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# A NEW ANTIINFLAMMATORY COMPOUND, TIMEGADINE* (*N*-CYCLOHEXYL-*N''*-4-[2-METHYLQUINOLYL]-*N'*-2-THIAZOLYLGUANIDINE), WHICH INHIBITS BOTH PROSTAGLANDIN AND 12-HYDROXYEICOSATETRAENOIC ACID (12-HETE) FORMATION

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**Abstract**—Timegadine (*N*-cyclohexyl-*N''*-4-[2-methylquinolyl]-*N'*-2-thiazolylguanidine), a new antiinflammatory agent, was found to be a potent, competitive inhibitor of prostaglandin synthetase derived from a variety of tissues, including bovine seminal vesicles, rabbit renal papillae, rabbit lung, rabbit platelets and rat brain. The concentration of timegadine required to obtain 50% inhibition ($IC_{50}$) varied from $5 \times 10^{-9}$M (washed rabbit platelets) to $2 \times 10^{-5}$M (rat brain). Timegadine was also found to be an inhibitor of lipoxygenase activity in the cytosol fraction of horse platelet homogenates, and in washed rabbit platelets. $IC_{50}$ was $1 \times 10^{-4}$M in both cases. These effects are discussed in the view of recent evidence, suggesting that dual inhibitors of arachidonate cyclo-oxygenase and lipoxygenase may have an improved profile of antiinflammatory activity.

Compounds which prevent both cyclo-oxygenase and lipoxygenase pathways of arachidonic acid metabolism may have advantages over selective cyclo-oxygenase inhibitors such as aspirin-like compounds in the treatment of inflammatory conditions. The final product of lipoxygenase pathway in platelets [1, 2] and possibly in other cells [3], 12-L-hydroxy-eicosatetraenoic acid (12-HETE) is a potent chemotactic agent for PMN's [4, 5] and macrophages [6] and it may be involved in the cellular component of the inflammatory response as suggested by recent (albeit indirect) evidence [7, 8]. In addition, the labile precursor of 12-HETE, 12-L-hydroperoxy-eicosatetraenoic acid (12-HPETE) may have important biological functions. For example, the release of anaphylactic mediators from guinea pig lungs is enhanced by hydroperoxy fatty acids [9, 10], and from rat isolated peritoneal cells by indomethacin [11]. Aspirin-like drugs have been found to give rise to increased levels of 12-HPETE in human platelet cytosol as a result of their ability to inhibit the enzymatic conversion of 12-HPETE to 12-HETE [12].

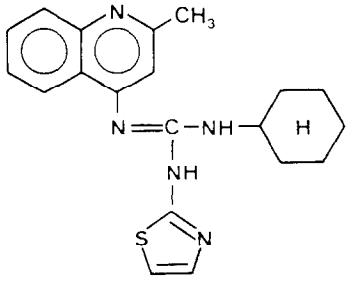


Fig. 1. Timegadine (*N*-cyclohexyl-*N''*-4-[2-methylquinolyl]-*N'*-2-thiazolylguanidine; SR 1368).

* Generic name assigned to the compound previously known as SR 1368.

We have recently described the potent antiinflammatory activity of timegadine (Fig. 1) [13]. Some aspects of the pharmacology of this compound [14] suggested an investigation of its effects on cyclo-oxygenase pathway of arachidonic acid metabolism in different tissues, on lipoxygenase pathway in horse platelet cytosol and on both pathways in intact washed rabbit platelets.

## MATERIALS AND METHODS

*Materials.* [1-$^{14}$C]Arachidonic acid, sp. act. 56.4 mCi/mmole (2.09 GBq/mmole) (The Radiochemical Centre, Amersham, U.K.), arachidonic acid (grade 1) and L-epinephrine bitartrate (Sigma Chemical Co., St. Louis, MO., U.S.A.), reduced glutathione (Boehringer Mannheim GmbH, F.R.G.), prostaglandin $E_2$ and prostaglandin $F_{2\alpha}$ (Unilever Research, Vlaardingen, The Netherlands), silica-coated aluminum sheets (0.2 mm DC-Alufolien Kieselgel 60 $F_{254}$, E. Merck, Darmstadt, F.R.G), SR 1368 (Chemical Research Department, Leo Pharmaceutical Products), Naproxene (Astra-Syntex, Södertälje, Sweden), Nictindole (L 8027) (S.A. Labaz, Bordeaux-Cédex, France), Flufenamic acid (Parke-Davis, Glostrup, Denmark), Indomethacin (Dumex A/S, Copenhagen, Denmark) and Chloroquine (Mecobenzon A/S, Copenhagen, Denmark). Dimilume® scintillation cocktail, Packard Instrument Co., ILL., U.S.A.

