_{av.}$ supernatant was passed through a small column of Sephadex G-25 to remove small molecules. The microsomal fractions and cell sap were then concentrated at 5°C on Amicon Centriflo membrane cones (grade 26-CF-50A) to yield concentrations of 20–30mg of microsomal protein and 50–60mg of cell sap protein/ml.

Phospholipase C treatment

This was generally done by incubating 2–6mg of microsomal protein/ml with 500μg of *Cl. perfringens* (*Clostridium welchii*) phospholipase C (1–5 nominal units, depending on the batch)/ml, or with 100μg of impure Makor *B. cereus* phospholipase C (2–5 nominal units)/ml, or with 10μl of pure *B. cereus* phospholipase C (14 nominal units)/ml. Incubation with phospholipase C was for 30–240min at 5° or 37°C in the presence of 10mM-Tris–10mM-maleate buffer (pH6.8) and 1mM-$CaCl_2$. *Cl. perfringens* phospholipase action was stopped by making incubations 2.0–4.0mM with respect to EGTA [ethanedioxybis(ethylamine)tetra-acetate]. This inhibited the *Cl. perfringens* enzyme completely. The action of *B. cereus* phospholipase C, or of both *B. cereus* and *Cl. perfringens* enzymes together, was stopped by adding an equal volume of chilled saturated 1,10-phenanthroline, dissolved in 0.05M-Tris–maleate, pH6.8, and containing 0.01M-EGTA. The effectiveness of stopping *B. cereus* phospholipase C was normally verified by adding sonically dispersed [^{14}C]choline-labelled liver lipids to the terminated incubation after all assay samples had been removed. This was then incubated for 10min at 37°C. In no case was more than 10% of the added radioactive phospholipid hydrolysed.

Assessment of phospholipid hydrolysis by phospholipase C

This was measured in every experiment by one of two methods: (*a*) a radiochemical glass-fibre-disc method previously described for measuring phosphatidylcholine hydrolysis (Hallinan *et al.*, 1969), but extended and further validated for measuring the hydrolysis of phosphatidylethanolamine, phosphatidylinositides and phosphatidylserine (B. R. Cater, P. Trivedi & T. Hallinan, unpublished work); (*b*) by determining lipid phosphate remaining in purified lipids quantitatively extracted from control and phospholipase C-treated microsomal fractions (Folch *et al.*, 1957) and wet-ashed for 2–3h at 180–220°C with 72% (w/v) $HClO_4$ (Allen, 1940).

Glucose 6-phosphatase assay

Glucose 6-phosphatase activity was assayed with 0.08M-glucose 6-phosphate in 0.05–0.15M-cacodylate

buffer, pH6.5, at 37°C. Non-specific hydrolysis of glucose 6-phosphate by phosphatases other than glucose 6-phosphatase was assessed by preincubating microsomal fractions for 10min at 37°C and pH5 before addition of an equal volume of 0.16M-glucose 6-phosphate in cacodylate buffer, pH6.5, (Beaufay & de Duve, 1954*a*). It averaged only 1.2% (range 0–2.5%) of the total glucose 6-phosphatase activity in five experiments. Mannose 6-phosphate phosphohydrolase and glucosamine 6-phosphate phosphohydrolase activities were also measured exactly as above but with 0.04M-sugar phosphates and 4–6mg of microsomal protein/ml. All enzyme activities were corrected by subtracting P_i present in microsomal fractions and substrates at zero time. No spontaneous hydrolysis of glucose 6-phosphate occurred in the absence of microsomal fractions. P_i was assayed by the method of Allen (1940).

Stimulation of glucose 6-phosphatase by destruction of latency

The latency of glucose 6-phosphatase was normally destroyed either by assaying in the presence of optimal concentrations of detergent (0.04–0.07% Triton X-100; 0.02% sodium deoxycholate; see the Results section), added with the substrate, or by incubating microsomal fractions (about 18mg of protein/ml) for 30min at 30°C with 0.09M-NH_3 (Stetten & Burnett, 1966).

Reactivation of glucose 6-phosphatase with phospholipid dispersions after phospholipase C treatment

After further phospholipase C action had been stopped, chilled ultrasonicated dispersions of phospholipids were added to delipidated microsomal fractions at weight ratios of 2–4mg of phospholipid/mg of microsomal protein; these were then left for 10min at 5°C before glucose 6-phosphatase was assayed at 37°C. Preliminary experiments with egg phosphatidylcholine and 1:1 (w/w) co-dispersions of phosphatidylcholine plus phosphatidylserine, showed that this large excess of phospholipid was necessary to obtain optimal restoration of glucose 6-phosphatase activity. Phospholipids were freed of solvent below 40°C and were dispersed in 0.01M-Tris–maleate buffer, pH6.8, by using a 50 or 150W MSE ultrasonic disintegrator at 0°C for a total of 3–5min, sonicating for 30s at a time and then cooling. In some experiments phospholipids were evaporated and sonicated under N_2, but omission of this precaution seemed to be without effect on their ability to reactivate glucose 6-phosphatase. After sonication, in two to four separate experiments for each phospholipid except phosphatidylinositol, phospholipid integrity was checked by t.l.c. (Skipski *et al.*, 1964); all were still chromatographically homogeneous. The above conditions yielded stable opalescent dispersions of all phospholipids used except phosphatidylethanolamine, which proved very difficult to disperse. More stable dispersions were obtained in 0.01M-Tris–HCl buffer, pH8.0, containing 0.001M-EDTA. Long-chain fatty acids were sonically dispersed in 0.1M-KCl after adjustment of a coarse dispersion to pH8–9 with NaOH. After sonication the fatty acid was buffered at pH8.0 with 0.01M-Tris buffer. All sonicated lipids were used within 2h of dispersion, phosphatidylethanolamine being used within 15min. Protein assays were performed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Materials

[*Me*-^{3}H]Choline (16.5Ci/mmol) or [*Me*-^{14}C]-choline (60mCi/mmol) and [2-^{3}H]ethanolamine (320mCi/mmol) or [2-^{14}C]ethanolamine (2–10mCi/mmol) and [2-^{3}H]*myo*-inositol (2Ci/mmol) were all purchased from The Radiochemical Centre, Amersham, Bucks., U.K. DL-[1-^{14}C]Serine (8.2mCi/mmol) and [1,2-^{14}C]ethanolamine (1.85mCi/mmol) were purchased from New England Nuclear Corp., Dreieichenhain, West Germany. Phospholipase C from *Cl. perfringens* (*Cl. welchii*) was purchased from Sigma (London) Chemical Co., London S.W.6, U.K. Its nominal specific activity ranged from 2 to 10units/mg of solid. Pure *B. cereus* phospholipase C was kindly given by Dr. Rob Zwaal, Department of Biochemistry, University of Utrecht, Utrecht, The Netherlands Its nominal specific activity was 1400units/ml and it was dissolved in 0.05M-Tris–HCl–5mM-$CaCl_2$–50% (v/v) glycerol, pH7.2. Impure *B. cereus* phospholipase C was purchased from Makor Chemical Co., P.O. Box 6570, Jerusalem, Israel. Its nominal specific activity ranged from 40 to 100units/mg of protein.

Pure phospholipid classes were either prepared in this department (egg-yolk phosphatidylcholine and phosphatidic acid prepared therefrom) and were generously given for this study by Dr. W. Tampion, or else were purchased from specialist suppliers of lipids: soya-bean phosphatidylinositol was purchased from the Hormel Institute, Austin, Minn., U.S.A.; phosphatidylcholine and phosphatidylserine from bovine spinal cord, and lysophosphatidylcholine and phosphatidylethanolamine from egg yolk, were all purchased from Lipid Products, South Nutfield, Surrey, U.K. All phospholipids were stored in solution under N_2 in sealed vials at −10°C. Vials were only opened immediately before use.

