*Biochemical Pharmacology*, Vol. 31, No. 23, pp. 3769–3773, 1982.
Printed in Great Britain.

0006-2952/82/233769-05 $03.00/0


# AN INTERACTION OF AMINOGLYCOSIDE ANTIBIOTICS WITH Ca BINDING TO LIPID MONOLAYERS AND TO BIOMEMBRANES

HEINZ LÜLLMANN and BENEDIKTA VOLLMER
Department of Pharmacology, Hospitalstrasse 4–6, 2300 Kiel, West Germany

(*Received* 3 *March* 1982; *accepted* 25 *May* 1982)

**Abstract**—Thirteen aminoglycoside antibiotics were investigated with respect to their ability to replace Ca from phosphatidylserine monolayers. The affinities of the glycosides for the Ca-binding sites depended on the pH and the $Ca^{2+}$ concentration of the aqueous subphase. At a $Ca^{2+}$ concentration of $1.2 \times 10^{-5}$ M and a pH of 7.5 the half maximum concentrations to replace Ca were found to range from $0.4 \times 10^{-6}$ (sisomicin) to $6 \times 10^{-6}$ M (streptomycin). The interaction between the streptomycins and Ca were of a competitive nature, whereas the aminoglycoside antibiotics with higher affinities displayed unusual steep dose–response curves suggesting positive cooperativity. The aminoglycoside antibiotics were also able to replace Ca from biomembranes (red cell ghosts and isolated sarcolemma of cardiac muscle) in a dose-dependent manner. The high affinity of aminoglycoside antibiotics to a phospholipid is considered to be involved in the toxic side effects of these antibiotics, e.g. nephrotoxicity and impairment of contractile force.

The aminoglycoside antibiotics exert (besides ototoxicity) a well-defined renal side effect, i.e. a toxic action upon proximal tubules. At the ultrastructural level, proximal tubule cells contain lamellated inclusion bodies the size and number of which depend upon the dosage and duration of treatment. Finally, the cells undergo necrosis accompanied by corresponding impairment of kidney function [1]. The lamellar bodies are membrane-bound and are derived from lysosomes. As judged from their ultrastructural appearance, the inclusion bodies in proximal tubule cells appear to consist, at least in part, of accumulated polar lipids. This has recently been confirmed by chemical analysis. Kaloyanides and Pastoriza-Munoz [2] reported that the phospholipid content of the renal cortex in rats is elevated upon treatment with aminoglycosides. In cultured fibroblasts, to which the aminoglycosides have rather slow access, the phospholipid content was also found to be raised, and lamellated inclusion bodies were present [3].

The accumulation of polar lipids in lysosomes to which aminoglycosides have gained access [4] stimulated us to investigate the possibility of whether or not a direct interaction between aminoglycosides and phospholipids does occur which then might intefere with the intralysosomal degradation of these lipids. To study a possible interaction we used the method of "Ca replacement" from lipid monolayers which has been successfully used previously for investigating the interaction between cationic amphiphilic drugs and polar lipids [5–7]. In addition, Ca replacement from biomembranes by aminoglycosides was investigated. The results obtained by both procedures suggest a direct interaction between aminoglycosides and polar lipids.

## METHODS

*Ca replacement from phosphatidylserine monolayers.* In principle the method makes use of the accumulation of Ca by a monolayer of acidic phospholipids spread on the surface of buffer solutions which contain different concentrations of $Ca^{2+}$ labelled by $^{45}Ca$ [8, 9]. Owing to the weak radiation of $^{45}Ca$, only the Ca concentration present at the surface will be measured by a methane flow counter placed on top of the buffer solution. In the presence of drugs which compete with Ca for the binding sites of the lipid layer the yield of radioactivity will decline accordingly.

In the present study phosphatidylserine—the phospholipid with the highest capacity for binding Ca—was used at a surface pressure of about 11 mN $m^{-1}$ corresponding to an occupation of about 60 $Å^2$ per molecule of phospholipid. The $Ca^{2+}$ concentrations varied between $3 \times 10^{-6}$ and $1 \times 10^{-4}$ M. The buffer solution had the following composition: 5 mM NaOH, 2 mM TES,* 2 mM histidine adjusted to pH 7.5 by HCl if not stated otherwise. The temperature was kept constant at 24°. A more detailed description has been given previously [7].