*Assay of prostaglandin synthetase (PGS) activity in bovine seminal vesicle microsomes.* A modification [15] of the method by Yanagi *et al.* [16] was used.

This method depends on the selective extraction of unmetabolized [$^{14}$C]arachidonic acid from the incubates after reaction for 5 min at 37°, followed by liquid scintillation counting of arachidonic acid metabolites which remain in the aqueous layer. The concentration of arachidonic acid was $4.1 \times 10^{-5}$M, 0.125 $\mu$Ci/ml, reduced glutathione and L-epinephrine both 0.4 mg/ml, and BSVM 0.95 mg protein/ml (protein was assayed by the method of Lowry *et al.* [17]), unless otherwise indicated.

Drugs were dissolved in dimethylsulphoxide (DMSO), and the final concentration of DMSO in the reaction mixture was 1%, which was shown not to interfere with PGS activity. Drugs were preincubated (unless otherwise indicated) for 5 min with enzyme prior to addition of radioactive substrate.

*Assay of PGS-activity in tissues other than BSV.* Mircosomes were prepared by homogenizing the tissue in the double vol. of 0.1 M Tris–HCl, pH 8.0 (Tris), filtering the homogenate through glass-wool, centrifuging the filtrate at 10,000 *g* for 10 min, followed by centrifugation of the supernatant for 1 hour at 100,000 *g*. The microsomal pellet was lyophilized and suspended at 5 mg of lyophilized powder/ml of Tris. 0.15 ml was incubated with 0.05 ml [$^{14}$C] arachidonic acid, $1.64 \times 10^{-4}$M, 0.50 $\mu$Ci/ml, at 37° for either 10 min (lung, renal papilla) or 60 min (brain). The reaction mixture was then adjusted to pH 3.5 with 1M glycin pH 3, containing 1M NaCl, and extracted twice with 10 vol. ethyl acetate containing, as carriers, 10 $\mu$g $PGE_2$ and $PGF_{2\alpha}$. The extract was subjected to thin-layer chromatography on silica-coated aluminum foil in 2 different solvent systems: (1) chloroform : methanol : acetic acid : water (90:9:1:0.65) and (2) The organic layer of ethyl acetate: iso-octane: acetic acid: water (55:25:10:50), and the products of arachidonic acid metabolism were identified and quantified as previously described [18].

*Assay of soluble lipoxygenase activity.* The cytosol fraction of horse platelets were prepared as previously described [1]. The high speed supernatant was diluted 10 × with Tris, and 100 $\mu$l was mixed with 2 $\mu$l drug solution (usually in DMSO) and preincubated for 5 min at 37°. Then 0.9 nmole ($10^5$ cpm) [1-$^{14}$C]arachidonate in 100 $\mu$l Tris was added, and the mixture was incubated for 30 min at 37°. The reaction was stopped by extraction with 2 ml ethyl acetate containing 1 $\mu$g/ml unlabelled arachidonic acid. More than 85 per cent of the radioactivity was extracted in one step. The extract was chromatographed in solvent mixture 1 (see above) on silica-coated aluminum-foil. Radioactive spots were localized by autoradiography, cut out and eluted with methanol before mixing with Dimilume® and counting in a liquid scintillation spectrometer. The identity of HETE was established as previously described [19].

