Br. J. Pharmac. (1979), **66**, 167–174

PHARMACOLOGICAL EFFECTS OF PHOSPHATIDYLSERINE LIPOSOMES: REGULATION OF GLYCOLYSIS AND ENERGY LEVEL IN BRAIN

E. BIGON, E. BOARATO*, A. BRUNI, A. LEON* & G. TOFFANO*

Institute of Pharmacology, University of Padova, Largo Meneghetti, 2, 35100 Padova, Italy, and Fidia Research Laboratories*, Abano Terme, Italy

1 The accumulation of glucose in the brain produced by the administration of phosphatidylserine liposomes into mice has been studied by measurement of the cerebral contents of glycolytic intermediates and high-energy compounds.

2 With a normal supply of oxygen to the brain, inhibition of glycolysis is indicated mainly at the phosphofructokinase step. The ratio of glucose-6-phosphate to fructose-1,6-diphosphate increased, whereas the levels of pyruvate and especially lactate decreased.

3 Under conditions of cerebral ischaemia, the administration of phosphatidylserine delays glycogen mobilization and ATP use. As a consequence of decreased energy utilization, the brain adenylate energy charge remains at a high level.

4 It is concluded that the phosphatidylserine-induced glucose accumulation in the brain is due to reduced energy expenditure and therefore to a decrease in carbohydrate consumption. The inhibition of glycolysis by the high level of adenylate energy charge is probably the control mechanism explaining the decreased carbohydrate utilization.

Introduction

Previous studies (Bruni, Leon & Boarato, 1976a; Bruni, Toffano, Leon & Boarato, 1976b) have shown that mixed brain phospholipids produce hyperglycaemia and accumulation of glucose in the brain. Phosphatidylserine alone reproduces these effects. In order to be effective, the phospholipids have to be injected intravenously and the size of their hydrated bilamellar structure (multilayer liposomes) has to be decreased by sonication. This procedure of administration has been useful in the detection of increased turnover of brain catecholamines (Toffano, Leon, Benvegnù, Boarato & Azzone, 1976; Leon, Benvegnù, Toffano & Massari, 1978) and the release of acetylcholine from rat brain cortex (Mantovani, Pepeu & Amaducci, 1976).

Pharmacological effects on brain glucose metabolism have been extensively investigated in small animals since it is possible to freeze the brain at sufficient speed to detect significant modifications of glycolytic intermediates. Investigations have been made after administration of barbiturates, general volatile anaesthetics, analgesics, and adrenoceptor agonists (Lowry, Passonneau, Hasselberger & Schulz, 1964; Brunner, Passonneau & Molstad, 1971; Nilsson & Siesjö, 1974; Tyce, 1976). Particular interest is connected with detection of changes in metabolic rates as they provide information on drug effects on regulatory mechanisms. For this purpose a convenient useful method is the 'closed system' introduced by Lowry *et al.* (1964). By the production of an abrupt transition from aerobic to anoxic conditions, brain glycolysis is stimulated several fold and glucose, glycogen, phosphocreatine and adenosine triphosphate (ATP) utilized as sources of energy. Their rate of utilization is dependent upon the concentration of critical compounds which reflect the metabolic requirement of the brain cell. Among these, the adenine nucleotides are of special interest as they are capable of regulating cellular energy production or utilization. The phosphorylation potential (Krebs, 1973) or the adenylate energy charge (Atkinson, 1968) both involving adenine nucleotides, have been proposed as important regulatory factors. The effect of phosphatidylserine liposomes under these conditions is described in this paper. Since phosphatidylserine administration is accompanied by efflux of adrenaline from the adrenal medulla (Bruni *et al.*, 1976b), experiments have been carried out with adrenaline in order to distinguish between the catecholamine-mediated and the direct effect of phospholipid. An additional control is provided by the administration of a concentrated solution of glucose in an amount sufficient to increase the

0007-1188/79/060167-08 $01.00

© Macmillan Journals Ltd 1979

blood and brain levels in the absence of other pharmacological stimuli.

