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CATIONIC AMPHIPHILIC DRUGS AS A POTENTIAL TOOL FOR MODIFYING PHOSPHOLIPIDS OF TUMOR CELLS. AN *IN VITRO* STUDY OF CHLORPROMAZINE EFFECTS ON KREBS II ASCITES CELLS

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Abstract—[^{32}P]orthophosphate and [U-^{14}C]glycerol incorporation into Krebs ascites cell lipids was studied *in vitro* in the presence of chlorpromazine (CPZ)*. At concentrations not exceeding 0.1 mM, the drug produced no cell damage within 3 hr incubation. Under these conditions, CPZ inhibited the incorporation of [^{32}P]orthophosphate into phosphatidylcholine and phosphatidylethanolamine and of [U-^{14}C]glycerol into phosphatidylcholine and triglycerides, in a dose-dependent manner. On the other hand, synthesis of phosphatidic acid and phosphatidylglycerol was greatly enhanced, whereas phosphatidylinositol showed no major change. These results are compatible with an inhibition of phosphatidate phosphohydrolase, redirecting phospholipid biosynthesis towards the anionic classes. *In vitro* treatment of cells for 3 hr induced profound changes of phospholipid composition, which displayed a relative increase of phosphatidylglycerol and phosphatidic acid at the expense of phosphatidylcholine and phosphatidylethanolamine. The use of amphipathic cationic drugs can thus offer an interesting model for studying the relationship between cell proliferation and membrane phospholipid biosynthesis.

Amphiphilic cationic drugs have been used to modify phospholipid biosynthesis in different mammalian cells [1–8]. Preferential formation of anionic phospholipids induced by these drugs seems to depend on the inhibition of phosphatidate phosphohydrolase, coupled to the stimulation of phosphatidate cytidylyltransferase by direct interaction with membrane components [9, 10]. Phosphatidate phosphohydrolase is a key enzyme in the glycerophospholipid biosynthesis, since it regulates the relative amounts of phosphatidate and diacylglycerol which are precursors to anionic and zwitterionic glycerophospholipids plus triglycerides, respectively [11].

Membrane biogenesis accompanying cellular growth needs an adapted phospholipid biosynthesis [12] and a higher rate of phospholipid turnover has been described in potentially malignant cells [13]. The use of amphiphilic cationic drugs could thus represent a means for disturbing membrane formation in rapidly growing tumoral tissues. Furthermore phospholipid modifications might be used to alter their membrane properties.

The present study deals with the action of chlorpromazine (CPZ) on the *in vitro* incorporation of radioactive precursors [^{32}P]orthophosphate and [U-^{14}C]glycerol into phospholipids of Krebs II ascites cells [14]. Moreover, the subsequent modifications of phospholipid composition are analysed.

MATERIALS AND METHODS

Materials. [^{32}P]Orthophosphate (sodium salt 2.4 Ci/mmole) was purchased from C.E.A., Gif sur Yvette, France. [U-^{14}C]glycerol (35 mCi/mmole) was a product of the Radiochemical Centre, Amersham, U.K.). The latter one was diluted with cold glycerol to a specific radioactivity of 2 mCi/mmole.

Cell suspensions. The Krebs II ascitic tumors [14] were maintained in female Swiss strain mice, aged between 9 and 12 weeks, by weekly intraperitoneal transfer of ascitic fluid. Cell counts were performed with a Nageotte counting cell. The viability of the ascites cells was estimated with the Trypan blue method. At the moment of the collect, 85 to 90 per cent of the cells exhibited a positive viability test. Cells were harvested by centrifugation at 250 *g* for 10 min and washed twice in Tyrode buffer (pH 7.35) containing 1 mM $MgCl_2$, 20 mM glucose, 0.2 mM EGTA, 0.35% bovine serum albumin (w/v), but NaH_2PO_4 was replaced by 5 mM HEPES. Final suspension was done in the same buffer with 2.5 mM $CaCl_2$, at a density of 4.2×10^6 cells/ml. The whole procedure was carried out at room temperature in siliconized glassware.

