The Effects of Phenazinemethosulfate and Methylene Blue on Human Platelet Phospholipid Synthesis[1] (36728)

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Active phospholipid synthesis is known to occur in the human platelet (1–4), the only peripheral blood cell capable of *de novo* phospholipid synthesis (1). Lipid precursors such as glycerol are primarily incorporated into platelet phosphatidylcholine (PC) *in vitro* (1, 2). It has been shown that *de novo* phospholipid synthesis is markedly altered when platelets are incubated with thrombin, which induces an absolute increase of glycerol incorporation into phosphatidylserine (PS), a relative increase into phosphatidylinositol (PI), and a relative decrease into phosphatidylethanolamine (PE) and PC (1). Although relatively small amounts of phospholipids are generated by *de novo* synthesis in platelets, the lipid produced may have an important functional role (1). An association between phospholipid synthesis and cellular physiological activity has been observed in phagocytosing leukocytes (5, 6). Since thrombin causes platelet aggregation and release of serotonin and ADP (7), the thrombin-altered phospholipid synthesis may be important for platelet physiological activity.

This study demonstrates that methylene blue (MB) and a related dye, phenazinemethosulfate (PMS), can alter platelet phospholipid synthesis. MB also induces the release of platelet serotonin.

Materials and Methods. Preparation of platelet-rich-plasma (PRP). Ten to 30 ml of blood were collected from healthy volunteers, anticoagulated with sodium heparin, 2 units/ml blood, and buffered with Tris–HCl buffer (15.4 m*M*), pH 7.3. PRP was prepared by centrifuging the blood at 300*g* for 15 min. Only plastic labwares were used. Cell counts were performed by phase-contrast microscopy. Erythrocyte contamination was less than 1 per 1000 platelets, and leukocytes were less than 1 per 5000 platelets. Platelet counts were done by the Brecher-Cronkite method and varied between 450,000 and 650,000/mm^3.

PMS (Sigma) and MB (M-291, Fisher Scientific) were dissolved in saline so that 0.1 ml of PMS or MB, when added to 2.4 ml of PRP, would result in the desired final concentration. MB was freshly prepared in the morning of the experiment, but PMS was made up immediately before the incubation and kept in the dark to avoid deterioration.

Glycerol-2-^{14}C (NEC-455, New England Nuclear) was added to the freshly prepared PRP, and about 0.5 μCi was present in 1 ml PRP. After adequate mixing, 2.4 ml aliquots of labeled PRP were transferred to 40 ml plastic tubes which contained 0.1 ml of MB, PMS, or saline. This marked the beginning of the incubation period.

Incubation. The plastic test tubes containing the incubation mixtures were agitated in a metabolic shaker at 37°. The incubation was stopped by rapidly filtering under suction 2 ml of the incubation mixture (about 10^9 platelets). The platelets collected on a GF/A filter (Whatman) were washed with 10 ml of cold 15.4 m*M* Tris–HCl buffer (pH 7.3). The filter was immediately placed in a chloroform–methanol solution (see below) for

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lipid extraction. Phase-contrast microscopy showed that the filtrate was free of platelets. An individual experiment usually included two to four incubation mixtures: a saline control and one to three tubes with different concentrations of MB and PMS, all representing aliquots of the same PRP specimen. Incubations were completed within 1 hr after blood collection. Phase-contrast microscopy showed that PMS and MB did not cause platelet clumping during the incubation period.

Platelet lipids were extracted by the method described by Folch, Lees, and Stanley (8). The chloroform–methanol mixture was prepared to contain 0.01 mg/100 ml butylated hydroxytoluene, an antioxidant, to prevent the peroxidation of lipids (9).

Separation and identification of the classes of phospholipids were done by thin-layer chromatography. Chromatoplates 20 × 20 cm, 250 μ thick, and precoated with silica gel G, were obtained from the Analtech Co. The developing solvents were chloroform/methanol/concentrated NH_4OH, 140/60/10 (v/v/v). PI and PS standards purchased from the Applied Science Laboratory, and extracted rat liver phospholipids, kindly provided by Dr. Lewis Gidez, were used to identify individual platelet phospholipids. Also Ninhydrin was used to identify PS and PE.