Methods

Drugs and reagents

Phosphatidylserine sodium salt was prepared from bovine brain as described by Lees (1957). Samples with low calcium content (approx. 0.01 μmol/μmol phospholipid) were used for preference because of the higher activity. Bovine brain phosphatidylethanolamine, phosphatidylinositol and phosphatidylcholine were obtained from General Biochemical, bovine heart diphosphatidylglycerol from Sigma. Synthetic phosphatidylcholines or phosphatidylglycerols were kindly donated by Dr P.W.M. Van Dijck (Dept. of Biochemistry, University of Utrecht). All phospholipids used in this study gave a single spot in thin layer chromatography with either a two-dimensional (Rauser, Kritchevsky, Yamamoto, Simon, Galli & Bauman, 1969) or a one-dimensional (Skipski & Barclay, 1969) system. Known amounts of phospholipid dissolved in organic solvents were carefully taken to dryness under a stream of nitrogen and the bilamellar organization was allowed to form by addition of an appropriate amount of 50 mM Tris HCl pH 7.8. The size of liposomes was then reduced by sonication at 0°C with a Branson sonifier (bursts of 2 min followed by cooling periods for a total time of 8 min). Phosphatidylcholines required a longer period of sonication to yield a transparent dispersion. The pH of the sonicated dispersion was carefully maintained at neutrality. When required, titanium dust was removed by centrifugation. Adrenaline was used as bitartrate, NADH (Sigma) was dissolved in 0.1 M carbonate buffer and stored at −30°C. NADH and ATP (Sigma) concentrations were frequently tested by spectrophotometric determinations at 340 and 259 nm respectively and by enzymatic analysis. Other reagents were from Sigma or Boehringer.

Treatment of animals

Young, fed male albino mice of 22 to 27 g were used throughout. Approximately 0.25 ml phospholipid dispersion was injected into the tail vein and the animals immersed quickly in liquid nitrogen 30 min later (unless stated otherwise). The control group received a corresponding amount of 50 mM Tris HCl pH 7.4. In order to produce brain anoxia, the animals were decapitated and the heads immersed in liquid nitrogen after the time-interval desired (usually 15 s) (Lowry *et al.*, 1964). The brain hemispheres were removed from the frozen heads and powdered with continuous addition of liquid nitrogen, together with 0.5 ml frozen 0.66 N perchloric acid. The samples were allowed to warm to 0°C and after determination of the weight, the concentration of perchloric acid was reduced to 0.33 N. After centrifugation, the clear supernatants were supplemented with 1.0 mM disodium edetate (EDTA) to remove traces of calcium and neutralized with 2 M $KHCO_3$. The insoluble material was then removed by centrifugation.

Analytical procedures

Glucose, lactate, ATP, ADP, AMP, phosphocreatine and creatine were determined by the enzymatic spectrophotometric procedures based on the oxidation of NADH or the reduction of NAD in coupled systems. Owing to their low concentration, glucose-6-phosphate, fructose-1,6-diphosphate, and pyruvate were determined by fluorimetric procedures (Lowry *et al.*, 1964; Lowry & Passonneau, 1972). Substrate-free bovine serum albumin was added to the incubation mixtures to prevent denaturation of diluted enzymes. Glycogen was determined as glucose after hydrolysis of the perchloric acid insoluble sediment in 1 N HCl for 2 h at 100°C (Lowry *et al.*, 1964). After hydrolysis, the suspension was neutralized with 2 M $KHCO_3$, supplemented with active charcoal powder and centrifuged. The cytosolic NAD/NADH ratio was calculated from the pyruvate/lactate ratio (Krebs, 1973). The brain energy state was expressed as adenylate energy charge according to Atkinson (1968). Protein was determined according to Lowry, Rosebrough, Farr & Randall (1951).

Results

Brain metabolism

Among several natural and synthetic phospholipids only phosphatidylserine increased the level of glucose in the blood and in the brain with consistent enhancement of brain/blood glucose ratio (Table 1). This effect of phospholipid was obtained at 10 to 20 mg/kg which corresponded to 0.013 to 0.026 mmol/kg on the basis of phosphorus content. When the negative polar head of phospholipids was formed by phosphorylinositol, diphosphorylglycerol and phosphorylglycerol the activity was not obtained, regardless of the length and saturation of acyl-chains. Isoelectric phospholipids were likewise inactive. These results demonstrated the prominent role of the specific configuration of the phosphatidylserine polar head and precluded certain non-specific effects due either to liposomal size, presence of negative charges, or to damaged brain circulation.

The comparison between the effects of administered phosphatidylserine, adrenaline and glucose on the

level of glycolytic intermediates with normal cerebral blood supply is shown in Table 2. The considerable effect of phosphatidylserine on glucose content (almost 200% increase) was decreased at the level of glucose-6-phosphate and reversed on fructose-1,6-diphosphate. Pyruvate and especially lactate concentrations were decreased. The large increase in the glucose/glucose-6-phosphate ratio and glucose-6-phosphate/fructose-1,6-diphosphate ratio indicated that hexokinase and phosphofructokinase limited the input of glucose.