*Arachidonic acid-metabolism in rabbit platelets.* 18 ml blood was obtained by heart-puncture of New Zealand White Rabbits, and was mixed immediately with 2 ml 3.8% Na-citrate. Plastic tubes were used throughout. Platelet-rich plasma was obtained by centrifugation at 200 *g* for 10 min at 20°, and the platelets were then pelleted at 2,000 *g* for 10 min (20°). The cells were washed by repeated resuspensions and centrifugations in 0.9% NaCl, $10^{-2}$M EDTA, pH 7.4, and were finally resuspended in 5 ml of this buffer at 20°. 150 $\mu$l platelet suspension was preincubated with 2 $\mu$l drug solution for 5 min at 30° in DMSO, or DMSO as control, before addition of 50 $\mu$l [$^{14}$C]arachidonic acid, $1.65 \times 10^{-4}$M, 0.5 $\mu$Ci/ml. After incubation with occasional shaking at 30° for 30 min, the tubes were cooled to < 4° on ice, and the cells were pelleted at 2,000 *g*, 10 min 4°. The radioactivity in an aliquot of the supernatant was determined by LSC. The rest was extracted twice with 2 ml ethyl acetate containing 2 $\mu$g/ml $PGE_2$ and $PGF_{2\alpha}$ and the extract, containing more than 80 per cent of the radioactivity not associated with the cells, was analyzed by t.l.c. as described above. The platelets were dissolved in 1 ml 1 N NaOH, ambient temperature, 4 hr, and 100 $\mu$l of the solution was

Table 1. Inhibition of prostaglandin synthetase from various tissues by timegadine, some nonsteroidal antiinflammatory drugs (NSAID's), and chloroquine

| Tissue | Inhibitor | Inhibition of the formation of $PGF_{2\alpha}$ | $PGE_2$ | $PGD_2$ | $TXB_2$ | 6-keto-$PGF_{1\alpha}$ | Inhibition of all arachidonate metabolism |
|---|---|---|---|---|---|---|---|
| Rabbit renal papilla | Timegadine | 5.1* | 6.1 | 5.6 | 4.2 | | 5.4 |
| | Indomethacin | 9.5 | 7.9 | 10.5 | 7.0 | | 8.2 |
| Rat brain | Timegadine | 20.2 | | | | | 20.2 |
| | Indomethacin | 2.8 | | | | | 2.8 |
| Rabbit lung | Timegadine | 2.5 | 2.6 | | 1.8 | 3.6 | 2.1 |
| | Indomethacin | 1.3 | 0.7 | | 0.8 | 1.7 | 1.3 |
| | Nictindole | 6.0 | 5.1 | | 2.5 | 6.3 | 4.0 |
| | Naproxene | 21.7 | 15.4 | | 18.0 | 30.5 | 27.3 |
| Bovine seminal vesicle | Timegadine | | | | | | 10.6 |
| | Indomethacin | | | | | | 1.1 |
| | Nictindole | | | | | | 9.7 |
| | Flufenamic | | | | | | 22.4 |
| | Naproxene | | | | | | 59.6 |
| | Chloroquine | | | | | | >100 |

* All values are $IC_{50}$'s ($\mu$M).

Only arachidonate metabolites which were identified are included in the table.

Due to the special assay for PGS in BSV (see Methods) only the overall arachidonate metabolism was determined in this tissue (main product is $PGE_2$).

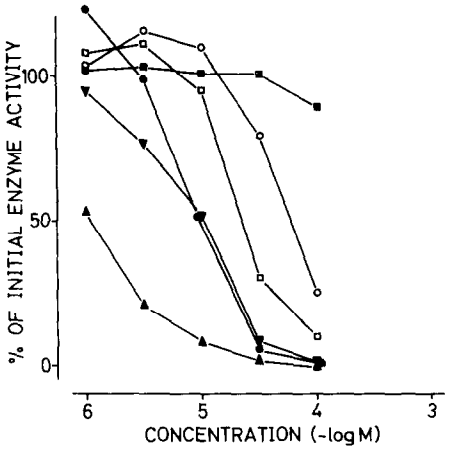


Fig. 2. Effect of timegadine (▼), indomethacin (▲), nictindole (●), flufenamic acid (□), naproxene (○) and chloroquine (■) on prostaglandin synthetase activity in bovine seminal vesicle microsomes.

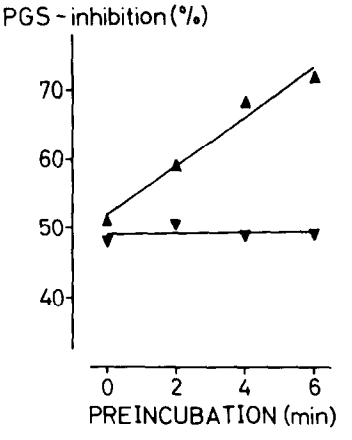


Fig. 4. Effect of preincubation on inhibition of BSVM-prostaglandin synthetase by timegadine, $3.0 \times 10^{-6}$M (▼) and indomethacin, $0.5 \times 10^{-6}$M (▲).

mixed with 10 ml Dimilume® scintillation cocktail and counted to determine the radioactivity in the cells.