Results

Inhibition of glucose 6-phosphatase by phospholipase C at 37° and 5°C

As shown in Fig. 1, digestion of rat liver microsomal fractions with *Cl. perfringens* phospholipase C at 37°C caused complete inhibition of glucose 6-phosphatase and hydrolysed 96% of the labelled membrane phosphatidylcholine and 76% of the phosphatidylethanolamine by 60min. Virtually complete inhibition of glucose 6-phosphatase was always seen after delipidation at 37°C, regardless of whether phospholipase C action was stopped with EGTA before glucose 6-phosphatase assay (see Fig. 4). The small residual capacity to hydrolyse glucose 6-phosphate, which averaged only 1.1% of the glucose 6-phosphatase of untreated microsomal fractions in 20 experiments (range 0–3.9%), can be ascribed to non-specific phosphatase.

The effect in Fig. 1 of phospholipase C treatment at 5°C on glucose 6-phosphatase is very different from that at 37°C. At 5°C after 60min of phospholipase C treatment, 31% of the glucose 6-phosphatase activity remained, irrespective of the continuing action of phospholipase C during glucose 6-phosphatase assay. This residual activity was fairly stable at 5°C, only decreasing a further 8% over the subsequent 3h treatment with phospholipase C. Nevertheless, phosphatidylcholine hydrolysis after 60min at 5°C was as extensive as at 37°C, and hydrolysis of labelled phosphatidylethanolamine at 5°C (85%) exceeded that at 37°C. Further, addition of *B. cereus* phospholipase C over the last 60min at 5°C only caused a small 7% further inhibition of glucose 6-phosphatase, though demonstrably hydrolysing all of the residual phosphatidylethanolamine. As shown in Fig. 2(*a*) partial (55–79%) inhibition of glucose 6-phosphatase was seen when microsomal fractions were treated at 5°C with *Cl. perfringens* phospholipase C. In these experiments phospholipase action was always stopped by adding EGTA, before glucose 6-phosphatase was assayed. Phosphatidylcholine hydrolysis ranged from 84 to 97% (mean 93%). In nine similar experiments *Cl. perfringens* phospholipase C at 5°C caused 60–85% hydrolysis of phosphatidylethanolamine (mean 69%). Examination of residual lipids by t.l.c. also showed substantial sphingomyelin hydrolysis by the *Cl. perfringens* enzyme, in agreement with Duttera *et al.* (1968). However, in other experiments (B. R. Cater, P. Trivedi & T. Hallinan, unpublished work), *Cl. perfringens* phospholipase C at 5° or 37°C caused little or no hydrolysis of the acidic phosphatidylserine and the phosphatidylinositides of microsomal fractions. Its net effect, as shown in three separate experiments in which phosphate in purified lipids extracted from control and 5°C phospholipase C-treated microsomal fractions was assayed, was to hydrolyse 75% (range 67–82%) of the total phospholipids and to produce altered membranes rich in acidic phosphatides.

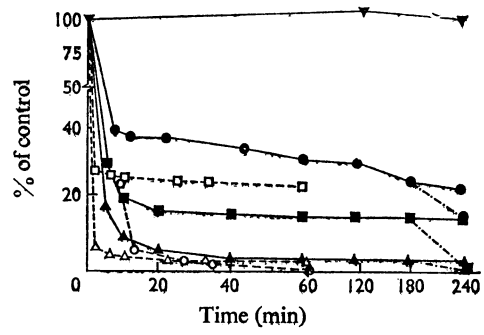

Fig. 1. *Phospholipid hydrolysis and inhibition of glucose 6-phosphatase by Cl. perfringens and B. cereus phospholipase C at 5° and 37°C*

Microsomal fractions were prepared as described in the Materials and Methods section from a rat injected with 100μCi of [*Me*-^{3}H]choline and 5μCi of [1,2-^{14}C]ethanolamine. These were treated at 37° or 5°C with 10 units of *Cl. perfringens* phospholipase C/ml and samples were removed, at the times indicated for assaying glucose 6-phosphatase and phospholipid hydrolysis (disc assay); phospholipase C action was not stopped before assay of glucose 6-phosphatase. After 180min at 5°C the incubation mixture was divided into two portions with a chilled pipette. One portion was incubated a further 60min at 5°C and assayed for glucose 6-phosphatase and phospholipid hydrolysis. To the other portion was added 5 units of impure *B. cereus* phospholipase C/ml and this was incubated for 60min at 5°C and assayed for glucose 6-phosphatase and phospholipid hydrolysis. In the control, which was incubated without phospholipase C, there was no detectable hydrolysis of phosphatidylcholine or phosphatidylethanolamine. Control glucose 6-phosphatase activity at zero time was 0.335μmol of glucose 6-phosphate hydrolysed/min per mg of microsomal protein. At zero time in controls mean ^{14}C radioactivity per disc was 487c.p.m.; mean ^{3}H radioactivity was 1886c.p.m. ▼——▼, Glucose 6-phosphatase activity in control; ●——●, glucose 6-phosphatase activity after *Cl. perfringens* phospholipase C treatment at 5°C; ●-·-·--●, glucose 6-phosphatase activity after addition of *B. cereus* phospholipase C at 5°C; ○----○, glucose 6-phosphatase activity after *Cl. perfringens* phospholipase C treatment at 37°C; ■——■, phosphatidylethanolamine hydrolysis by *Cl. perfringens* phospholipase C at 5°C; ■-·-·-■, phosphatidylethanolamine hydrolysis after adding *B. cereus* phospholipase C at 5°C; □----□, phosphatidylethanolamine hydrolysis at 37°C by *Cl. perfringens* phospholipase C: ▲——▲, phosphatidylcholine hydrolysis by *Cl. perfringens* phospholipase C at 5°C; ▲-·-·-▲, phosphatidylcholine hydrolysis after adding *B. cereus* phospholipase C at 5°C; △----△, phosphatidylcholine hydrolysis by *Cl. perfringens* phospholipase C at 37°C.

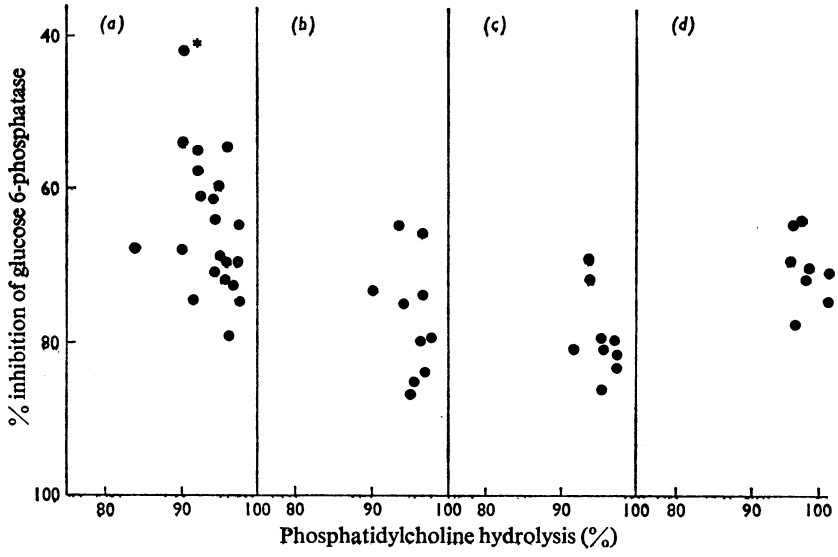

Fig. 2. *Hydrolysis of phosphatidylcholine and inhibition of glucose 6-phosphatase by phospholipase C at 5°C*

Microsomal fractions from rats pre-labelled with [^{3}H]- or [^{14}C]-choline were treated at 5°C for 45–120min with the following phospholipases: (*a*) *Cl. perfringens*; (*b*) impure *B. cereus*; (*c*) pure *B. cereus*; (*d*) *Cl. perfringens* + *B. cereus*. Phospholipid hydrolysis was monitored by disc counting and glucose 6-phosphatase was assayed after stopping further phospholipase C action. In some experiments *Cl. perfringens* phospholipase C was stopped by pipetting an equal volume of ice-cold 0.16M-glucose 6-phosphate+0.004M-EGTA in cacodylate buffer, pH6.5, into phospholipase C incubation tubes. These were then immediately shifted to a 37°C bath to initiate the glucose 6-phosphatase assay. In controls, glucose 6-phosphatase activity was 0.275–0.41 μmol of glucose 6-phosphate hydrolysed/min per mg of microsomal protein. The starred result in (*a*) is considered anomalous and was omitted when calculating the inhibition of glucose 6-phosphatase.