From the concentration of pyruvate and lactate, a high value of cytosolic NAD/NADH ratio was calculated. This was taken as an indication (Krebs, 1973) of unimpeded availability of oxygen and that an elevated phosphorylation of the cytosolic adenine nucleotide system was associated with the phosphatidylserine-induced effect. Modifications similar to those produced by the phospholipid were observed after glucose administration. This confirmed that in the phosphatidylserine group, the input of glucose exceeded energy requirement. After phosphatidylserine treatment the NAD/NADH ratio was increased whereas decreased activation of glycogen synthase was more evident after exogenous glucose administration. In contrast, administration of adrenaline produced significant changes only in glucose and lactate concentrations. A slight increase in glycogen content suggested that in this case part of the excess glucose was converted to glycogen.

As expected in view of previous experiments with anaesthetics (Nilsson & Siesjö, 1974), little change was detected in the content of cerebral high-energy compounds (Table 3). In this case it is especially difficult

Table 1 Specificity of phosphatidylserine

Phospholipid (50 mg/kg)	*Blood glucose (μmol/ml)*	*Brain glucose (μmol/g wet wt.)*	*Brain/blood ratio*
None (14)	7.91 ± 0.19	0.94 ± 0.04	0.12
Brain phosphatidylserine (prepared) (10)	13.34 ± 0.68	3.69 ± 0.16	0.28
Brain phosphatidylserine (commercial) (4)	12.28 ± 1.15	4.31 ± 0.32	0.35
Brain phosphatidylethanolamine (5)	7.82 ± 0.33	1.04 ± 0.19	0.13
Brain phosphatidylinositol (4)	6.13 ± 0.36	1.17 ± 0.07	0.19
Brain phosphatidylcholine (4)	8.00 ± 0.48	0.96 ± 0.09	0.12
Heart diphosphatidylglycerol (4)	7.47 ± 0.42	0.89 ± 0.13	0.12
Synth. dimyristoylphosphatidylglycerol (2)	7.04 ± 0.24	1.16 ± 0.24	0.16

Other synthetic phospholipids tested in groups of four animals and found inactive were dipalmitoylphosphatidylglycerol, dielaidoylphosphatidylglycerol, dilauroylphosphatidylcholine, dielaidoylphosphatidylcholine, dipalmitoylphosphatidylcholine. Mean ± s.e. mean; number of determinations given in parentheses. Bovine brain phosphatidylserine was prepared according to Lees (1957), a commercial sample from General Biochemicals was used for comparison.

Table 2 Effects of phosphatidylserine (PS) on glycolytic intermediates under aerobic conditions

Substrate (μmol/g wet wt.)	*Control*	*PS*	*Adrenaline bitartrate*	*Glucose*
Glycogen	3.014 ± 0.100	2.994 ± 0.093	3.307 ± 0.125	2.437 ± 0.090*
Glucose	1.668 ± 0.075	4.471 ± 0.234*	3.654 ± 0.163*	2.961 ± 0.216*
Glucose-6-phosphate	0.067 ± 0.002	0.085 ± 0.005*	0.067 ± 0.003	0.084 ± 0.003*
Fructose-1,6-diphosphate	0.130 ± 0.008	0.097 ± 0.006*	0.128 ± 0.010	0.092 ± 0.007*
Pyruvate	0.139 ± 0.008	0.122 ± 0.006	0.139 ± 0.006	0.111 ± 0.008
Lactate	2.387 ± 0.121	1.553 ± 0.061*	2.092 ± 0.146*	1.785 ± 0.074*
Glucose-6-P/fructose-1,6-P ratio	0.51	0.88	0.52	0.91
Cytosolic NAD/NADH ratio	524	708	598	560

Forty mg/kg sonicated liposomes of bovine brain phosphatidylserine was given intravenously; 0.1 mg/kg adrenaline bitartrate or 0.3 ml 20% glucose per mouse was given subcutaneously. The mice were killed 30 min later by rapid immersion of the entire body in liquid nitrogen. Mean ± s.e. mean of 6 to 30 determinations. *P* values of difference between treated and control animals determined by Student's *t* test. * Indicates $P < 0.01$.

to distinguish between absolute changes and variation in metabolic rate during the few seconds of anoxia preceding brain freezing. It should be stressed that these determinations did not allow us to distinguish between brain cell compartments, particularly the relative distribution of ATP between the cytosolic and mitochondrial pools. Considering that the rate of phosphocreatine use was not modified by phosphatidylserine treatment (see below), the increased level of this high energy compound probably reflected a real change *in vivo*.