## RESULTS

*Effect on prostaglandin synthetase.* The effect of timegadine, some NSAIDs, and chloroquine on BSVM-PGS is shown in Fig. 2. All compounds except chloroquine showed significant inhibition at concentrations below $10^{-4}$M, and the order of potency was indomethacin > nictindole = timegadine > flufenamic acid > naproxene. $IC_{50}$ values are listed in Table 1.

In order to classify the mechanism of PGS-inhibition by timegadine, the effect of arachidonic acid concentration on inhibition by timegadine was investigated. A Lineweaver–Burk graph was plotted for the uninhibited reaction and the reaction inhibited by timegadine (3.0 $\mu$M) and indomethacin (0.5 $\mu$M) (Fig. 3). Maximum velocity, $V_{max}$ of the reaction was 0.46 nmoles PGE formed/min, and $K_m$ was $5.36 \times 10^{-5}$M. The action of both inhibitors was markedly suppressed by increasing the concentration of arachidonic acid, and the presence of a common intercept on the $1/V$-axis for the enzyme reaction in the presence and absence of drugs indicated competitive inhibition with both timegadine and indomethacin. The inhibitor constants, $K_i$, were $5.79 \times 10^{-6}$M and $0.91 \times 10^{-6}$M, respectively.

The amount of inhibition obtained with indomethacin was clearly time-dependent, but inhibition with timegadine was constant with respect to time (Fig. 4).

Comparative studies of the effect of timegadine and other antiinflammatory agents on PGS's derived from different animal tissues were performed (Table 1). With rabbit renal papillary microsomes arachidonic acid was metabolized mainly into $PGF_{2\alpha}$, $PGE_2$ $PGD_2$, $TXB_2$ [15]. Both timegadine and indomethacin inhibited the formation of all metabolites with $IC_{50}$-values of the same order of magnitude for all metabolites. Timegadine was 1.3–1.9 times more potent than indomethacin in this system. Microsomes derived from rat brain cortex produced $PGF_{2\alpha}$ from arachidonic acid at an extremely slow rate, and it

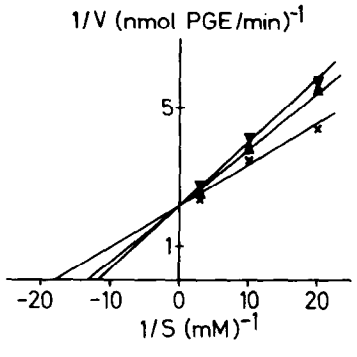


Fig. 3. Bovine seminal vesicle microsomal prostaglandin synthetase: Lineweaver–Burk plot of uninhibited reaction (×), and reaction inhibited by timegadine, $3.0 \times 10^{-6}$M (▼) and indomethacin, $0.5 \times 10^{-6}$M (▲).

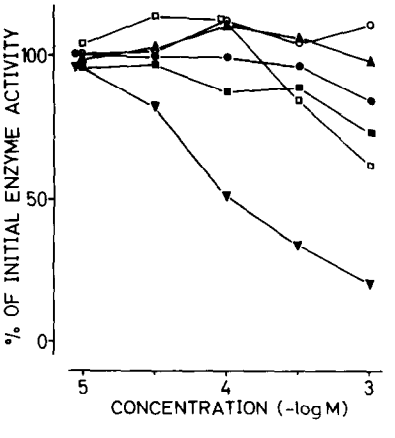


Fig. 5. Effect of compounds on horse platelet cytosol lipoxygenase activity. For explanation of symbols, see legend of Fig. 2.