Effects of pure and impure B. cereus phospholipase C

By contrast with the *Cl. perfringens* enzyme both pure and impure phospholipase C from *B. cereus* hydrolysed almost all of the microsomal phosphatidylethanolamine and phosphatidylcholine as well as about 80% of the phosphatidylinositides and about 50% of the phosphatidylserine at 5°C, or 37°C, though no evidence of spingomyelin hydrolysis was seen (B. R. Cater, P. Trivedi & T. Hallinan, unpublished work). The net effect at 5°C, in three separate experiments determining lipid phosphate, was the hydrolysis of 90% (range 86–95%) of the total phospholipids, leaving membranes enriched in sphingomyelin and acidic phospholipids. However, as shown in Figs. 2(*b*) and 2(*c*), inhibition of glucose 6-phosphatase (65–87%) was still incomplete at 5°C, though on average [78±6.4% (S.D.)] it somewhat exceeded that caused by *Cl. perfringens* phospholipase C [mean 67±9.6% (S.D.)]. Both pure and impure *B. cereus* phospholipase C appeared to hydrolyse phosphatidylcholine and to inhibit glucose 6-phosphatase to similar extents. Finally, complete inhibition of glucose 6-phosphatase was still not achieved even when microsomal fractions were treated at 5°C with both *Cl. perfringens* and *B. cereus* phospholipase C together (Figs. 1 and 2*d*), though these appeared to hydrolyse 95% of the total microsomal phospholipids.

Restoration of glucose 6-phosphatase with phospholipid dispersions after phospholipase C treatment at 5°C

Addition of ultrasonicated dispersions of different pure phospholipid classes to microsomal fractions that had been delipidated at 5°C caused reactivation of glucose 6-phosphatase. Though quite variable, reactivation was often substantial but seemed to indicate little specificity in the phospholipid requirements of the enzyme. Thus phosphatidylcholine and phosphatidylserine both restored activity well, though 1:1 (w/w) co-dispersions of these two phosphatides always seemed slightly more effective, and 1:1 (w/w) co-dispersions of phosphatidylethanolamine plus phosphatidylserine were also very effective.

The column 'Control+phospholipid' of Table 1 shows the effect of the phosphatide dispersions on the activity of glucose 6-phosphatase in control microsomal fractions (see also the Discussion section). This was determined in every reactivation experiment to help assess any stimulatory effect the phosphatides might exert on uninhibited glucose 6-phosphatase.

Table 1. *Restoration of glucose 6-phosphatase with phospholipid dispersions after phospholipase C treatment at 5°C without bovine serum albumin*

Choline-labelled microsomal fractions were treated at 5°C with different C-type phospholipases, phospholipid hydrolysis being measured radiochemically. After 60–120min phospholipase action was stopped. Glucose 6-phosphatase was then assayed to give the 'phospholipase C-treated' activity shown in vertical column 4. To control microsomal fractions (often with inactive phospholipase C) and to phospholipase C-treated microsomal fractions, sonically dispersed phospholipids were then added at phospholipid/protein ratios of 2–4: after being left for 10min at 5°C glucose 6-phosphatase was assayed to give the 'control+phospholipid' and 'phospholipase C-treated+phospholipid' glucose 6-phosphatase values shown in vertical columns 5 and 6. In untreated controls, glucose 6-phosphatase activity was 0.29–0.37 μmol of glucose 6-phosphate hydrolysed/min per mg of microsomal protein. Results are reported as the means ± the average deviations from the means, with the range in parentheses. Numbers in the parentheses in vertical column 3 indicate the numbers of experiments. PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; lyso-PC, 1-acyl glycerylphosphorylcholine; PI, phosphatidylinositol; PA, phosphatidic acid.

Phospholipase	% phosphatidylcholine hydrolysed	Phospholipid dispersion	Phospholipase C-treated	Glucose 6-phosphatase (% of control): Control+phospholipid	Glucose 6-phosphatase (% of control): Phospholipase C-treated+phospholipid	% reactivation
Cl. perfringens	92±2 (89–94)	PC (4)	42±4 (38–50)	103±6 (94–110)	84±6 (73–90)	41±8 (26–50)
	93 (92–94)	PS (2)	39 (28–50)	99 (97–102)	92 (84–99)	53 (49–56)
	90	PE (4)	38±6 (31–44)	144±6 (138–150)	93±9 (81–111)	57±10 (44–67)
	95 (94–96)	PS+PC (2)	40 (29–50)	99 (97–100)	109 (96–122)	70 (67–72)
	90	Lyso-PC (2)	50 (49–51)	162 (157–166)	66 (64–67)	16 (13–18)
Pure *B. cereus*	95	PC	18	102	64	46
	95	PS	18	100	60	42
	95	PS+PC	18	106	80	62
Impure *B. cereus*	94	PC (2)	19 (18–19)	107 (100–113)	49 (47–50)	29 (26–31)
	96±3 (93–99)	PE (3)	30±6 (22–36)	107±2 (105–110)	51±7 (39–60)	19±12 (3–38)
	94	PS	19	100	52	33
	94	PS+PC	19	100	72	53
	96	PI	26	125	72	46
	96	PI+PC	26	122	70	44
	97	PA (2)	28 (22–34)	66 (60–72)	22 (19–24)	Inhibits
Cl. perfringens+	95	PC (2)	29 (22–36)	112 (111–113)	59 (56–62)	30 (26–34)
pure *B. cereus*	95	PS	36	97	93	57
	95	PS+PC	36	97	98	62
Cl. perfringens+	97 (96–97)	PC (3)	36±2 (33–38)	98±4 (93–103)	34±2 (31–36)	None
impure *B. cereus*	97	PS+PE (2)	37 (36–38)	107 (98–115)	97 (94–100)	60 (58–62)
	97	PS+PC	36	96	88	52
	97	PS	36	102	81	45

This is most important for deciding whether an increase in enzyme activity, when phospholipids are restored, could be due to reactivation of enzyme inhibited by phospholipid removal or merely to stimulation of residual glucose 6-phosphatase which survived delipidation uninhibited. From the results obtained it appears that most of the increase in activity elicited by lysophosphatidylcholine, and part of the effects of phosphatidylethanolamine and phosphatidylinositol, may be due to stimulation of residual glucose 6-phosphatase surviving delipidation. However, the increased activity produced by phosphatidylcholine and especially by phosphatidylserine and by co-dispersions containing that phosphatide are clearly not caused by stimulation of residual enzyme. It must therefore represent reactivation of glucose 6-phosphatase inhibited on phospholipid hydrolysis.

A clear-cut specificity effect is shown by phosphatidic acid, which inhibited glucose 6-phosphatase in both control and phospholipase C-treated microsomal fractions. This may contribute to the inability to reactivate glucose 6-phosphatase with phospholipid dispersions after treating microsomal fractions with phospholipase D (Lumper *et al.*, 1969) and appears further to contraindicate the use of this phospholipase.

Paradoxically, in three experiments, where *Cl. perfringens* and one individual batch of impure *B. cereus* phospholipase C were used together or in sequence at 5°C to induce very extensive hydrolysis of microsomal phospholipids, we found that glucose 6-phosphatase could no longer be restored with phosphatidylcholine dispersions, though dispersions of phosphatidylserine or co-dispersions containing

that phosphatide still reactivated quite effectively (Table 1, lowest set of results). It is impossible at present to explain this observation, which may be an unusual artifact specific to the particular batch of impure *B. cereus* phospholipase C used, as it was not reproduced with another batch of the impure enzyme (of lower specific activity) or with pure *B. cereus* phospholipase C, used alone or together with the *Cl. perfringens* enzyme (Table 1). Unfortunately no measurements were made on the activity of this phospholipase batch against acidic phospholipids.