Changes in brain energy state and in the relative metabolic rates were clearly manifest when Lowry's closed system (Lowry *et al.*, 1964) was used to induce 15 s of ischaemia (Table 4). Under these conditions stimulation of glycolysis was obtained in all groups of animals. The high initial level of glucose produced by phosphatidylserine, adrenaline and glucose administration sustained a higher concentration of glucose-6-phosphate and fructose-1,6-diphosphate. The ratio between these two intermediates rapidly approached an almost uniform value. The possibility of using more glucose to meet energy requirements was reflected in the cases of adrenaline and of glucose administration by the maintenance of high lactate phosphocreatine and ATP levels and by the decreased produc-

Table 3 Effects of phosphatidylserine (PS) on brain high-energy compounds under aerobic conditions

Substrate (*μmol/g wet wt.*)	*Control*	*PS*	*Adrenaline bitartrate*	*Glucose*
ATP	2.301 ± 0.038	2.340 ± 0.068	2.241 ± 0.047	2.445 ± 0.040
ADP	0.756 ± 0.015	0.641 ± 0.025*	0.756 ± 0.029	0.710 ± 0.024
AMP	0.199 ± 0.013	0.151 ± 0.014	0.169 ± 0.014	0.145 ± 0.016
P-Creatine	2.107 ± 0.063	2.576 ± 0.069*	2.215 ± 0.152	2.439 ± 0.067*
Creatine	8.365 ± 0.172	7.843 ± 0.183	8.573 ± 0.243	7.791 ± 0.200
Total adenylate	3.256	3.132	3.166	3.300
Adenylate energy charge	0.82	0.85	0.83	0.85

Experimental conditions described in Table 2. The adenylate energy charge was calculated (Atkinson, 1968) from ATP + 0.5 ADP/ATP + ADP + AMP. Mean ± s.e. mean of 6 to 20 determinations. * Indicates $P < 0.01$ with respect to control group.

Table 4 Effects of phosphatidylserine (PS) on glycolytic intermediates and high energy compounds after anoxia

Substrate (*μmol/g wet wt.*)	*Control*	*PS*	*Adrenaline bitartrate*	*Glucose*
Glycogen	2.291 ± 0.088	2.868 ± 0.119*	2.233 ± 0.183	1.715 ± 0.121*
Glucose	0.457 ± 0.025	3.228 ± 0.172*	1.806 ± 0.116*	1.107 ± 0.124*
Glucose-6-phosphate	0.039 ± 0.002	0.071 ± 0.003*	0.062 ± 0.003*	0.053 ± 0.005
Fructose-1,6-diphosphate	0.084 ± 0.005	0.170 ± 0.009*	0.161 ± 0.007*	0.114 ± 0.007*
Pyruvate	0.167 ± 0.008	0.175 ± 0.005	0.208 ± 0.007*	0.163 ± 0.010
Lactate	5.464 ± 0.165	4.275 ± 0.143*	5.953 ± 0.242	5.208 ± 0.109
ATP	1.760 ± 0.035	2.145 ± 0.091*	1.976 ± 0.036*	1.968 ± 0.085
ADP	1.001 ± 0.018	0.782 ± 0.032*	0.917 ± 0.017*	0.867 ± 0.028*
AMP	0.454 ± 0.018	0.214 ± 0.015*	0.311 ± 0.022*	0.321 ± 0.022*
P-creatine	0.743 ± 0.031	1.277 ± 0.051*	0.928 ± 0.022*	1.046 ± 0.027*
Creatine	10.630 ± 0.256	9.826 ± 0.330	10.148 ± 0.296	9.513 ± 0.270*
Glucose-6-P/fructose-1,6-P ratio	0.46	0.42	0.38	0.46
Cytosolic NAD/NADH ratio	275	369	315	282
Total adenylate	3.215	3.141	3.204	3.156
Adenylate energy charge	0.70	0.81	0.76	0.76

Experimental conditions described in Table 2. After treatment with phosphatidylserine, adrenaline and glucose the mice were decapitated and the heads left 15 s at room temperature before immersion in liquid nitrogen (Lowry's closed system). Mean ± s.e. mean of 6 to 20 determinations. * Indicates $P < 0.01$ with respect to control group.