*Ca replacement from biomembranes.* The method of determining the Ca replacement from biomembranes involves three steps: (1) preparation of the membranes in a Ca-free medium, (2) refilling of the binding sites with Ca, and (3) replacement of Ca by aminoglycoside antibiotics.

Preparations of red cells ghosts (open sheets) were obtained according to the methods of Dodge *et al.* [10] and Hanahan and Ekholm [11]. A plasmalemmal fraction from guinea pig heart muscle was obtained as described by Lüllmann and Peters [12]. Both methods yield membranes which are nearly equivalent to intact biomembranes with respect to lipid composition, protein distribution, and function of enzymes. During the preparation processes the

* TES = *N*-tris (hydroxymethyl)-methyl-2-aminoethane-sulfonic acid.

media contained no Ca so that the membranes were free of Ca. The presence of a chelating agent was avoided in order to preserve the plasmalemmal structure [13, 14].

In a second step, the Ca content was refilled by incubating the membranes for 5 hr in a medium with Ca (1 mM) labelled with $^{45}Ca$. After centrifugation of the membranes (red cells ghosts at 25,000 *g* for 1 hr, and plasmalemma at 45,000 *g* for 1 hr, respectively) the Ca content was determined in a liquid scintillation counter and corrected for the extravesicular space, which was estimated by the difference of the volume of distribution between $[^3H]H_2O$ and [$^{14}$C]EDTA. The Ca binding per milligram of protein [15] was taken as 100%.

To investigate the Ca replacement by aminoglycoside antibiotics, the membranes loaded with Ca were incubated in the medium described earlier containing the antibiotics at different concentrations. The aminoglycosides investigated were dibekacin, amikacin and streptomycin. The Ca content was determined in a liquid scintillation counter as earlier, and the decrease was set in relation to the drug-dependent Ca replacement. When $^{45}Ca$ and the antibiotics were simultaneously applied to the membrane fractions the same results were obtained. To examine the mechanism of Ca replacement, different Ca concentrations ranging from $3 \times 10^{-5}$ to $10^{-3}$ M were applied.

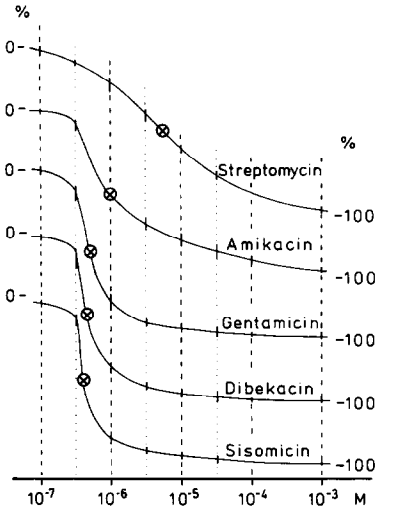


Fig. 1. Replacement of Ca from phosphatidylserine (PS) monolayers by different aminoglycoside antibiotics at a $Ca^{2+}$ concentration of $1.2 \times 10^{-5}$ M. Ordinate: Percentage of $^{45}Ca$ replaced, 100% refers to complete replacement, 0% reflects 86 pmoles of Ca bound per square centimetre; abscissa: molar concentrations of drugs. Vertical bars represent range of data ($n = 5$–7). The curves are projected upon each other by shifting the ordinate. Concentrations of drugs replacing 50% Ca bound to phospholipid monolayers ($ID_{50}$s) are marked by ⊗. The dotted vertical lines indicate the decades and half-decades.

## RESULTS

### *Replacement of* Ca *from monolayer binding sites by aminoglycosides*

Thirteen aminoglycoside antibiotics were investigated for their potency to interact with Ca bound to phosphatidylserine under standard conditions (pH 7.5, $Ca^{2+}$ concentration $1.2 \times 10^{-5}$ M, 24°). The results are summarized in Table 1 and presented in simplified dose–response curves for some of the antibiotics in Fig. 1. As can be taken from the curves, the antibiotics were able to completely replace Ca bound to the monolayer. Most compounds displayed threshold concentrations below $3 \times 10^{-7}$ M and half maximum concentrations of about $5 \times 10^{-7}$ M, but reached their maximum effect only at concentrations of about $10^{-5}$ M, thus yielding asymmetrically shaped dose–response curves. In contrast to the majority of compounds, streptomycin and dihydrostreptomycin were found to be less active in replacing Ca; their symmetrical dose–response curves were found to be markedly flatter than those obtained with other aminoglycosides. In Table 1, the different slopes are characterized by the $ID_{75}/ID_{25}$ ratios.