Incubations. Cell suspensions (12.5 ml) were added to tubes containing [^{32}P]orthophosphate (50 μCi) or [U-^{14}C]glycerol (2.5 μCi) and CPZ at the desired concentrations. No pH change was observed under the conditions used. Incubations were performed for different times at 37° under gentle stirring. Cells were then centrifuged at 250 *g* for 10 min, the supernatants were collected and cell lysis was determined as described elsewhere [15] by measuring lactate dehydrogenase activity [16]. The pellets were resuspended in 2 ml of ice-cold EDTA (15 mM, pH 7.4).

Phospholipid analysis. Lipids were immediately extracted according to Bligh and Dyer [17]. Phospholipids were separated by bidimensional thin-layer chromatography as described by Rouser *et al.* [18].

* Abbreviations: CPZ, chlorpromazine; HEPES, 2-(*N*-2-hydroxyethylpiperazine-*N'*-yl) ethane sulphonic acid.

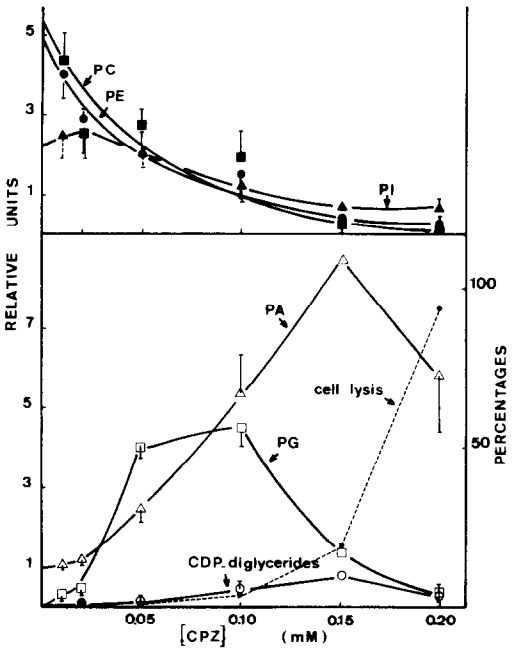

Fig. 1. Effect of increasing concentrations of chlorpromazine on the incorporation of [^{32}P]orthophosphate into phospholipids of Krebs II cells. 5.2×10^7 cells were incubated for 3 hr at 37°, in the presence of [^{32}P]orthophosphate (50 μCi) and various concentrations of chlorpromazine, under a final volume of 12.5 ml. Cell lysis (----) is expressed as percent of total lactate dehydrogenase activity. Radioactivity is given in relative units, taking the radioactivity of phosphatidic acid in the controls as a 1 value, the radioactivity corresponding to the reference value of phosphatidate being of 30101 ± 3766 dpm/10^7 cells. Values are the mean ± S.E.M. of 3 experiments except for 0.15 mM CPZ. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; PG, phosphatidylglycerol.

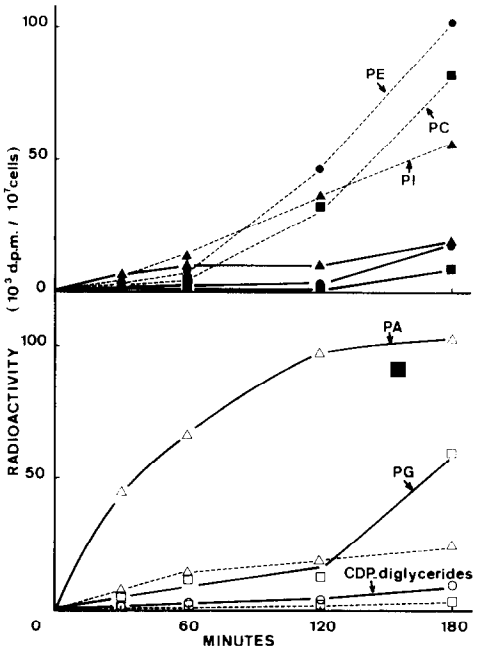

Fig. 2. Time–course of [^{32}P]orthophosphate incorporation into phospholipids of Krebs II ascites cells at a non lytic concentration of chlorpromazine (0.1 mM). 5.2×10^7 cells were incubated in the presence of [^{32}P]orthophosphate (50 μCi) without (----) or with (——) 0.1 mM chlorpromazine. Radioactivity was measured in phosphatidic acid – △ –, phosphatidylglycerol – □ –, CDP-diglycerides – ○ –, phosphatidylethanolamine – ● –, phosphatidylcholine – ■ – and phosphatidylinositol – ▲ –. Data represent mean of two identical experiments.