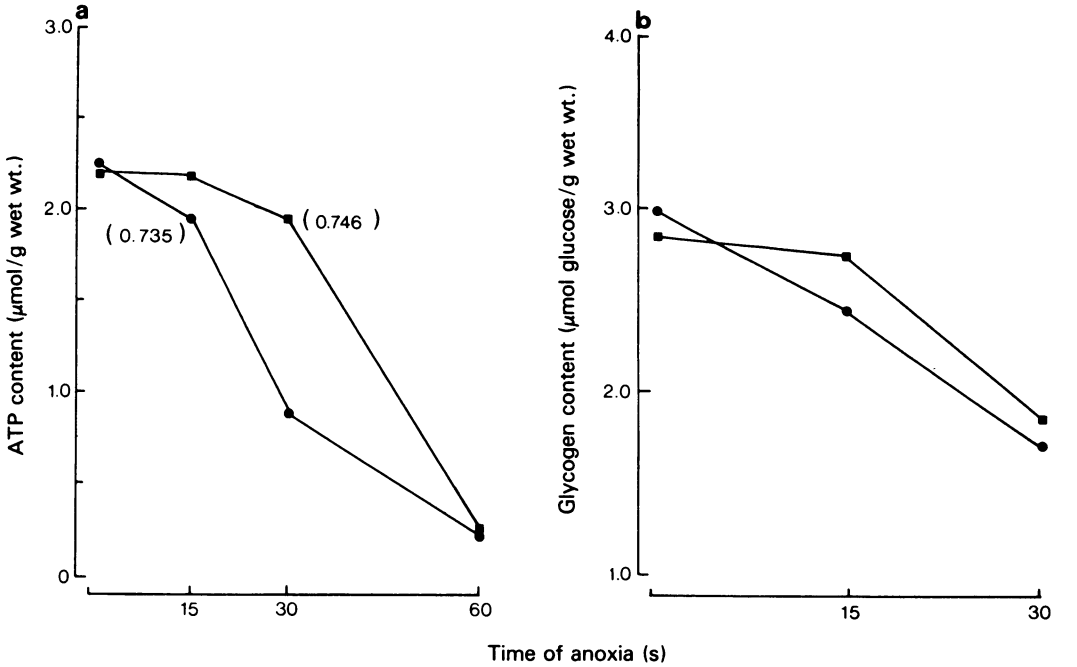

Figure 1 Effect of phosphatidylserine (PS) treatment on the utilization of (a) ATP and (b) glycogen reserves in anoxia: (■) PS-treatment; (●) control. Experimental conditions as in Table 4. After decapitation, the heads were kept at room temperature for the indicated time before immersion in liquid nitrogen. The numbers in parentheses refer to the value of adenylate energy charge at specified time of anoxia.

tion of ADP and AMP. This resulted in a sustained adenylate energy charge. By contrast, in the animals treated with phosphatidylserine the levels of glucose and glycogen were significantly higher but the level of lactate was lower, showing that in this case the rate of glycolysis was not stimulated as in the other groups. In spite of lesser carbohydrate utilization and lactate formation, the concentration of ATP was maintained and that of ADP and AMP greatly reduced. The value of adenylate energy charge was in this case the highest recorded.

Large changes in the [ATP] [AMP]/[ADP]^2 ratio were not detected among the groups, indicating that the equilibrium of adenylate kinase reaction was not altered. As a result of anoxia the [ATP] [creatine]/[ADP] [phosphocreatine] ratio was increased by 90 to 100% in all groups. Total adenylates remained constant indicating that activation of AMP-deaminase did not ensue.

Metabolic rates could be calculated from the difference between the levels of metabolites at the onset (Tables 2 and 3) and at the end of anoxia (Table 4). The most striking modifications were observed in the case of glycogen and ATP utilization which were reduced by 82 and 64% respectively by the administration of phosphatidylserine. At the same time, the AMP and the ADP formation was 75 and 42% inhibited.

In order to exclude the possibility that the inhibition of glycogen and ATP utilization was due to a direct influence of phosphatidylserine on the relative enzymatic steps, the period of anoxia was prolonged. The experiment shown in Figure 1 demonstrated that the reaction sequences were not affected since ATP and glycogen were also promptly utilized after phosphatidylserine treatment when the energy state of brain required their mobilization. At the onset of the rapid ATP decline the adenylate energy charge was about the same in both control and phosphatidylserine-treated animals. This showed that the initial high value of adenylate energy charge and no phosphatidylserine was responsible for the delayed ATP and glycogen utilization.

Interaction with blood constituents

During the investigation it became clear that those preparations of phosphatidylserine containing a high amount of calcium (approx. 0.25 μmol/μmol phospholipid) showed considerably less activity. The possibility was considered that the bound cation prevented the phospholipid from forming a metal-lipid complex in the blood. The alteration of blood ionic equilibria and particularly the variation in the content of calcium or magnesium could become relevant after the intravenous injection of phosphatidylserine. Indirect

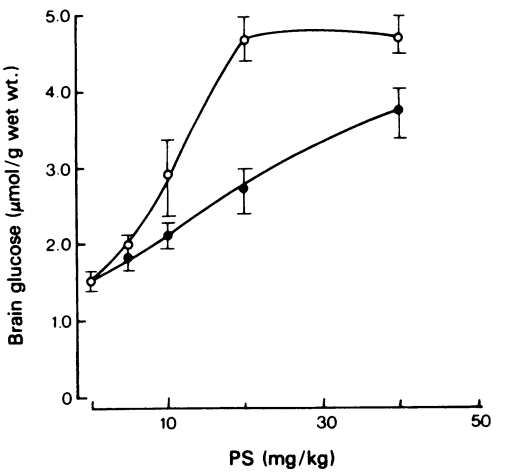

Figure 2 Activation of phosphatidylserine by rat serum. Liposomes were prepared in Tris HCl buffer (●) or rat serum (○) as described in Table 5 and injected intravenously into groups of five mice.