Search for non-specific inhibition of glucose 6-phosphatase

Complete inhibition of glucose 6-phosphatase was not seen even when microsomal phosphatides were very extensively hydrolysed and neither its inhibition pattern nor the specificity of its reactivation appeared to correlate with any major phosphatide class. Therefore appreciating that the microsomal fractions themselves are most heterogeneous and would be contaminated by degradative enzymes and that the impure phospholipase preparations used in many experiments would contain proteinases and other degradative enzymes (Möllby *et al.*, 1973) a number of control experiments were done to exclude the following artifacts.

(*a*) Non-linear assay curves following delipidation: release of phosphate from 80mM-glucose 6-phosphate was linear with time over at least 25min at 37°C and 120min at 5°C in both control and delipidated microsomal fractions.

(*b*) A changed pH optimum for glucose 6-phosphatase: the pH-dependence of the delipidated enzyme at 80mM-glucose 6-phosphate was not significantly different from the control over pH5.8–6.5, though the decrease in activity from pH6.5–8.0 appeared less steep for the delipidated enzyme.

(*c*) Ca^{2+} inhibition: Garland & Cori (1972) stated that the use of phospholipases to study glucose 6-phosphatase was contraindicated because glucose 6-phosphatase is inhibited by Ca^{2+}, which is required for phospholipase action. In six experiments in this study, incubation of microsomal fractions for 60–245min at 5°C with 1mM-$CaCl_2$ caused negligible glucose 6-phosphatase inhibition (mean, 0.1% activation; range 5% inhibition–4% activation). On the other hand *Cl. perfringens* phospholipase C effectively hydrolyses microsomal phosphatides and inhibits glucose 6-phosphatase without added Ca^{2+}. Presumably, endogenous enzyme-bound cations support phospholipase C activity (Zwaal *et al.*, 1971; Möllby *et al.*, 1973).

(*d*) Inhibition by the products of phospholipase C action: Duttera *et al.* (1968) and Snoke & Nordlie (1972) showed that the water-soluble base phosphates formed by phospholipase C do not inhibit glucose 6-phosphatase; since their evidence appears unequivocal, this question was not reinvestigated. Both of the above authors also showed, by adding exogenous diglycerides to microsomal fractions, that these too do not inhibit glucose 6-phosphatase. However, because of possible ambiguities arising from the failure of added diglycerides to obtain access to potential inhibitory sites, we instead destroyed the diglyceride formed endogenously, by using *Rhizopus arrihizus* lipase (Boehringer Corp., London W.5, U.K.) at 5°C and removed at least 98% of the resultant fatty acids from the microsomal fractions by two washes with 30mg of bovine serum albumin/ml, separating the bovine serum albumin and microsomal fractions on a Sepharose 6B column. However, this failed to alleviate the 45% inhibition of total glucose 6-phosphatase activity which accompanied phospholipase C treatment.

(*e*) Non-specific proteolytic or other damage to glucose 6-phosphatase: in 22 experiments in which microsomal fractions were treated for 30–120min at 5° or 37°C with *Cl. perfringens* phospholipase C, in the presence of EGTA to remove the necessary cofactor for phospholipase action, the mean activity of glucose 6-phosphatase remained 97% of the corresponding zero-time controls (range 89–103%). The absence of significant non-specific inhibition was confirmed in three other ways. Microsomal fractions were treated, in the presence of free Ca^{2+} with *Cl. perfringens* phospholipase C plus the antibody (Wellcome Research Laboratories, Beckenham, Kent, U.K.) against this enzyme. Neither hydrolysis of phospholipid nor inhibition of glucose 6-phosphatase occurred. Microsomal fractions were treated in the presence of free Ca^{2+} with *Cl. perfringens* phospholipase C in the presence of 0.125mg of lysophosphatidylcholine/mg of microsomal protein. This is a very versatile control because lysophosphatidylcholine inhibits this phospholipase C competitively and by virtue of its detergent effect also stimulates glucose 6-phosphatase in the presence of 80mM-glucose 6-phosphate. It might also be expected to rupture lysosomes effectively and to facilitate access of degradative enzymes to regions of the membranes unavailable in untreated microsomal fractions but possibly available on phospholipase C treatment. Nevertheless, the activated state of glucose 6-phosphatase (157% of control) was not inhibited with phospholipase C and Ca^{2+} present for 60min at 5°C: only 0.7% of the phosphatidylcholine was hydrolysed. Finally, glucose 6-phosphatase is always inhibited substantially by pure *B. cereus* phospholipase C, though this inhibition can be prevented at 5°C with *o*-phenanthroline, which instantly inhibits this phospholipase C (though *o*-phenanthroline itself can cause up to 15% inhibition of glucose 6-phosphatase at 5°C in the absence of substrate).

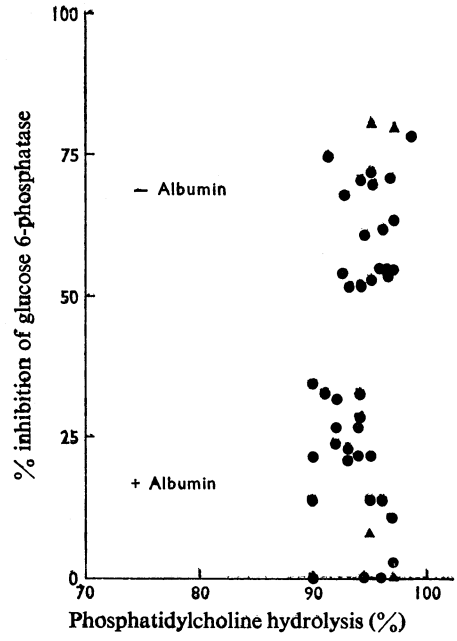

Fig. 3. *Protection of glucose 6-phosphatase by albumin during phospholipase C treatment at 5°C*

Choline-labelled microsomal fractions were treated with *Cl. perfringens* (●) or pure *B. cereus* phospholipase C (▲) at 5°C for 60–120min at a final sucrose concentration of 0.25M and in the presence and absence of 30–200mg of bovine serum albumin/ml. Phospholipid hydrolysis and glucose 6-phosphatase was assayed as in Fig. 2. In most experiments normal bovine serum albumin was used though defatted bovine serum albumin and defatted bovine serum fraction V was used in some experiments. In controls, glucose 6-phosphatase activity was 0.275–0.37μmol of glucose 6-phosphate hydrolysed/min per mg of microsomal protein.

Protection of glucose 6-phosphatase by bovine serum albumin at 5°C

With the use of sufficient phospholipase C to avoid impairing phospholipid hydrolysis (phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine were tested), microsomal fractions were delipidated in the presence of 10–200mg of bovine serum albumin/ml. This substantially protected glucose 6-phosphatase from inhibition by *Cl. perfringens* or by pure *B. cereus* phospholipase C in Fig. 3, so that in some experiments there was no detectable loss of enzyme activity compared with the untreated controls. The mean inhibition was only 18% and the maximum inhibition observed was 35%. This compared with inhibitions from 52–81% when the same preparations of microsomal fractions were phospholipase C-treated in the absence of albumin. The difference between the glucose 6-phosphatase activity remaining in microsomal fractions delipidated in the presence of albumin and those delipidated in its absence in Fig. 3, is highly significant ($t = 13.6$; $P < 0.001$).