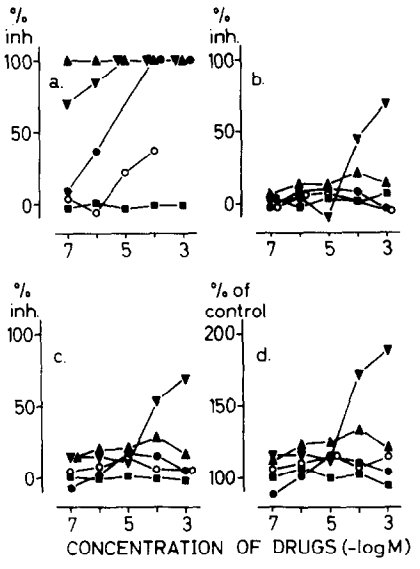


Fig. 6. Arachidonic acid metabolism in washed rabbit platelets (for explanation of symbols, see legend of Fig. 2). (a) Effect on cyclooxygenase activity, measured as the formation of 12-hydroxy-heptadecatrienoic acid (HHT) and $TXB_2$. (b) Effect on 12-hydroxyeicosatetraenoic acid (12-HETE) formation. (c) Effect on total metabolism of arachidonic acid. (d) Effect on cell-associated radioactivity after 30 min of incubation in presence of exogenous [$^{14}$C]arachidonic acid. Control tubes contained DMSO at the same concentration (1%) as tubes containing drugs.

was necessary to incubate for 1 hour to achieve measurable quantities of $PGF_{2\alpha}$. No other metabolites were formed. In this system indomethacin was 10 times more potent than timegadine, which was weaker as brain PGS-inhibitor than observed with other enzymes.

Rabbit lung microsome produced $PGF_{2\alpha}$, $PGE_2$, $TXB_2$ and 6K-$PGF_{1\alpha}$ [18]. Again, all compounds inhibited the formation of all metabolites, the order of potency being indomethacin > timegadine> nictindole> naproxene.

*Effect on soluble lipoxygenase.* The effect of timegadine, indomethacin, flufenamic acid, naproxene, nictindole and chloroquine on soluble lipoxygenase activity from horse platelets was also investigated (Fig. 5). Significant inhibition occurred only with timegadine ($IC_{50}$ $1.07 \times 10^{-4}$M). All other compounds were inactive at concentrations up to $10^{-3}$M.

*Effect on arachidonic acid metabolism in washed rabbit platelets.* Three major radioactive metabolites of [$^{14}$C]-AA, [$^{14}$C]-$TXB_2$, [$^{14}$C]-HHT and [$^{14}$C]-HETE, and some minor metabolites (unidentified) were formed by washed rabbit platelets in the presence of exogenous AA. Under the experimental conditions described under Materials and Methods, in the absence of drugs, 40–60 per cent of the radioactivity added to the cell suspension was associated with the cellular pellet after incubation. Of the five drugs tested only timegadine changed significantly the ratio of cell-associated radioactivity vs. soluble radioactivity (Fig. 6d). Thus, at $10^{-4}$M timegadine more than 80 per cent of the radioactivity was present in the cells, possibly as arachidonic acid incorporated into the phospholipid membrane.

Indomethacin, timegadine and nictindole were potent inhibitors of platelet cyclo-oxygenase, naproxene was less potent ($IC_{50}$ $>10^{-4}$M; due to interference with extraction and t.l.c. it was not possible to use higher concentrations of naproxene than $10^{-4}$M), and chloroquine had no effect on cyclooxygenase (Fig. 6a). The inhibition of intact platelet-lipoxygenase by timegadine (Fig. 6b) was of the same order of magnitude as observed with soluble horse platelet lipoxygenase (Fig. 5). None of the other compounds significantly changed the activity of this enzyme.

## DISCUSSION

These results suggest that the profile of activity of timegadine on arachidonic acid metabolism is clearly different from that of other NSAIDs.