evidence against this possibility came from the observation that other acidic phospholipids were inactive (Table 1). As shown in a number of previous papers (Dawson & Hauser, 1970; Rand & Sengupta, 1972) all acidic phospholipids bind divalent cations in an amount and with an affinity which is dependent upon the net negative charge on their polar head. Since the amino group of phosphatidylserine becomes deprotonated at pH 9, this phospholipid at blood pH behaves like phosphatidylglycerol and phosphatidylinositol. In addition, it was found that the intravenous administration of up to 0.04 mmol/kg of EGTA or EDTA did not reproduce the effect of phosphatidylserine. Further experiments pertaining directly to this possibility are summarized in Table 5. When the liposomes of calcium-free phosphatidylserine were prepared in the presence of an equimolar concentration of magnesium to saturate the cation binding sites, the phospholipid retained full activity. In contrast, the addition of calcium severely reduced the effect of phosphatidylserine. The ineffectiveness of magnesium was consistent with the lower ability of this cation to influence the thermotropic phase transition of phosphatidylserine and the structure of relative liposomes (Papahadjopoulos, Vail, Newton, Nir, Jacobson, Poste & Lazo, 1977). With calcium, gross alteration of liposomal structure was clearly manifested by aggregation and precipitation. For this reason, no conclusion could be drawn as the effect of phosphatidylserine is known to require a well-dispersed lamellar structure (Bruni *et al.*, 1976a). In order to reproduce as closely as possible the conditions *in vivo*, the interaction with the cations was also studied in blood serum (rat and mouse). In this case the presence of equimolar concentrations of either calcium or magnesium failed to modify the effect of phosphatidylserine. The addition of calcium under these conditions was not followed by extensive liposome aggregation, indicating that the calcium-phosphatidylserine interaction was modified by the

Table 5 Interaction of phosphatidylserine (PS) with divalent cations or serum protein

Preparation injected	*Dose (mg/kg)*	*Brain glucose (μmol/g wet wt.)*	*Variation*
None	—	1.7 ± 0.05	—
PS in Tris buffer	40	4.4 ± 0.21	+2.7
PS in Tris buffer plus Mg^{2+}	40	4.5 ± 0.18	+2.8
PS in Tris buffer plus Ca^{2+}	40	2.4 ± 0.11	+0.7
PS in rat serum	40	4.5 ± 0.12	+2.8
PS in rat serum plus Mg^{2+}	40	4.6 ± 0.15	+2.9
PS in rat serum plus Ca^{2+}	40	4.3 ± 0.18	+2.6
PS in Tris buffer	20	2.6 ± 0.09	+0.9
PS in rat serum	20	4.2 ± 0.12	+2.5
PS in dialysed rat serum	20	4.8 ± 0.15	+3.1
PS in fractionated rat serum	20	5.0 ± 0.11	+3.3

Liposomes were prepared (see methods) from 8 mM phosphatidylserine in 50 mM Tris HCl buffer pH 7.8 or in blood serum from male albino rats (pH 7.8, protein concentration 100 mg/ml). When indicated, a sufficient amount of $CaCl_2$ or $MgCl_2$ was added to reach an equimolar ratio of cation to phosphatidylserine.

Dialysed rat serum: rat serum was dialysed 24 h at 2°C with a change at 5 h against 100 volumes of 50 mM Tris HCl, pH 7.8. Fractionated rat serum: rat serum was fractionated with solid ammonium sulphate at 0°C and the fraction between 30 and 60% saturation was collected in Tris buffer and dialysed as above; final protein concentration, 50 mg/ml. Mean ± s.e. mean of 10 determinations.

presence of blood protein (cf. Juliano, Kimelberg & Papahadjopoulos, 1971).

Since these experiments did not support a role of blood divalent cations in the effect of phosphatidylserine, it was concluded that the reduced activity observed in the phospholipid preparations containing calcium was due to the change induced by this cation on the properties of liposomes (Papahadjopoulos *et al.*, 1977). The shift in the thermotropic phase transition to higher temperatures and the charge neutralization may result in a large modification of liposome distribution and interaction with protein.

That the formation of a phosphatidylserine–protein complex might become a necessary condition for the liposome activity was indicated by the observation that injection of a small amount of the phospholipid sonicated in rat serum strongly increased the activity (Table 5). The same activation was obtained after the preparation of liposomes in Tris buffer and a subsequent short incubation (10 min at 37°C) with rat serum. Only negligible activation followed the preparation of phosphatidylserine liposomes in mouse serum.