Protection of glucose 6-phosphatase by bovine serum albumin was never seen when microsomal fractions were delipidated with any preparations of impure *B. cereus* phospholipase C, though bovine serum albumin protects just as effectively against pure *B. cereus* or *Cl. perfringens* phospholipase C. This anomalous behaviour is illustrated in Table 2. Here the same microsomal preparation was treated separately with each of the three phospholipase C preparations in the presence and absence of bovine serum albumin. Albumin still failed to protect glucose 6-phosphatase when microsomal fractions were treated with the impure *B. cereus* enzyme, which had been heated to 56°C in the presence of Ca^{2+} to destroy any residual 'direct lytic factor',

Table 2. *Effect of three different phospholipase C preparations on glucose 6-phosphatase in the presence and absence of bovine serum albumin*

Samples of [^{3}H]choline-labelled microsomal fractions were treated in the presence or absence of 100mg of bovine serum albumin/ml, for 120min at 5°C with 10 units of *Cl. perfringens* phospholipase C (1ml), 14 units of pure *B. cereus* phospholipase C (1ml) or 5 units of impure *B. cereus* phospholipase C (1ml). Phosphatidylcholine hydrolysis was determined radiochemically and glucose 6-phosphatase activity, which was 0.32μmol of glucose 6-phosphate hydrolysed/min per mg of protein in control microsomal fractions, was assayed without stopping phospholipase C action.

Phospholipase C preparation	% phosphatidylcholine hydrolysed		% inhibition of glucose 6-phosphatase	
	−Bovine serum albumin	+Bovine serum albumin	−Bovine serum albumin	+Bovine serum albumin
Cl. perfringens	96	96	79	0
Pure *B. cereus*	97	98	80	0
Impure *B. cereus*	97	98	80	80

Table 3. *Inhibition of total glucose 6-phosphatase activity by phospholipase C plus bovine serum albumin at 5°C*

In experiments denoted (A) the latency of glucose 6-phosphatase was first destroyed with the agent indicated before total enzyme activity and activity surviving 60–120min after subsequent treatment with phospholipase C+50mg of bovine serum albumin/ml at 5°C were assayed. In (B)-type experiments intact microsomal fractions (not exposed to disruptive agents) were similarly treated with phospholipase C plus bovine serum albumin and the activity remaining was compared with the total (latency fully destroyed) activity in samples from the same microsomal fractions+bovine serum albumin. Phosphatidylcholine hydrolysis was determined radiochemically ([^{3}H]choline labelling). Activities are generally expressed as μmol of glucose 6-phosphate hydrolysed/min per mg of microsomal protein, but those denoted (*) are expressed as μmol of glucose 6-phosphate hydrolysed/min per ml of assay mixture. In experiments denoted (†) both phospholipase C treatment and assay of glucose 6-phosphatase were performed at 5°C.

Experimental conditions	% phosphatidylcholine hydrolysed	Agent destroying latency	Total glucose 6-phosphatase activity	Glucose 6-phosphatase activity after phospholipase C treatment	% inhibition
Pure *B. cereus* (A)	98	Triton X-100	0.57	0.285	50
	98	Alkali	0.040†	0.0205†	49
Pure *B. cereus*+*Cl. perfringens* (A)	98	Triton X-100	0.57	0.27	53
Cl. perfringens (A)	96	Deoxycholate	1.1*	0.75*	32
	94	Triton X-100	0.57	0.38	33
	97.5	Alkali	0.40	0.206	48
(Two experiments)	91–93.5	Alkali	0.034–0.039†	0.018–0.0195†	47–50
Cl. perfringens (B)	91	Alkali	0.034†	0.0215†	37
	92	Triton X-100	0.52	0.33	36
(Seven experiments)	(90–94)		(0.46–0.60)	(0.32–0.36)	(31–44)

(Sabban *et al.*, 1972); however, phospholipase activity survived this procedure.

Different preparations of bovine serum albumin showed some variability in the protection they conferred on glucose 6-phosphatase, with the less pure plasma fraction V being least effective. Neither the presence of 1mM-dithiothreitol nor defatting nor prolonged dialysis of the bovine serum albumin enhanced its protection, but this protection appears somewhat specific to bovine serum albumin and was not shown by other proteins and macromolecules tested. Thus haemoglobin, dextran (average molecular weight 100000–200000), CM-cellulose, egg albumin, cytochrome *c* and lysozyme at 25–50mg/ml all failed to protect glucose 6-phosphatase significantly in microsomal fractions delipidated at 5°C with *Cl. perfringens* phospholipase C, nor did maintenance of a high concentration of microsomal fraction plus cell sap, about 60% of that in intact liver. Serum albumin also confers some protection against thermal inactivation of glucose 6-phosphatase after delipidation with *Cl. perfringens* phospholipase C. However, it is unable to protect the enzyme after the more extensive effects of *B. cereus* phospholipase V treatment (see below).

Effect of phospholipase C plus bovine serum albumin on the total activity of glucose 6-phosphatase

Treating microsomal fractions at 5°C with phospholipase C plus bovine serum albumin after prior destruction of glucose 6-phosphatase latency with Triton X-100, sodium deoxycholate or NH_3 caused 30–50% inhibition of glucose 6-phosphatase (Table 3). This inhibition is not caused by destabilization of the enzyme during assay at 37°C, as it was also seen when both delipidation and assay were done at 5°C, and the 37°C assay was linear with time. Nor is it caused by the agents used to destroy latency inhibiting in the delipidated state. This is clear because similar inhibition was seen when the activity remaining after direct phospholipase C treatment of microsomal fractions (whose latency was not first destroyed), was compared with the total (latency fully destroyed) activity in these particles not exposed to phospholipase C (B-type experiments, Table 3). Not unexpectedly, phospholipase C treatment seems to destroy the latency of glucose 6-phosphatase, as well as inhibiting it.

Reactivation of glucose 6-phosphatase by phospholipid dispersions after phospholipase C treatment at 5°C plus bovine serum albumin

As shown in Table 4 added phospholipid dispersions restored much more of the total activity of glucose 6-phosphatase (latency fully destroyed) when microsomal fractions were delipidated in the presence of albumin; reactivation averaged 94% (range 88–97.5%), when optimal amounts of phospholipid were used. As in Table 1, co-dispersions of phosphatidylserine plus phosphatidylcholine (1:1, w/w), reactivated very efficiently; however, Triton X-100 was not very effective. Comparison of the

Table 4. *Reactivation of glucose 6-phosphatase with phospholipid dispersions after delipidation at 5°C (+bovine serum albumin)*

Choline-labelled microsomal fractions were treated for 120min at 5°C with *Cl. perfringens* phospholipase C in the presence of 60mg of bovine serum albumin/ml. After phospholipase action had been stopped with EGTA, phospholipid dispersions were added and the activity of glucose 6-phosphatase in control microsomal fractions+phospholipid and in phospholipase C-treated microsomal fractions+phospholipid was measured. In two experiments Triton X-100 was also added to a final concentration of 0.07% to phospholipase C-treated microsomal fractions to test the efficacy of this detergent for reactivating phospholipase C-treated glucose 6-phosphatase. The total (latency fully destroyed) activity of 6-phosphatase was determined by assaying controls+bovine serum albumin in the presence of 0.07% Triton X-100. Phospholipid hydrolysis was determined radiochemically. The values in parentheses represent the ratios of phospholipid added to microsomal protein. PC, phosphatidylcholine; PS, phosphatidylserine.

	Glucose 6-phosphatase activity (% of control)		
	Expt. 1	Expt. 2	Expt. 3
Control+bovine serum albumin+phospholipid	107 (PC) 108 (PC+PS)	100 (PC+PS)	98 (PC+PS)
Control+bovine serum albumin+Triton X-100	166	148	158
Phospholipase C-treated	38	28	—
Phospholipase C-treated+bovine serum albumin	93	93	96
Phospholipase C-treated+bovine serum albumin+Triton X-100	—	94	100
Phospholipase C-treated+bovine serum albumin+phospholipid	146 (PC) 162 (PC+PS)	143	92 (0.14) 110 (0.49) 114 (0.93) 124 (2.1) 144 (3.3) 148 (4.6)
% phosphatidylcholine hydrolysed	91	93	94
Glucose 6-phosphatase activity in controls	0.35	0.36	0.38

stimulatory effects of the phospholipid dispersions on glucose 6-phosphatase in control microsomal fractions and the extent of reactivation indicates that stimulation of residual uninhibited enzyme cannot account for the reactivations seen.