Liquid scintillation counting. The individual phospholipid bands, visualized with iodine vapor, were scraped into plastic vials containing a thixotropic mixture (POPOP, PPO, toluene, and Cab-O-Sil). Radioactivity was determined in a Packard Tri-carb liquid scintillation counter.

Lipid phosphorus was determined by the method described by Norton and Autilio (10).

Platelet serotonin release was determined by modification of the method described by Kattlove and Spaet (11). Whole blood was anticoagulated with 3.2 g % trisodium citrate (1 ml citrate/9 ml blood). Instead of preparing platelet-poor-plasma by centrifugation, platelets were separated by rapidly filtering the platelet suspension through GF/A glass fiber filters under suction. Since only 8% of the added ^{14}C-serotonin was present in platelet-free-plasma in control experiments, the collection of platelets on glass fiber filters did not result in excessive loss of platelet serotonin.

Results. The lipid phosphorus present in each of the five major platelet phospholipids is expressed in Fig. 1. The results are in agreement with those reported by Marcus, Ullman and Safier (12). The lipid phosphorus extracted from 10^9 platelets was found to be 9.5 and 9.3 μg in two separate experiments and was comparable to the amounts originally applied to the plate.

The pattern of glycerol incorporation into each of the five major platelet phospholipids in control experiments is shown to have been reproducible, as shown in Fig. 2. The present data for normal platelets are in agreement with those reported by Lewis and Majerus (1). Since glycerol is primarily incorporated into platelet phospholipids, diversion into other lipids does not represent a complicating problem. The starting pH was 7.3 and rose to 7.8 after 30 min incubation, but the elevated pH did not alter incorporation of glycerol into platelet phospholipids in control

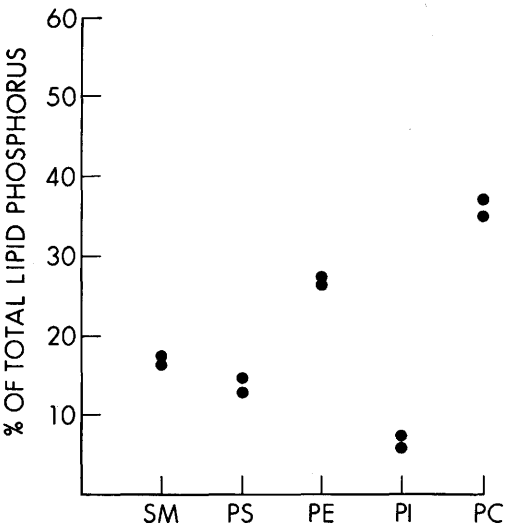

FIG. 1. Phospholipids in human platelets. Total lipid phosphorus represents the amount of phosphorus present in normal 10^9 platelets. SM = sphingomyelin; PS = phosphatidylserine; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PC = phosphatidylcholine.

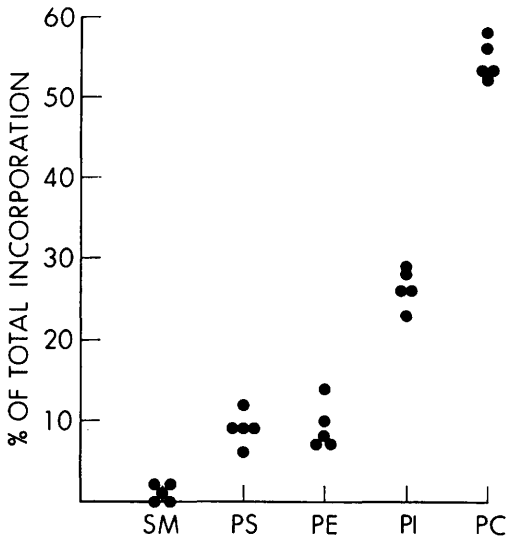

FIG. 2. Glycerol-2-^{14}C incorporation into control human platelets. Total incorporation refers to the glycerol incorporated into the five major platelet phospholipids during 30 min incubation periods. SM = sphingomyelin; PS = phosphatidylserine; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PC = phosphatidylcholine.

experiments.