Table 1. Half maximum concentrations of the aminoglycoside antibiotics investigated to replace Ca from phosphatidylserine monolayers at a $Ca^{2+}$ concentration of $1.2 \times 10^{-5}$ M, expressed as $ID_{50}$ ($\times 10^{-7}$ M)

| | $ID_{50}$ ($\times 10^{-7}$ M) | $ID_{75}/ID_{25}$ |
|---|---|---|
| Streptomycin | 60 | 17 |
| Dihydrostreptomycin | 60 | 30 |
| Amikacin | 10 | 19 |
| Ribostamycin | 7 | 4 |
| Kanamycin | 6 | 5 |
| Paromomycin | 6 | 2 |
| Tobramycin | 6 | 2 |
| Netilmicin | 5.5 | 2 |
| Neomycin | 5 | 2 |
| Framycetin | 5 | 2 |
| Gentamicin | 5 | 2 |
| Dibekacin | 4.5 | 2 |
| Sisomicin | 4 | 2 |

To characterize the slope of the dose–response curves the $ID_{75}/ID_{25}$ ratios are given.

### *Replacement of* Ca *by aminoglycosides at different pH*

To check whether or not the degree of protonation plays a role in the potency of aminoglycoside antibiotics to replace Ca from phosphatidylserine monolayers, experiments were performed at different pH values of the aqueous phase. In the case of dibekacin, which contains five amino groups, the ability to compete with Ca increased with increasing $H^+$ concentrations, reaching a maximum at pH 7.5 (Fig. 2a). A further increase to pH 6.0 and 4.0 (the latter not shown) did not influence the replacement curve. Streptomycin, which besides carrying only one amino group has two guanidyl groups, which are rather strong bases, was found to be fairly independent of the pH (Fig. 2b) in the range between pH 8.5 and 4.0.

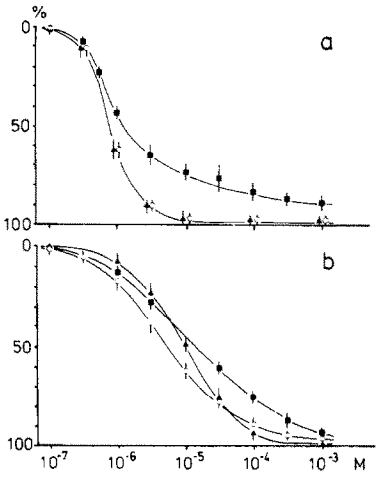


Fig. 2. Replacement of Ca from PS monolayers by dibekacin (a) and streptomycin (b) dependent upon the pH of the aqueous phase at a $Ca^{2+}$ concentration of $1.2 \times 10^{-5}$ M. Ordinates: Percentage of $^{45}Ca$ replaced; abscissa: molar concentrations of the drug. Symbols represent means ± S.E.M. ($n = 5$–7). pH 8.5 (■), pH 7.5 (○), pH 6.0 (▲). The curve obtained at pH 4.0 (not shown) was identical with that at pH 6.0.