The different phospholipids were identified by comparison with authentic pure standards in various chromatographic systems [19–21]. Neutral lipids were separated by monodimensional thin-layer chromatography according to Derksen and Cohen [22]. The different spots were scraped off and analysed for radioactivity, by counting in 10 ml Instafluor (Packard), or for phosphorus by the method of Böttcher *et al.* [23].

RESULTS

Incorporation of [^{32}P]*orthophosphate into phospholipids of Krebs II ascites cells. Effect of increasing concentrations of CPZ*

In the absence of CPZ, ^{32}P-incorporation occurred essentially into phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol (34 ± 1.7% 34 ± 0.8% and 16 ± 1% of total lipid radioactivity respectively, mean ± S.E.M., 9 experiments). Phosphatidic acid labelling was lower (9 ± 1% of total), while only traces of radioactivity were detected in cardiolipin, phosphatidylglycerol and phosphatidylserine. No radioactivity was found in sphingomyelin.

Phosphatidylcholine and phosphatidylethanolamine radioactivity fell progressively upon increasing CPZ concentration (Fig. 1). Maximal incorporation was observed into phosphatidic acid and phosphatidylglycerol. Phosphatidylinositol showed an intermediary pattern, with a slight increase at low CPZ concentrations, followed by a decrease with higher amounts of the drug. However, these differences were not significant, except at 0.2 mM CPZ ($P < 0.05$). An accumulation of ^{32}P was also observed into cardiolipin and CDP-diacylglycerol, but these compounds represented only 10% of phosphatidic acid radioactivity. Phosphatidylserine and sphingomyelin did not show any significant change. Cell lysis occurred at the highest CPZ concentrations used (0.15 and 0.20 mM), as checked by lactate dehydrogenase determination.

Time course of CPZ effects on phospholipid metabolism

Incubations were performed at the highest CPZ concentration (0.1 mM) giving a maximal effect on phospholipid metabolism without inducing lysis. As shown in Fig. 2, the stimulation of phosphatidic acid labelling occurred very rapidly and levelling off was observed between 2 and 3 hr, while phosphatidylglycerol increase was the highest in the same time. On the other hand, incorporation of ^{32}P into phosphatidylcholine, phosphatidylethanolamine and

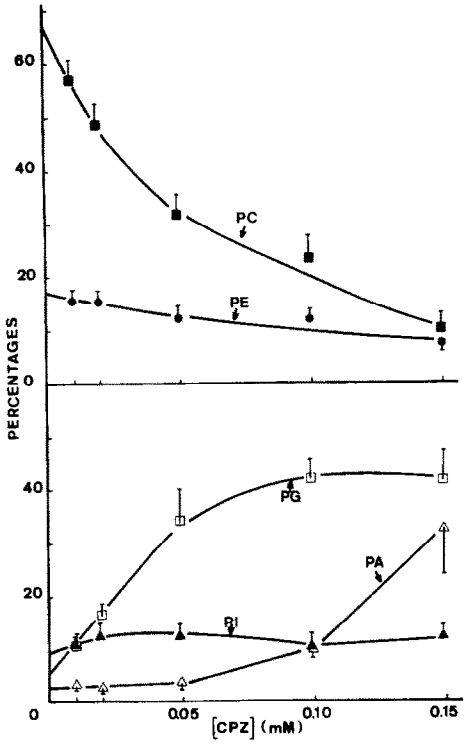

Fig. 3. Incorporation of [U-^{14}C]glycerol into phospholipids of Krebs II ascites cells. Effect of increasing concentrations of chlorpromazine. 5.2×10^7 cells were incubated for 3 hr in the presence of [U-^{14}C]glycerol (2.5 μCi) and increasing concentrations of chlorpromazine. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PA, phosphatidic acid. Results are expressed as percentage of total phospholipid radioactivity (mean ± S.E.M., 4 experiments). Total radioactivity of phospholipids in the controls was: 4158 ± 695 dpm/10^7 cells. This value was not significantly different in the assays.