The total glycerol incorporation into platelet phospholipids was influenced by the presence of MB or PMS, as shown in Fig. 3. PMS (6×10^{-5} *M*) caused a 140%, and MB (1×10^{-4} *M*) caused a 50% increase in total glycerol incorporation. Higher concentrations depressed the incorporation of glycerol.

The pattern of glycerol incorporation into individual platelet phospholipids in relation to the concentration of PMS or MB is shown in Fig. 4. Increasing concentrations of PMS and MB caused an increase of incorporation of glycerol into PS, but a relative decrease of incorporation of radioactivity into PC and PE. Glycerol incorporation into phosphatidylinositol was maximal at 6×10^{-5} *M* PMS. The effects of PMS on the pattern of glycerol incorporation into platelet phospholipids were more pronounced than those of MB.

Table I shows the effect of PMS or MB on platelet serotonin release. Increasing concentrations of MB induced a progressively greater loss of platelet serotonin. PMS did not cause the release of serotonin.

Discussion. The present study demonstrates that platelet phospholipid synthesis can be altered by PMS and MB. The incorporation of radioactive glycerol into platelet phospholipids was initially enhanced but eventually depressed as the concentrations of the dyes were increased. MB and PMS influenced the pattern as well as the degree of incorporation of radioactivity into the individual platelet phospholipids. Incorporation into PS was increased whereas incorporation into PC and PE was decreased. PMS induced a marked increase of glycerol incorporation into PI. The observed MB and PMS-induced alteration in platelet phospholipid synthesis resembles the pattern which occurred in platelets incubated with thrombin (1) an agent which caused platelet aggregation and release of platelet ADP and serotonin (7).

Since exchange of the glycerol backbone of phospholipids does not occur (1), glycerol incorporation into platelet phospholipids represents *de novo* lipid synthesis but not necessarily increased net synthesis. The MB and PMS effects on platelet phospholipid synthesis may have been compensatory to membrane modification, perhaps as a result of photochemical oxidation (13). Platelet phospholipid synthesis can be altered by trypsin, possibly by membrane modification (1). An alternate possibility is that the dyes stimulated phospholipid synthesis. The pathways for PS biosynthesis have not been as completely investigated as those for PI syn-

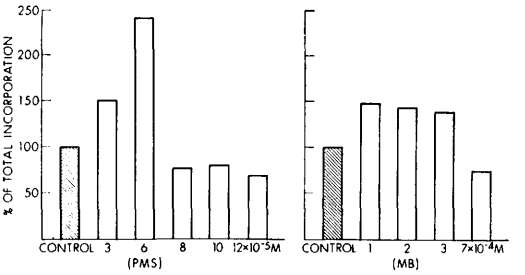

FIG. 3. Incorporation of glycerol-2-^{14}C into platelet phospholipids in relation to the concentration of PMS and MB. Total incorporation refers to the glycerol incorporated into the five major platelet phospholipds during 30 min incubation periods. The incorporation of glycerol into platelet phospholipids in control experiments is expressed as 100%.

thesis in platelets (3, 14). It is known that diverse factors such as hyposmolality (3) and succinate (15) can stimulate platelet PI synthesis. This study shows that PMS and MB have a marked effect on platelet phospholipid synthesis, including the augmentation of PS and PI synthesis.

MB in the present study also induced the release of platelet serotonin, whereas PMS had no such effect. An association between cellular physiological activity and phospholipid synthesis has been demonstrated. Elsbach (5) showed that synthesis of PC from lysophosphatidylcholine is increased in alveolar macrophages and leukocytes during phagocytosis. Incorporation of lipid precursors into PI and/or PS has been observed in the phagocytosing leukocyte (6), the neuron, and secretory cells (hepatocyte and the pancreatic cell) (16). Hokin (17) speculated that increased turnover of PI occurs during cellular secretion. He considered the phospholipid turnover to be necessary for the formation of new membranes involved in reverse endocytosis and resultant extrusion of intracellular substances. The present study did not establish whether PMS or MB stimulated platelet membrane formation, since it was not possible to localize the newly synthesized phospholipids in the intact platelet.