Separate controls showed that rat serum injected alone did not produce any change in the brain metabolism of mice. Prolonged dialysis or fractionation with ammonium sulphate did not decrease the activation, strongly implicating the protein components of serum or a component closely associated with protein. Other phospholipids (phosphatidylcholine, phosphatidylinositol, diphosphatidylglycerol) subjected to the conditions described in Table 5 remained without activity. The efficiency of rat serum in the enhancement of the phosphatidylserine effect at brain level indicated that a limited activation reduced the effectiveness of the phospholipid in mice. Accordingly, when the liposomes were prepared in rat serum, the effect of phosphatidylserine was fully manifest at surprisingly low doses (Figure 2).

Discussion

When the glucose supply to the brain is in excess of energy demand, efficient control at phosphofructokinase and hexokinase steps prevents unnecessary input of substrates into the glycolytic system (Lowry *et al.*, 1964). The modifications induced by phosphatidylserine on brain carbohydrate metabolism under aerobic conditions are consistent with inhibition of glycolysis occurring at these control points. Thus, the main effect of phosphatidylserine seems to produce an imbalance between the carbohydrate supply and the energy requirement of brain tissue. Cerebral glucose accumulation follows as a consequence of diminished carbohydrate consumption. Hexokinase inhibition might result from the elevated levels of glucose-6-phosphate. Although the concentration of this substrate is apparently not greatly increased, it is likely that the brain sampling method was not rapid enough to avoid a significant reduction of its actual value.

The inhibition at phosphofructokinase might result from an increased ratio between the concentration of substrates producing inhibition (ATP, phosphocreatine) and those producing activation (AMP, ADP, fructose-1,6-diphosphate) since all of the former tended to be increased and the latter decreased. Citrate levels were not determined in this study. The imbalance between glucose intake and energy demand in the case of phosphatidylserine is further indicated by the results showing similar changes after administration of exogenous glucose. In contrast, the adrenaline-induced hyperglycaemia does not result in significant modifications of the concentrations of brain glycolytic intermediates, suggesting that in this case the greater intake of glucose is in equilibrium with the activity of phosphofructokinase. This is especially important because it shows that the phospholipid effect on the adrenal medulla does not contribute to the modification of brain metabolism. This is consistent with the finding that increase of brain glucose content also occurred when the phosphatidylserine-induced hyperglycaemia was prevented by the administration of the adrenoceptor antagonist, dihydroergotoxine (see also Bruni *et al.* 1976a, b). Under anoxic conditions, complete mobilization of brain carbohydrate reserves is delayed by phosphatidylserine treatment. Calculation of the anoxic rates of glycogen plus glucose mobilization gives values corresponding to 11.7 and 10.3 μmol glucose min^{-1} g^{-1} wet wt. in animals treated with adrenaline and glucose, although only a fraction appeared as lactate. The corresponding value for mice receiving phosphatidylserine is 5.5, a decrease of approximately 50%. The evaluation of adenylate energy charge indicates that this decrease in carbohydrate mobilization is due to decreased ATP expenditure. The energy charge of the adenylate pool remains for an appreciable period of time at a level which does not greatly activate energy-producing reactions. The 75% decrease in AMP production was sufficient to reduce the stimulation of carbohydrate metabolism by this regulator.

In accordance with a lower energy demand, it was observed that the phosphatidylserine-treated animals were less active than controls. The measurement of rectal temperature during the time of the experiment showed a decrease from the initial 39°C to 37.5°C. This limited fall in body temperature was unlikely to modify brain metabolism (Brunner, *et al.*, 1971). It was thought to have resulted rather from the lower physical activity. Accordingly, the phosphatidylserine effect was not prevented by appropriate warming.

Since the massive entrance of sonicated liposomes

into the brain has not been demonstrated (Tyrell, Heath, Colley & Ryman, 1976; Steger & Desnick, 1977), it is an open question how phosphatidylserine liposomes can influence brain metabolism. The increased activity observed upon incubation of liposomes with rat serum suggests that an activation step occurs in the blood before the pharmacological stimulus reaches the central nervous system. Although firm evidence is available only for rats and mice, it is apparent that the extent of activation varies in the animal species. The activation of phosphatidylserine may result from the formation of an active lipoprotein complex or from interaction with a lipid transporting system. A metabolic transformation of phosphatidylserine is also possible since phospholipase activity has been detected in rat serum (Paysant, Bitran, Etienne & Polonovski, 1969). These possibilities are at present under investigation.

We are grateful to Dr P. Gonzato (Fidia Res. Lab.) for calcium analysis of phosphatidylserine preparations.

References

ATKINSON, D.E. (1968). The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifier. *Biochemistry*, **7**, 4030–4034.