The microsomal fractions in Expt. 2 were also simultaneously phospholipase C-treated without bovine serum albumin and glucose 6-phosphatase was reactivated. The restored activity without bovine serum albumin, but with the same phospholipid co-dispersion as was used when bovine serum was present, was only 96% of the partly latent activity in the control microsomal fractions. Although typical of reactivations obtained under the same conditions (without bovine serum albumin) in Table 1, this represents only 65% of the total (latency fully destroyed) activity of the enzyme, or 32% less reactivation than was elicited after delipidation (plus bovine serum albumin).

Ability of detergents to reactivate glucose 6-phosphatase after phospholipase C treatment plus bovine serum albumin

Further data relating to the question of whether detergents can substitute for phospholipids in restoring glucose 6-phosphatase activity in delipidated microsomal fractions are shown in Table 5. The detergents Triton X-100 and deoxycholate substantially stimulated glucose 6-phosphatase activity where this had been extensively inhibited by phospholipase C treatment in the absence of bovine serum albumin. Final concentrations of 0.04% Triton X-100 and 0.02% deoxycholate, which were found to be optimal, increased activity more than twofold in some cases, which is as effective as dispersions of some phospholipids under these conditions (Table 1). A final concentration of 0.07% Triton X-100 was found to be optimal after phospholipase C treatment of microsomal fractions in the presence of bovine serum albumin; however, the detergent was less efficient at restoring enzyme activity under these conditions (Tables 4 and 5) than were phospholipid dispersions (Table 4), though bovine serum albumin did not interfere with the ability of Triton to destroy the latency of glucose 6-phosphatase and stimulate it (Table 4).

Removal of fatty acids from phospholipase C-treated microsomal fractions with bovine serum albumin

The specificity of bovine serum albumin in protecting glucose 6-phosphatase on phospholipase C treatment suggested that bovine serum albumin might protect glucose 6-phosphatase by removing long-chain fatty acids. These are formed in microsomal fractions (Cater & Hallinan, 1971) and other membranes (Michell & Coleman, 1971; Michell *et al.*, 1973) by endogenous membrane lipase(s) acting on the diglyceride products formed by

Table 5. *Efficacy of detergents for reactivating glucose 6-phosphatase*

Choline-labelled microsomal fractions were treated with *Cl. perfringens* or pure *B. cereus* phospholipase C for 60–120min at 5°C. In some experiments 100mg of bovine serum albumin/ml was present. Optimum concentrations of Triton X-100 (0.04% without bovine serum albumin; 0.07%+bovine serum albumin) or of sodium deoxycholate (0.02%) were added with the glucose 6-phosphate during assay. (Standing for 10min at 5°C with detergent before the addition of glucose 6-phosphate gave the same stimulation of glucose 6-phosphatase.) Phosphatidylcholine hydrolysis was determined radiochemically: glucose 6-phosphatase activity of untreated controls was 0.275–0.35 μmol of glucose 6-phosphate hydrolysed/min per mg of protein. Results reported are means with the ranges in parentheses: the numbers of experiments performed are in parentheses in vertical column 1.

Phospholipase C preparation and conditions	% phosphatidylcholine hydrolysed	Glucose 6-phosphatase (% of control)		
		Phospholipase C-treated	Phospholipase C-treated +detergent	Control+ detergent
Cl. perfringens (+bovine serum albumin)				
Triton X-100 (4)	92 (90–93)	94 (88–98)	100 (94–115)	138 (135–140)
Cl. perfringens (No bovine serum albumin)				
Triton X-100 (4)	93 (92–94)	27 (25–32)	73 (65–80)	142 (135–153)
Deoxycholate (1)	93	32	62	176
Pure *B. cereus* (No bovine serum albumin)				
Triton X-100	96	28	49	153
Deoxycholate (1)	96	28	70	176

phospholipase C. In support of this, substantial sequestration of long-chain fatty acids from phospholipase C-treated microsomal fractions was demonstrated in 12 experiments (results not shown).

Effect of exogenous fatty acids at 5° and 37°C on glucose 6-phosphatase

To probe further the mechanism of protection of glucose 6-phosphatase by bovine serum albumin, dispersions of long-chain fatty acids were added to control microsomal fractions and their effects at 5° and 37°C on glucose 6-phosphatase were studied. Palmitate, oleate, linoleate and linolenate, present at concentrations from 0.1–0.4 μmol/mg of protein, all (separately) caused substantial inhibition of glucose 6-phosphatase when incubated at 37°C with microsomal fractions in the absence of glucose 6-phosphate (Cater & Hallinan, 1971). However, these four fatty acids, alone or in equimolar mixtures, only poorly inhibit, if preincubated with microsomal fractions at 5°C, before assay of glucose 6-phosphatase at 37°C. In 12 experiments, exposure of microsomal fractions for 30–120min at 5°C to 0.1–0.4 μmol of fatty acid/mg of protein caused 7±6% (S.D.) inhibition of glucose 6-phosphatase (range 0–18%). Inhibitions above 10% were only seen in three experiments. Further, cooling microsomal fractions to 5°C instantly stopped fatty acid inhibition of glucose 6-phosphatase in progress at 37°C, regardless of whether a small or a large proportion of the enzyme's activity had been destroyed (results not shown). Paradoxically therefore, long-chain fatty acids appear to be poor inhibitors of glucose 6-phosphatase at 5°C in microsomal fractions containing their normal phospholipid complement.

Instability of glucose 6-phosphatase in phospholipase C-treated microsomal fractions: glucose 6-phosphate protects and phosphatides restore stability

Though 80mM-glucose 6-phosphate appears to protect the enzyme completely, it was shown in 20 experiments that if microsomal fractions, delipidated at 5°C, are incubated at 37° or 20°C in the presence of physiological concentrations of glucose 6-phosphate (0.1–0.4mM) or without substrate, glucose 6-phosphatase is rapidly inactivated. This is illustrated in Fig. 4. Glucose 6-phosphatase is virtually completely inactivated if delipidated microsomal fractions are incubated for 10min at 37°C. Instability is seen regardless of which phospholipase is used and whether phospholipase C action is stopped or not (compare Fig. 1 with Fig. 4). In two experiments maintenance of a high concentration of microsomal fraction plus cell sap, about 60% of that in intact liver, failed to enhance significantly the stability of glucose 6-phosphatase on delipidation, but in five experiments ultrasonic dispersions of phosphatidylcholine or phosphatidylethanolamine plus phosphatidylserine (1:1, w/w), added after stopping phospholipase action, to microsomal fractions treated at 5°C with *Cl. perfringens* phospholipase C, substantially restored thermal stability to