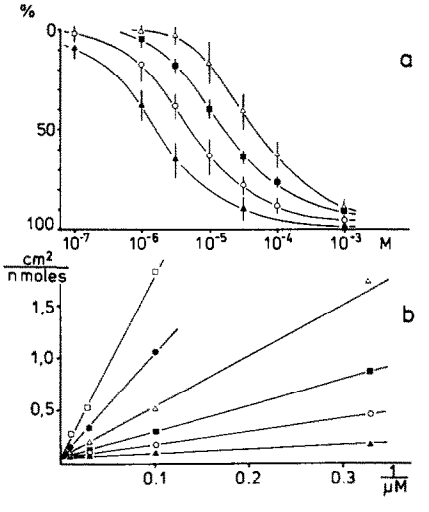


Fig. 3. Replacement of Ca from PS monolayers by streptomycin. (a) Dose–response curves were obtained at different $Ca^{2+}$ concentrations: $1 \times 10^{-4}$ M (△), $3 \times 10^{-5}$ M (■), $1 \times 10^{-5}$ M (○) and $3 \times 10^{-6}$ M (▲). Ordinates: Percentage of Ca replaced; 0% reflects 164 pmoles of Ca bound per square centimetre at a $Ca^{2+}$ concentration of $1 \times 10^{-4}$ M, 135 pmoles at $3 \times 10^{-5}$ M, 86 mmoles at $1 \times 10^{-5}$ M, and 56 pmoles at $3 \times 10^{-6}$ M, respectively; abscissa: molar concentrations of the drug. Symbols represent means ± S.E.M. ($n = 5$). (b) The same data presented as a Lineweaver–Burk plot. Drug concentrations $3 \times 10^{-4}$ M (□), $1 \times 10^{-4}$ M (●), $3 \times 10^{-5}$ M (△), $1 \times 10^{-5}$ M (■), $3 \times 10^{-6}$ M (○) and control (▲). Ordinate: Inverse $Ca^{2+}$ concentrations; abscissa: reciprocal of the amount of Ca bound (nmoles) per square centimetre of monolayer.

*Replacement of* Ca *by aminoglycosides at different* $Ca^{2+}$ *concentrations*

The earlier results were obtained at a standard $Ca^{2+}$ concentration of $1.2 \times 10^{-5}$ M in the aqueous phase. To study the type of interaction between Ca and aminoglycosides the $Ca^{2+}$ concentration was varied between $3 \times 10^{-6}$ and $10^{-4}$ M, and dose–response curves for some aminoglycosides were evaluated. The results obtained with streptomycin are depicted in Fig. 3a. With increasing $Ca^{2+}$ concentration, the dose–response curves were parallel-shifted to the right. Analysis of these data in a Lineweaver–Burk plot (Fig. 3b) suggests that the interaction between Ca and streptomycin is of a competitive nature. The results yielded with dibekacin demonstrate a more complex interaction. In this case, again, the $ID_{50}$ values were shifted to the right with increasing $Ca^{2+}$ concentrations, but the shape of the dose–response curves markedly changed (Fig. 4). At low $Ca^{2+}$ concentrations the curves were unusually steep; at a high $Ca^{2+}$ concentration they were unusually flat, covering about 3.5 decades. The complex type of interaction becomes particularly evident when the experimental data are plotted according to Scatchard (Fig. 4, inset). The resulting lines demonstrate convex curvature which has been proposed to indicate positive cooperativity (e.g. [16–18]).

*Replacement of* Ca *from biomembranes by aminoglycoside antibiotics*

Three of the aminoglycosides were studied with respect to their potency to replace Ca bound to biomembranes. Figure 5 shows the results obtained with dibekacin. From both biomembranes, i.e. red

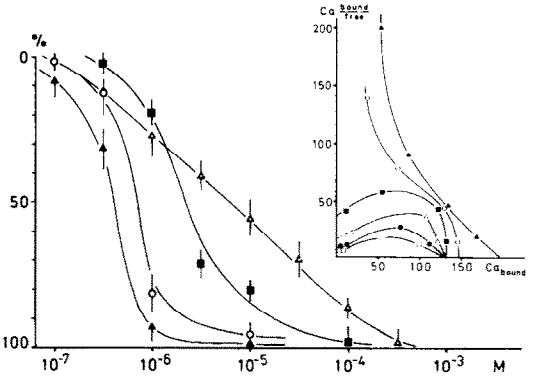


Fig. 4. Replacement of Ca from PS monolayers by dibekacin. Dose–response curves were obtained at different $Ca^{2+}$ concentrations: $1 \times 10^{-4}$ M (△), $3 \times 10^{-5}$ M (■), $1 \times 10^{-5}$ M (○) and $3 \times 10^{-6}$ M (▲). Ordinate: Percentage of $^{45}Ca$ replaced; abscissa: molar concentrations of the drug. Symbols represent means ± S.E.M. ($n = 5$). Inset: The same data presented as a Scatchard plot. Control (▲), dibekacin concentrations: $3 \times 10^{-7}$ M (○), $6 \times 10^{-7}$ M (■), $1 \times 10^{-6}$ M (△), $1.8 \times 10^{-6}$ M (●) and $3 \times 10^{-6}$ M (□). Ordinate: Ratio Ca bound (pmoles per square centimetre of monolayer) divided by free $Ca^{2+}$ concentration (moles of Ca per millilitre of aqueous phase); abscissa: Ca bound (pmoles) per square centimetre of monolayer.