BRUNI, A., LEON, A. & BOARATO, E. (1976a). Effect of polar lipids on cerebral content of free glucose in mice. *Adv. exp. Med. Biol.* **72**, 271–283.

BRUNI, A., TOFFANO, G., LEON, A. & BOARATO, E. (1976b). Pharmacological effects of phosphatidylserine liposomes. *Nature, Lond.* **260**, 331–333.

BRUNNER, E.A., PASSONNEAU, J.V. & MOLSTAD, C. (1971). The effect of volatile anaesthetics on levels of metabolites and on metabolic rate in brain. *J. Neurochem.*, **18**, 2301–2316.

DAWSON, R.M.C. & HAUSER, H. (1970). Binding of calcium to phospholipids. In *Calcium and Cellular Function*. ed. Cuthbert, A.W., pp 17–41. London: MacMillan.

JULIANO, R.L., KIMELBERG, H.K. & PAPAHADJOPOULOS, D. (1971). Synergistic effect of a membrane protein (Spectrin) and Ca^{2+} on the Na^{+} permeability of phospholipid vesicles. *Biochim. biophys. Acta*, **241**, 894–905.

KREBS, H.A. (1973). Pyridine nucleotides in rate control. *Symp. Soc. exp. Biol.*, **27**, 299–318.

LEES, M.B. (1957). Preparation and analysis of phosphatides. *Meth. Enzymol.* **3**, 328–345.

LEON, A., BENVEGNÙ, D., TOFFANO, G. & MASSARI, P. (1978). Effect of brain cortex phospholipids on adenylate-cyclase activity of mouse brain. *J. Neurochem.* **30**, 23–26.

LOWRY, H.O. & PASSONNEAU, J.V. (1972). A Flexible System of Enzymatic Analysis. New York, London: Academic Press.

LOWRY, O.H., PASSONNEAU, J.V., HASSELBERGER, F.X. & SCHULZ, D.W. (1964). Effect of ischemia on known substrates and cofactors of the glycolytic pathway in brain. *J. biol. Chem.*, **239**, 18–30.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin Phenol Reagent. *J. biol. Chem.*, **193**, 265–275.

MANTOVANI, P., PEPEU, G. & AMADUCCI, L. (1976) Investigation into the relationship between phospholipids and brain acetilcholine. *Adv. exp. Med. Biol.* **72**, 285–292.

NILSSON, L. & SIESJÖ, B.K. (1974). Influence of anaesthetics on the balance between production and utilization of energy in the brain. *J. Neurochem.*, **23**, 29–36.

PAPAHADJOPOULOS, D., VAIL, W.J., NEWTON, C., NIR, S., JACOBSON, K., POSTE, G. & LAZO, R. (1977). Studies on membrane fusion III. The role of calcium-induced phase changes. *Biochim. biophys. Acta*, **465**, 579–598.

PAYSANT, M., BITRAN, M., ETIENNE, J. & POLONOVSKI, J. (1969). Phospholipase A du plasma sanguin de rat. Cinétique et propriétés. Existence d'un précurseur inactif. *Bull. Soc. Chim. biol.* **51**, 863–873.

RAND, R. P. & SENGUPTA, S. (1972). Cardiolipin forms hexogonal structures with divalent cations. *Biochim. biophys. Acta*, **255**, 484–492.

RAUSER, G., KRITCHEVISKY, G., YAMAMOTO, A., SIMON, G., GALLI, C. & BAUMAN, A.J. (1969). Diethylaminoethyl and triethylaminoethyl cellulose column chromatographic procedure for phospholipids, glycolipids and pigments. *Meth. Enzymol.*, **14**, 272–317.

SKIPSKI, V.P. & BARCLAY, M. (1969). Thin-layer chromatography of lipids. *Meth. Enzymol.*, **14**, 530–597.

STEGER, L.D. & DESNICK, R.J. (1977). Enzyme therapy VI: comparative *in vivo* fates and effects on lysosomal integrity of enzyme entrapped in negatively and positively charged liposomes. *Biochim. biophys. Acta*, **464**, 530–546.

TOFFANO, G., LEON, A., BENVEGNÙ, D., BOARATO, E. & AZZONE, G.F. (1976). Effects of brain cortex phospholipids on catecholamine content of mouse brain. *Pharmac. Res. Commun.*, **8**, 581–590.

TYCE, G.H. (1976). The effect of L-dopa and an inhibitor of peripheral decarboxylation on glucose metabolism in brain. *J. Neurochem.*, **27**, 1397–1403.

TYRELL, D.A., HEATH, T.D., COLLEY, C.M. & RYMAN, B.E. (1976). New aspect of liposomes. *Biochim. biophys. Acta.* **457**, 259–302.

(Received January 25, 1978. Revised September 28, 1978.)