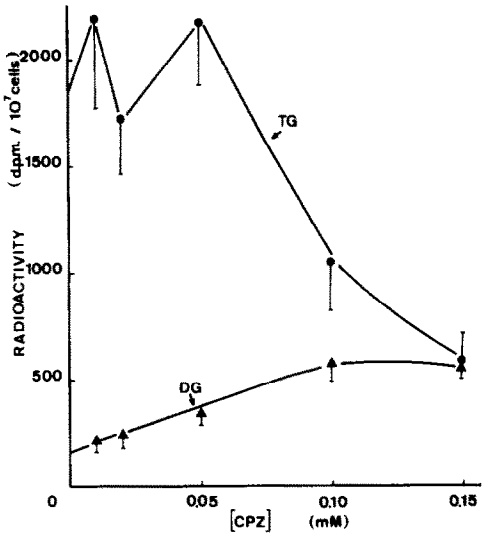

Fig. 4. Effect of increasing concentrations of chlorpromazine on the incorporation of [U-^{14}C]glycerol into neutral lipids of Krebs II ascites cells. Cells were incubated for 3 hr in the presence of [U-^{14}C]glycerol (2.5 μCi) and various concentrations of chlorpromazine. Radioactivity was measured in diglycerides (DG) and triglycerides (TG). The data are the means ± S.E.M. of 4 experiments.

phosphatidylinositol remained very low over a 3-hr-incubation in the presence of CPZ, compared to the controls.

These effects were then compared to those induced by lytic concentrations of CPZ. At 0.2 mM CPZ, lysis started only at 60 min and no ^{32}P-incorporation occurred into phosphatidylglycerol (not shown). It was thus verified that the trace amounts of radioactivity found for phosphatidylglycerol at 0.2 mM CPZ (Fig. 1) corresponded to a lack of ^{32}P-incorporation and not to hydrolysis of the phospholipid induced by cell lysis. Also, the stimulation of ^{32}P-incorporation into phosphatidic acid was less important than with 0.1 mM CPZ. These results indicate that cell integrity is necessary for a maximal effect of CPZ on phospholipid metabolism.

Incorporation of [U-^{14}C] *glycerol into lipids of Krebs II ascites cells. Effects of increasing concentrations of CPZ*

In order to better follow CPZ effects on *de novo* synthesis of phospholipids and neutral lipids, [U-^{14}C]glycerol was used as a radioactive precursor (Fig. 3). In the absence of CPZ, phosphatidylcholine displayed the highest labelling (67 ± 2 per cent of total phospholipid radioactivity, mean ± S.E.M., 4 experiments), which dramatically decreased in a dose-dependent manner upon CPZ addition. However, no significant change of phosphatidylethanolamine radioactivity was observed. An accumulation of [^{14}C]glycerol into phosphatidylglycerol occurred at low doses of CPZ, levelling off being obtained at 0.05 mM of drug, whereas that observed for phosphatidic acid started only at 0.05 mM and increased further linearly. A slight stimulation was measured for phosphatidylinositol.

In Fig. 4 are given the variations of neutral lipid metabolism. Triacylglycerol displayed a biphasic response, with a small increase for low CPZ concentrations, followed by a decrease at the highest drug concentrations. But differences were significant at 0.15 mM CPZ only. On the other hand, the radioactivity incorporated into diglycerides slightly increased in the presence of CPZ.

Changes in phospholipid composition of CPZ-treated cells

When considering the percentages of phospholipid composition, CPZ induced no significant changes of sphingomyelin + phosphatidylserine and cardiolipin. On the other hand, phosphatidylcholine and phosphatidylethanolamine were reduced (9 and 16% respectively, $P < 0.001$ according to Student's t test), whereas phosphatidylglycerol and phosphatidic acid showed a counter-balancing increase. Phosphatidylinositol content became somewhat higher.

In order to obtain more quantitative data on these compositional changes, absolute amounts of

Table 1. Amounts of phospholipids present in Krebs cells after a 3 hr-incubation with or without chlorpromazine (0.1 mM)*

Phospholipids	Controls		Assays		P†
	Mean	S.D.	Mean	S.D.	
Sphingomyelin + phosphatidylserine	75.2	11.4	84	14	<0.01
Phosphatidylinositol	29.4	4.1	35	5.2	<0.01
Phosphatidylcholine	191	28.7	191	39.4	NS
Phosphatidylethanolamine	102.2	17.2	95.3	21.9	<0.02
Phosphatidylglycerol	5.8	2.4	19	3.4	<0.001
Phosphatidic acid	6.25	1.9	19.6	2.8	<0.001
Cardiolipin	8.1	2.4	10.2	2	<0.02