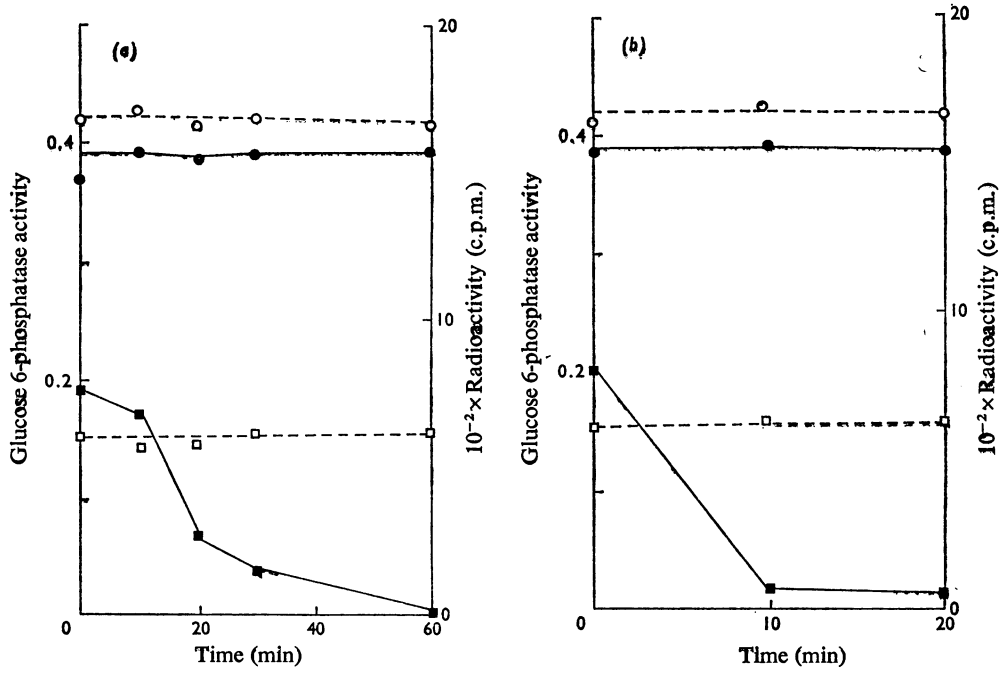

Fig. 4. *Instability of 6-phosphatase in microsomal fractions treated with phospholipase C*

Identical samples of microsomal fractions, labelled with [2-^{3}H]ethanolamine, were exposed at 5°C to 2 units of *Cl. perfringens* phospholipase C/ml; 0.004 M-EGTA was present in one case to prevent phospholipase action. The other sample was treated with active phospholipase C for 60 min. This caused 50% inhibition of glucose 6-phosphatase and 64% hydrolysis of phosphatidylethanolamine (disc array). Phospholipase action was now stopped in this sample by making it 0.0033 M with respect to EGTA and it was divided into two portions with a chilled pipette. One portion was further incubated at 20°C (*a*) and the other at 37°C (*b*), samples being withdrawn at the times indicated for assay of glucose 6-phosphatase and to confirm absence of phosphatidylethanolamine hydrolysis. No further significant hydrolysis of phosphatidylethanolamine was seen in either case. (In other experiments absence of phosphatidylcholine and phosphatidylserine and of total phospholipid hydrolysis were also demonstrated.) With the microsomal fractions exposed to inactive phospholipase C for 60 min at 5°C, subsequent incubation at either 37° or 20°C caused no significant inhibition of glucose 6-phosphatase or phosphatidylethanolamine hydrolysis. Glucose 6-phosphatase activity at zero time in the control was 0.383 μmol of glucose 6-phosphate hydrolysed/min per mg of microsomal protein. Glucose 6-phosphatase activity in the 'inactive phospholipase' controls (●); radioactive phosphatidylethanolamine in controls (○) or phospholipase C-treated samples postincubated with EGTA present (□); glucose 6-phosphatase activity in phospholipase C-treated samples postincubated at 20° or 37°C with EGTA present (■).

glucose 6-phosphatase. Only 0–20% inhibition occurred during 10 min incubation at 37°C without glucose 6-phosphate (results not shown). Thus we confirm the original finding of Beaufay & De Duve (1954*b*) that phospholipase C treatment destabilizes glucose 6-phosphatase, but are at variance with Duttera *et al.* (1968) and Zakim (1970), both of whom reported that unlike phospholipase A_2, phospholipase C treatment did not produce an unstable form of the enzyme.

Finally, none of the agents tested, including dispersions of phosphatidylcholine, lysophosphatidylcholine, deoxycholate, Triton X-100, cetyltrimethylammonium bromide, long-chain fatty acids, bovine serum albumin or cytochrome *c*, were able to restore glucose 6-phosphatase activity in microsomal fractions which had been delipidated and then incubated for 10 min at 37°C without substrate to inactivate the enzyme. Hence this inactivation must presently be regarded, at least operationally, as irreversible.

Discussion

Interpretation of the effects of phospholipid hydrolysis (delipidation) on the activity of glucose 6-phosphatase in such a complex system as microsomal fractions is difficult and demands caution.

Phospholipase C destroys the latency of lysosomal acid hydrolases (Lewis *et al.*, 1974) and also of two enzymes concentrated in the same subfraction of endoplasmic-reticular membranes as glucose 6-phosphatase (Beaufay *et al.*, 1974), namely nucleoside diphosphatase (Kuriyama, 1972) and *p*-nitrophenyl UDP-glucuronyltransferase (Berry *et al.*, 1974). It seems essential therefore to consider the effects of phospholipase C on the latency of glucose 6-phosphatase.

Latency of glucose 6-phosphatase in microsomal fractions

Glucose 6-phosphohydrolase appears to exist in a partly latent state in microsomal fractions with about 35% of the activity requiring prior disruption of the membranes for expression, though some other phosphohydrolase activities and all of the phosphotransferase activities, also attributed to glucose 6-phosphatase, exhibit much greater latency, up to 90% (Arion *et al.*, 1972*b*). Membrane disruption, allowing full expression of glucose 6-phosphatase activity, can be achieved with a variety of detergents (e.g. Arion *et al.*, 1972*a*) or by exposure to pH9.5–10.0 (Stetten & Burnett, 1966) and sonication or preincubation with pyrophosphate are also reported by Arion *et al.* (1972*b*) to destroy latency. In the present study, optimum concentrations of Triton X-100, deoxycholate or lysophosphatidylcholine, or alkali treatment, were used in 22 experiments to remove the latency of glucose 6-phosphatase; the total glucose 6-phosphate–phosphohydrolase activity averaged 154% of that in untreated control microsomal fractions, indicating a mean latency of 35% (range 26–46%). Arion *et al.* (1972*a*,*b*) seem to attribute physiological significance to the latency of glucose 6-phosphatase in microsomal fractions, whereas Gunderson & Nordlie (1973) maintain that its latency is an artifact generated when the endoplasmic reticulum is fragmented. We have not yet studied the structural basis for latency, but complete inversion of microsomal fractions, at least, seems a highly improbable cause. The enzyme is concentrated in microsomal vesicles bearing attached ribosomes (Beaufay *et al.*, 1974), where it exhibits latency (Stetten *et al.*, 1969), but ribosomes are virtually never seen located within microsomal vesicles.

Delipidation substantially inhibits several activities of glucose 6-phosphatase if these are measured after removal of latency

If microsomal fractions were treated with phospholipase C at 5°C (plus albumin) and glucose 6-phosphatase activity remaining was compared with the partly latent activity of the enzyme in untreated control microsomal fractions, extensive phospholipid hydrolysis apparently caused no inhibition in some experiments and the average inhibition was only 18% (Fig. 3). It is thought that this is caused by the enzyme being inhibited by 30–50% owing to phospholipid depletion and being simultaneously stimulated by 26–46% by destruction of its latency, so a residual fraction of the total non-latent activity of glucose 6-phosphatase is expressed in phospholipase C-treated microsomal fractions. Evidence in support of this view was obtained by comparing the activity surviving delipidation with the total activity assayed in the same microsomal fractions after the removal of latency. If microsomal fractions were delipidated at 5°C (plus albumin), after prior destruction of the latency of glucose 6-phosphatase, then 30–50% inhibition of the enzyme's total activity was seen (A-type experiments, Table 3). A similar inhibition was seen if microsomal fractions were delipidated without previously destroying latency and the activity remaining was compared with the total glucose 6-phosphatase activity (i.e. latency fully destroyed) in the same microsomal fraction (B-type experiments, Table 3). This inhibition is almost completely reversible by phospholipid dispersions, 1:1 (w/w) co-dispersions of phosphatidylcholine plus phosphatidylserine being especially effective.