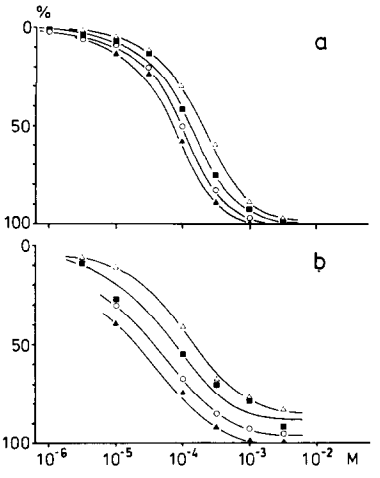


Fig. 5. Replacement of Ca by dibekacin from red cell ghosts (a) and plasmalemma from guinea pig heart muscles (b). Dose–response curves were obtained at different $Ca^{2+}$ concentrations: $1 \times 10^{-3}$ M (△), $3 \times 10^{-4}$ M (■), $1 \times 10^{-4}$ M (○) and $3 \times 10^{-5}$ M (▲). Ordinates: Percentage decrease of the replaceable Ca bound to the membranes; abscissa: molar concentrations of the drug. For sake of clarity the S.E.M. were omitted; S.E.M. were in the range 5–10% (a) and 10–15% (b).

cell ghosts and cardiac plasmalemma, Ca could be replaced by the aminoglycoside in a dose-dependent manner irrespective of the $Ca^{2+}$ concentration (range $3 \times 10^{-5}$–$10^{-3}$ M). Amikacin proved to be similarly potent, whereas streptomycin was less active. At low $Ca^{2+}$ concentrations, the $ID_{50}$ of debekacin amounted to about $10^{-4}$ M for the red cell ghosts and to about $3 \times 10^{-5}$ M for the plasmalemma. The aminoglycoside concentrations necessary to replace Ca from biomembranes are thus higher than those replacing Ca from PS monolayers at similar $Ca^{2+}$ concentrations. In principle, aminoglycoside antibiotics also interfere with the Ca binding to biomembranes.

## DISCUSSION

Aminoglycoside antibiotics specifically damage the epithelial cells of the renal proximal tubules. This toxic action involves three steps: the uptake of the antibiotics into the cells, the intralysosomal lipid storage, and finally cell necrosis. Concerning the first step, the aminoglycoside antibiotics are highly water-soluble compounds which hardly penetrate into any cell except the proximal tubule cells. As elucidated by Just *et al.* [4] and Just and Habermann [19], the proximal tubular epithelium possesses a polybase "transport" essential for reuptake of basic oligopeptides. The polybasic aminoglycosides fit into the "transport" system and are taken up by receptor-mediated endocytosis. By fusion with endocytotic vesicles aminoglycoside antibiotics are transferred to the lysosomes. In the present paper we report on an interaction between aminoglycoside antibiotics and a polar lipid. The results are thought to offer an explanation for the lipid accumulation within the lysosomal apparatus observed in proximal tubule cells upon systemic treatment by these antibiotics. In addition, an interaction of aminoglycosides with biomembranes could also be demonstrated. The interaction might facilitate the disruption of lysosomal membranes, which then may lead to cell necrosis.

As shown by dose–response curves (Fig. 1), all 13 aminoglycoside antibiotics investigated are able to totally replace the Ca bound to the PS monolayer. Under standard conditions the half maximum concentrations ($ID_{50}$s) range between $3 \times 10^{-7}$ and $10^{-6}$ M except for the streptomycins which display $ID_{50}$s of about $6 \times 10^{-6}$ M. Thus, the affinities of the aminoglycosides are rather high at a $Ca^{2+}$ concentration of $1.2 \times 10^{-5}$ M which might mimic the intralysosomal $Ca^{2+}$ concentrations. When the protonation of the aminoglycosides is promoted by decreasing the pH of the subphase, the affinities increase until pH values are reached at which the protonation can be considered complete. In contrast to the bulk of aminoglycosides, the affinities of streptomycins were nearly independent of pH changes (Fig. 2); this is in accordance with the presence of only one amino group and the strongly basic properties of the two guanidyl groups.