* Results are expressed in nmoles lipid phosphorus/10^7 cells and are the means of 12 determinations.
† Probability of significance according to Student's paired t test.

phospholipids were then measured. Total phospholipids were slightly increased: 422 ± 22 to 470 ± 25 nmoles/10^7 cells (mean ± S.E.M). This difference was significant when using the paired t test. The data from Table 1 show that the concentrations of phosphatidic acid and phosphatidylglycerol were three times higher in the assays. Moreover, phosphatidylinositol, cardiolipin and sphingomyelin plus phosphatidylserine also showed a slight increase. On the other hand, phosphatidylethanolamine slightly decreased whereas phosphatidylcholine displayed no variation.

DISCUSSION

The most important modifications of phospholipid metabolism observed in the present study are compatible with an inhibition of phosphatidate phosphohydrolase combined to a stimulation of phosphatidate cytidylyltransferase reported by others and due to a direct complexation of the cationic amphiphilic drug CPZ with phosphatidate [9]. However, our results differ significantly from those of Allan and Michell [4], who found an important stimulation of phosphatidylinositol biosynthesis in pig lymphocytes under similar conditions. These discrepancies are observed by other authors [2, 5] and might be due to differences existing between tissues or to variations in the incubation conditions. Freinkel *et al.* [5] suggested that free intracellular inositol could regulate the rate of phosphatidylinositol biosynthesis in rat pancreatic islets incubated with tetracaine. But under our conditions, we failed to find any specific stimulation by CPZ of phosphatidylinositol biosynthesis, in the presence of cold inositol, which also enhanced the labelling of the other anionic phospholipids. It should be noticed that tumoral transformation could be accompanied by an increase of mitochondrial phosphatidylglycerol synthesis, since Hostetler *et al.* [24] reported a 360 per cent increase of CTP: phosphatidic acid cytidylyltransferase activity in mitochondria from hepatoma 7777 compared to normal liver mitochondria. On the other hand, besides stimulating biosynthesis of anionic phospholipids, CPZ could also impair their further transformation probably by complexation with them [25–29].

This study also shows important differences between [^{32}P]phosphate and [^{14}C]glycerol incorporations into phosphatidylethanolamine, with or without CPZ. As discussed by Waku *et al.* [30], a large part of phosphatidylethanolamine in Ehrlich cells are l-alkenyl- or l-alkyl-species, whose synthesis could imply an exchange reaction of the phosphorylated base, which might explain the higher labelling by ^{32}P compared to [^{14}C]glycerol. On the other hand, the lack of inhibition of glycerol entry into phosphatidylethanolamine by CPZ would suggest that phosphatidylethanolamine is synthesized through a phosphatidic acid pool (l-alkylated) which would have less affinity for CPZ. It is worthy of note that incorporation of [^{3}H]glycerol into phosphatidylethanolamine of rat pineal gland was not depressed by 0.1 mM propranolol either [8]. The decrease of triglyceride biosynthesis induced by CPZ is also compatible with an inhibition of phosphatidate phosphohydrolase. It should also be noticed that diglyceride labelling was always found increased by CPZ. Similar results were reported by Brindley and Bowley [3] using derivatives of fenfluramine. As discussed by these authors, diglyceride utilization might be inhibited or a pool of diglycerides could originate from a metabolic pathway stimulated by amphiphathic cationic drugs.

Interestingly, these metabolic changes are followed by a rapid modification of the phospholipid composition of Krebs II ascites cells, the relative amounts of anionic phospholipids augmenting at the expense of zwitterionic phospholipids. However, as already mentioned, CPZ increases total phospholipids of Krebs cells, which is essentially due to the newly formed phosphatidylglycerol and phosphatidic acid. Consequently, when considering the absolute amounts of zwitterionic glycerophospholipids, no significant changes are observed for phosphatidylcholine and phosphatidylethanolamine, respectively. That would mean that the catabolism of phosphatidylcholine and phosphatidylethanolamine is too low to obtain significant decrease of these phospholipids during the relatively short incubation times used in this study. Moreover, glycerophospholipid degradation could also be lowered under our conditions, since inhibition of phospholipases by cationic amphiphilic drugs has been reported [26–29].