Direct evidence of destruction of enzyme latency by phospholipase C treatment was readily obtained with the mannose 6-phosphate phosphohydrolase and glucosamine 6-phosphate phosphohydrolase activities of glucose 6-phosphatase, which can reportedly be more than 90% latent (Wallin & Arion, 1972; Arion *et al.*, 1972*b*). In three experiments mannose 6-phosphatase was stimulated 278–406% (mean 358%), and glucosamine 6-phosphatase was stimulated 200% by *Cl. perfringens* phospholipase C at 5°C (plus albumin). Added phospholipid dispersions further increased these activities after phospholipase C treatment, so they approached the total non-latent values assayed in the same microsomal fractions after exposure to Triton X-100 or NH_3; these were 600–700% of the highly latent activities in intact control microsomal fractions. If latency was destroyed before treatment with *Cl. perfringens* phospholipase C at 5°C (plus albumin) then total mannose 6-phosphatase activity was inhibited by 21–26% (mean 23%) and glucosamine 6-phosphatase was inhibited by 29%. There seems no possibility therefore that these activities of glucose 6-phosphatase are constrained by phospholipids. Our findings on the effects of added phospholipids on glucose 6-phosphate phosphohydrolase in control microsomal fractions also differ from those of Zakim (1970) and do not favour phospholipid constraint. Thus in 28 experiments, various phospholipids exerted no marked effect on glucose 6-phosphatase activity (less than 10% stimulation or

inhibition), whereas in 13 experiments they stimulated markedly. Only phosphatidic acid, which is a very minor component of rat liver microsomal phospholipids (Kleinig, 1970; Colbeau *et al.*, 1971), substantially inhibited glucose 6-phosphatase, inhibition being 30–40%.

Phospholipid dependence of glucose 6-phosphatase

All three phosphohydrolase activities of glucose 6-phosphatase examined in this study were further stimulated by added phospholipid dispersions after delipidation (plus albumin) and values approaching their total non-latent activity could be restored in this way. We consider that this indicates that the enzyme is at least partly phospholipid-dependent, and its complete dependence on phospholipids is not excluded. Indeed it could conceivably be dependent on a single phospholipid class, phosphatidylserine, since about 50% of this phosphatide evades hydrolysis when microsomal fractions are treated with the *B. cereus* enzyme or even with both *B. cereus* + *Cl. perfringens* phospholipases together, at 5° or 37°C.

Possible inhibition of glucose 6-phosphatase by proteolysis or by fatty acids in delipidated microsomal fractions

A much more ambiguous and poorly understood aspect of this study is the extensive inhibition of glucose 6-phosphatase when microsomal fractions are treated with phospholipase C in the absence of bovine serum albumin. This inhibition, around 60–80% of the glucose 6-phosphatase activity of untreated control microsomal fractions, exceeds that seen when the same microsomal fractions are treated in the presence of bovine serum albumin by about 50%. (Fig. 3). A possible explanation of this effect is that bovine serum albumin serves as a sacrificial substrate for microsomal proteinase(s) acting on apolar regions of membrane proteins, which are only accessible in phospholipid-depleted membranes (inhibition by proteinase contaminants in phospholipases seems to be adequately excluded). However, such an explanation does not seem to account for other proteins or cell sap failing to protect glucose 6-phosphatase significantly. It is also poorly consistent with the ability of phospholipid dispersions partly to reactivate glucose 6-phosphatase after phospholipase treatment at 5°C in the absence of bovine serum albumin. Nevertheless, it is very difficult to exclude completely a proteolytic mechanism of inhibition, especially if proteolysis of glucose 6-phosphatase was stimulated by long-chain fatty acids.

An alternative explanation attributes the extensive loss of glucose 6-phosphatase activity to mixed inhibition caused by both phospholipid depletion and long-chain fatty acids, formed by microsomal lipase(s) from the diglyceride products of phospholipase C. However, evidence for this, although suggestive, is only indirect and some points require further exploration. Thus it is unclear why bovine serum albumin fails to prevent this extensive inhibition of glucose 6-phosphatase when microsomal fractions are delipidated with impure *B. cereus* phospholipase C, even when the weight ratio of albumin/phospholipase C is 400:1 (Table 2). Presumably the impure phospholipase may contain some very active contaminant which interferes with bovine serum albumin protection. However, a much more serious difficulty for a simple 'mixed inhibition' model is the following. When added to untreated control microsomal fractions, the amount of long-chain fatty acids which accumulated during phospholipase C treatment for 60min at 5°C (<0.1 μmol/mg of microsomal protein; Cater & Hallinan, 1971) is quite insufficient alone to cause any substantial inhibition of glucose 6-phosphatase at 5°C. Therefore an additional postulate must be made that phospholipid depletion sensitizes glucose 6-phosphatase to inhibition by fatty acids. It is therefore possible that in addition to their other roles phospholipids serve non-specifically as de-inhibitors against long-chain fatty acids for glucose 6-phosphatase. This may also be how detergents can substantially reverse the large inhibition of glucose 6-phosphatase caused by phospholipase C in the absence of bovine serum albumin (when fatty acids accumulate in the membrane), though they fail to reverse effectively the smaller inhibition caused by phospholipase C in the presence of bovine serum albumin.

Could phospholipase C inhibit phospholipid-dependent enzymes without destroying the specific phosphatides that they require?

It has been implicitly assumed that the action pattern of phospholipases against individual phospholipid classes, together with the effectiveness of different phosphatides in restoring the reactivity of a phospholipid-dependent enzyme after delipidation, would provide a reliable guide to the specificity of that enzyme's phospholipid requirement. By these criteria glucose 6-phosphatase exhibits a fairly general non-specific phospholipid dependence, which can be satisfied, at least in part, by phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol, with 1:1 (w/w) mixtures of phosphatidylserine plus phosphatidylcholine or plus phosphatidylethanolamine restoring activity most effectively. We wish to conclude this report by considering whether these criteria will necessarily give an accurate indication of the phospholipid requirement of an enzyme which might depend on

a relatively minor component of its phospholipid environment.

In microsomal fractions (Trump *et al.*, 1970; Finean & Martonosi, 1965) and many other membranes also, phospholipase C treatment not only cleaves the base phosphate from the membrane phospholipids but also leads to the relocation of their apolar moieties, which self-associate in large lipid droplets on the membrane periphery. It must be presumed that such a major rearrangement would generate a large number of apolar interaction sites on intrinsic membrane proteins, which were formerly occupied by phospholipids and are only partly satisfied by the increased apolar protein–protein interaction coincident with lateral contraction of the membrane (Coleman *et al.*, 1970). In a membrane where 75% of the phospholipids have been hydrolysed, as when microsomal fractions are treated at 5°C with *Cl. perfringens* phospholipase C, such sites might be sufficiently abundant to cause a significant competition for and relocation of the residual phospholipids. For relatively minor phosphatides such as phosphatidylserine, which constitutes 2–4% of the phospholipids in microsomal fractions (Gurr *et al.*, 1965; McMurray & Dawson, 1969; Kleinig, 1970) but is not significantly hydrolysed by *Cl. perfringens* phospholipase C at 5°C (B. R. Cater, P. Trivedi & T. Hallinan, unpublished work), the frequency of weak interactions with 'competitor sites' might be such as to decrease interaction with some phosphatidylserine-dependent enzymes below the minimum needed to fully satisfy the latter's phospholipid requirements. Partial inhibition of those enzymes with least affinity for phosphatidylserine would then be seen, although none of their essential phosphatide had in fact been hydrolysed. In such a situation, phosphatidylserine would presumably interact less strongly with the 'competitor sites' than with its 'natural' interaction sites, since the 'competitor sites' would normally accommodate other phospholipids. Hence, in a reactivation experiment, when phosphatidylcholine dispersion was added in large amounts, this could fully restore the activity of phosphatidylserine-dependent enzymes, simply by occupying 'competitor sites' and removing their competition for phosphatidylserine.

In the light of the discussion above, we wish to reserve judgement on the true specificity of the phospholipid-requirement of the several part-reactions of glucose 6-phosphatase, until it is possible to hydrolyse acidic phosphatides of microsomal fractions more extensively, without inactivating this very labile enzyme.

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