The effects of timegadine on cycloxygenase pathways in different tissues are not qualitatively different from those of other NSAIDs used in comparative assays. In most of these assays its potency is comparable to that of indomethacin. The distinct lipoxygenase pathway [1, 2] leading to the formation of 12-HETE, not affected by NSAIDs, is concentration-dependently inhibited by timegadine both in intact washed rabbit platelets ($IC_{50} = 1.58 \times 10^{-4}$M) and in horse platelet cytosol ($IC_{50} = 1.07 \times 10^{-4}$M). In the latter assay Higgs *et al.* [7] have found that the structural analogue of phenidone BW 755 C, an inhibitor of leukocyte migration in sponge exudate, has an $IC_{50} = 1.70$ μg/ml ($7.5 \times 10^{-6}$M). The amount of 12-HETE determined in our assays is likely to be the sum of 12-HPETE and 12-HETE, as in the chromatographic system we used the two products co-chromatograph [20] and in assay mixtures 12-HPETE is unstable [1]. In view of this it seems likely that timegadine inhibits the early steps of the lipoxygenase pathway. Whether an inhibition of the peroxidase activity which catalyzes the transformation of 12-HPETE to 12-HETE [20] also takes place cannot be assessed on the basis of present investigations. This has been reported to occur in intact or lysed human platelets with NSAIDs and it has been found to be accompanied by a remarkable increase of 12-HPETE levels [12, 20]. However, an enhancement of this step by timegadine is also a theoretical possibility, due to the weak reducing properties of the thiazole-ring. Lipoxygenase inhibition by timegadine occurred not only in lysed, isolated preparations of lipoxygenase essentially free of cyclo-oxygenase, but also to a similar extent in intact rabbit platelets. In this assay system the inhibition of cyclooxygenase by timegadine was at least two orders of magnitude greater than that observed in microsomal preparation of bovine seminal vesicles. As a result the total rate of conversion of the exogenous arachidonic acid in intact platelets is decreased.

The mechanism of the inhibitory effect of timegadine on the turnover-rate of arachidonic acid therefore appears to differ from that of the antiinflammatory steroid dexamethasone which does not inhibit cyclo-oxygenase of lipoxygenase *in vitro* [7] but probably prevents the production of arachidonic acid metabolites through inhibition of phospholipase $A_2$ activity [21]. The effect of timegadine on the

synthesis of other lipoxygenase products with pro-inflammatory properties, such as 5-HPETE and leukotrienes, remains to be investigated.

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## REFERENCES

1. D. H. Nugteren, *Biochim. biophys. Acta* **380**, 299 (1975).
2. M. Hamberg and B. Samuelsson, *Proc. natn. Acad. Sci. U.S.A.* **71**, 3400 (1974).
3. S. Hammarström, M. Hamberg, B. Samuelsson, E. A. Duell, M. Stawiski and J. J. Voorhees, *Proc. natn. Acad. Sci. U.S.A.* **72**, 5130 (1975).
4. S. R. Turner, J. A. Tainer and W. S. Lynn, *Nature* **257**, 680 (1975).
5. E. J. Goetzl, J. M. Woods and R. R. Gorman, *J. clin. Invest.* **59**, 179 (1977).
6. J. A. Tainer, S. R. Turner and W. S. Lynn, *Am. J. Pathol.* **81**, 401 (1975).
7. G. A. Higgs, R. J. Flower and J. R. Vane, *Biochem. Pharmac.* **28**, 1959 (1979).
8. J. R. Walker and W. Dawson, *J. Pharm. Pharmac.* **31**, 778 (1979).
9. J. J. Adcock, L. G. Garland, S. Moncada and J. A. Salmon, *Prostaglandins* **16**, 163 (1978).
10. J. J. Adcock, L. G. Garland, S. Moncada and J. A. Salmon, *Prostaglandins* **16**, 179 (1978).
11. J. F. Burka and R. J. Flower, *Br. J. Pharmac.* **65**, 35 (1979).
12. M. I. Siegel, R. T. McConnell, N. A. Porter and P. Cuatrecasas, *Proc. natn. Acad. Sci. U.S.A.* **77**, 308 (1980).
13. S. Rachlin, E. Bramm, I. Ahnfelt-Rønne and E. Arrigoni-Martelli, *J. Med. Chem.* **23**, 13 (1980).
14. E. Bramm and E. Arrigoni-Martelli, in preparation.
15. I. Ahnfelt-Rønne, *Prostaglandins* **16**, 711 (1978).
16. Y. Yanagi and T. Komatsu, *Biochem. Pharmac.* **25**, 937 (1976).
17. O. H. Lowry, N. S. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem* **193**, 265 (1951).
18. I. Ahnfelt-Rønne and E. Arrigoni-Martelli, *Agents Actions* (suppl.) **4**, 110 (1979).
19. I. Ahnfelt-Rønne, and E. Arrigoni-Martelli, *Agents Actions* in press.
20. M. I. Siegel, R. T. McConnell and P. Cuatrecases, *Proc. natn. Acad. Sci. U.S.A.* **76**, 3774 (1979).
21. R. J. Flower and G. J. Blackwell, *Nature, Lond.* **278**, 456 (1979).