An important question raised by the present study concerns the physiological consequences for tumor cells of the phospholipid changes induced by CPZ. The use of cationic amphipathic drugs could thus represent an interesting model for studying the coupling between phospholipid synthesis and cell growth. Further works are now in progress on cultured cells in this perspective.

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REFERENCES

1. R. H. Michell, D. Allan, M. Bowley and D. N. Brindley, *J. Pharm. Pharmac.* **28**, 331 (1976).
2. J. Eichberg and G. Hauser, *Biochim, biophys. Res. Commun.* **60**, 1460 (1974).
3. D. N. Brindley and M. Bowley, *Biochem. J.* **148**, 461 (1975).
4. D. Allan and R. H. Michell, *Biochem. J.* **148**, 471 (1975).
5. N. Freinkel, C. El Younsi and R. M. C. Dawson, *Eur. J. Biochem.* **59**, 245 (1975).
6. A. A. Abdel-Latif and J. P. Smith, *Biochem. Pharmac.* **25**, 1697 (1976).
7. N. G. Bazán, M. G. Ilincheta de Boschero, N. M. Giusto and H. E. Pascual de Bazán, *Adv. exp. Med. Biol.* **72**, 139 (1976).
8. J. Eichberg, J. Gates and G. Hauser, *Biochim. biophys. Acta* **573**, 90 (1979).
9. R. G. Sturton and D. N. Brindley, *Biochem. J.* **162**, 25 (1977).
10. M. Bowley, J. Cooling, S. L. Burditt and D. N. Brindley, *Biochem. J.* **165**, 447 (1977).
11. H. Van den Bosch, *Ann. Rev. Biochem.* **43**, 243 (1974).
12. S. K. Malhotra, in *Mammalian Cell Membranes* (Eds. G. A. Jamieson and D. M. Robinson), Vol. 1, pp. 224–243. Butterworths, London (1976).
13. C. A. Pasternak, in *Tumor Lipids, Biochemistry and Metabolism* (Ed. R. Wood), pp. 66–74. American Oil Chemists' Society Press, Champaign (1973).
14. T. S. Hauschka and A. Levan, *J. natn. Cancer Inst.* **21**, 77 (1958).
15. H. Chap, R. F. A. Zwaal and L. L. M. Van Deenen, *Biochim. biophys. Acta* **467**, 146 (1977).
16. F. Wroblewski and J. S. La Due, *Proc. Soc. exp. Biol.* **90**, 210 (1955).
17. E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.* **37**, 911 (1959).
18. G. Rouser, G. Kritchevsky and A. Yamamoto, in *Lipid Chromatographic Analysis* (Ed. G. V. Marinetti), Vol. 1, pp. 99–162. Marcel Dekker, New York (1967).
19. V. P. Skipski, F. F. Peterson and M. Barclay, *Biochem. J.* **90**, 374 (1964).
20. R. E. Anderson, M. B. Maude and G. L. Feldman, *Biochim. biophys. Acta* **187**, 345 (1969).
21. P. Cohen and A. Derksen, *Br. J. Haemat.* **17**, 359 (1969).
22. A. Derksen and P. Cohen, *J. biol. Chem.* **248**, 7396 (1973).
23. C. J. F. Böttcher, C. M. Van Gent and C. Pries, *Anal. Chim. Acta* **24**, 203 (1961).
24. K. Y. Hostetler, B. D. Zenner and H. P. Morris, *Biochem. biophys. Res. Commun.* **72**, 418 (1976).
25. H. Lüllmann, R. Lüllmann-Rauch and O. Wassermann, *Biochem. Pharmac.* **27**, 1103 (1978).
26. Y. Matsuzawa and K. Y. Hostetler, *J. biol. Chem.* **255**, 5190 (1980).
27. M. Waite and P. Sisson, *Biochemistry* **11**, 3098 (1972).
28. G. L. Scherphof, A. Scarpa and A. Van Toorenenbergen, *Biochim. biophys. Acta* **270**, 226 (1972).
29. H. Kunze, E. Bohn and N. Vogt, *Biochim. biophys. Acta* **360**, 260 (1974).
30. K. Waku, Y. Nakazawa and W. Mori, *J. Biochem.* **80**, 711 (1976).