It is clear that thrombin (1) and MB cause a profound alteration in platelet phospholipid synthesis, particularly of PS, as well as the release of platelet serotonin. The role of phospholipids in platelets (cellular) physiological activity is not well understood.

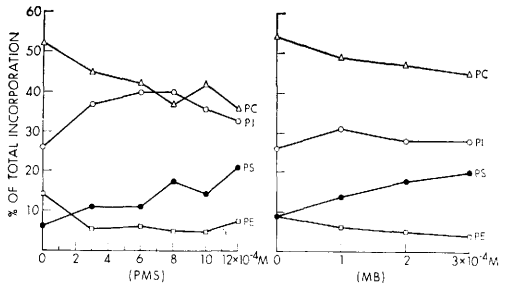

FIG. 4. Pattern of glycerol-2-^{14}C incorporation into platelet phospholipids in relation to the concentration of PMS and MB. Total incorporation refers to the glycerol incorporated into the five major platelet phospholipids during 30 min incubation periods. PS = phosphatidylserine; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PC = phosphatidylcholine.

TABLE I. The Effects of PMS and MB on the Release of Platelet ^{14}C-Serotonin.

Conen		Serotonin released (%) during 30 min (av of 5 expts.)
Bovine thrombin (25 units)		84
Control		9
MB	$1 \times 10^{-4} M$	37
	$2.5 \times 10^{-4} M$	59
	$4.0 \times 10^{-4} M$	70
PMS	$3.0 \times 10^{-5} M$	10
	$6.0 \times 10^{-5} M$	8
	$1.2 \times 10^{-4} M$	8
	$2.4 \times 10^{-4} M$	9

Summary. 1. In the present study phenazinemethosulfate (PMS) and methylene blue (MB) were shown to induce a pattern of glycerol incorporation into platelet phospholipids which resembles the pattern that Lewis and Majerus (1) observed when platelets were incubated with thrombin.

2. MB induced the release of platelet serotonin, but PMS did not cause the release of this amine.

3. The thrombin and MB-enhanced synthesis of platelet phosphatidylserine (PS) and associated release of platelet serotonin may be interrelated.

1. Lewis, N., and Majerus, P. W., J. Clin. Invest. **48**, 2114 (1969).

2. Cohen, P., Derksen, A., and Van den Bosch, H., J. Clin. Invest. **49**, 128 (1970).

3. Cohen, P., Broekman, M. J., Verkley, A., Lisman, J. W. W., and Derkson, A., J. Clin. Invest. **50**, 762 (1971).

4. Elsbach, P., Pettis, P., and Marcus, A., Blood **37**, 675 (1971).

5. Elsbach, P., J. Clin. Invest. **47**, 2217 (1968).

6. Karnovsky, M. L., and Wallach, D. F. H., J. Biol. Chem. **236**, 1895 (1961).

7. Holmsen, H., Day, H. J., and Stormorken, H., Scand. J. Haematol., Suppl. **n8** (1969).

8. Folch, J., Lees, M., and Stanley, G. H. S., J. Biol. Chem. **226**, 497 (1957).

9. Skipski, V. P., and Barclay, B., *in* "Methods in Enzymology" (J. M. Lowenstein, ed.), Vol. 15, p. 537. Academic Press, New York (1969).

10. Norton, W. T., and Autilio, L. A., J. Neurochem. **13**, 213 (1966).

11. Kattlove, H. E., and Spaet, T. H., Blood **35**, 659 (1970).

12. Marcus, A. J., Ullman, H. L., and Safier, L. B., J. Lipid Res. **10**, 108 (1969).

13. Rippa, M., Picco, C., and Pontremoli, S., J. Biol. Chem. **245**, 4977 (1970).

14. Lucas, C. T., Call, F. L., II, and Williams, W. J., J. Clin. Invest. **49**, 1949 (1970).

15. Grossman, C. M., and Bartos, F., Arch. Biochem. Biophys. **128**, 231 (1968).

16. Hokin, L. E., Ann. N.Y. Acad. Sci. **165**, 695 (1969).

17. Hokin, L. E., *in* "Handbook of Physiology" (C. Code, ed.), p. 935. Amer. Physiol. Soc., Washington, DC (1967).

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