By studying the interaction of aminoglycosides with the PS monolayer over a large range of $Ca^{2+}$ concentrations ($3 \times 10^{-6}$–$10^{-4}$ M) a competitive antagonism was revealed in the case of streptomycin (Fig. 3), whereas dibekacin, an example of an aminoglycoside with a high affinity, demonstrated a rather complex interaction (Fig. 4). Both the shape of the dose–response curve and the Scatchard analysis suggest positive cooperativity, i.e. the replacement of small amounts of Ca by dibekacin facilitates further Ca substitution by the aminoglycoside.

The degradation of polar lipids by lysosomal lipases is critically dependent upon the micellar structure and the surface charge of the substrate [20, 21]. It is conceivable that the binding of aminoglycoside antibiotics to polar lipids—instead of Ca—alters the physicochemical properties of the lipid as substrates and renders them indigestible for the lysosomal lipases. In fact, this has recently been demonstrated by Hostetler and Hall [22]. Thus it becomes understandable that polar lipids accumulate within the lysosomes of proximal tubule cells upon treament with aminoglycoside antibiotics, as it has extensively demonstrated for cationic amphiphilic compounds inducing a generalized lysosomal storage of polar lipids [23, 24].

Our study has been extended to possible interference of aminoglycosides with Ca binding to biomembranes. As shown with red cells ghosts and cardiac sarcolemma, the antibiotics were able to displace Ca from these structures. The effect proved to be dose-dependent (see Fig. 5). Increasing the $Ca^{2+}$ concentration of the medium resulted in a parallel shift of the dose–response curves to the right. As shown in an additional experiment with intact cells, the interaction between aminoglycosides and Ca led to functional consequences. When the sedimentation rate of red cells was determined in heparinized blood, the presence of aminoglycoside antibiotics retarded the sedimentation rate, dibek-

acin being effective in the range $10^{-4}$–$10^{-5}$ M. In the case of isolated guinea pig atria (temperature 32°, $Ca^{2+}$ concentration 1.8 mM, stimulation frequency 3 Hz), the contractile force could be reduced by dibekacin and streptomycin in a dose-dependent manner. The threshold concentration was estimated to be about $10^{-4}$ M. A strong negative inotropic effect of the aminoglycosides could completely be overcome by addition of Ca yielding a final concentration of 2.8 mM. We conclude from these results that aminoglycoside antibiotics inferfere not only with the Ca binding to lipid monolayers but also with Ca binding to biomembranes resulting in functional disturbances.

The nephrotoxic action of aminoglycoside antibiotics is accompanied by a loss of brush border enzymes suggesting damage to the luminal plasmalemma, and by disruption of cytosomal membranes leading to necrosis of tubular epithelial cells. As shown in the present work, aminoglycosides interact with biomembranes as indicated by Ca replacement. Most recently Williams *et al.* [25] reported on a decrease in the Ca content and binding of the basolateral membranes of tubular cells of rats treated by gentamicin. It is tempting to speculate that the drug–membrane interaction might participate in the damage of cellular membranes occurring in the nephrotoxic processes induced by treatment with aminoglycoside antibiotics.

In conclusion, aminoglycoside antibiotics possess high affinities for polar lipids. The complex formation is thought to be responsible for the lipid accumulation of polar lipids in lysosomes of proximal tubule cells (lamellar inclusion bodies). This interaction can also be demonstrated to occur with biomembranes which may result in functional disturbances. It is conceivable that the replacement of Ca from cellular membranes by aminoglycosides promotes the disruption of membranes of storage-affected lysosomes, which are exposed to high concentrations of aminoglycosides, but still contain active enzymes. The result will be cell necrosis.

*Acknowledgements*—This work was supported by a grant of the Deutsche Forschungsgemeinschaft (Lu 31/25-3), Bonn-Bad Godesberg. We are grateful to the drug firms which kindly supplied the antibiotics.

## REFERENCES

1. A. M. Walter and L. Heilmeier, in *Antibiotika-Fibel* (Eds H. Otten, M. Plempel and W. Siegenthaler), p. 362. Thieme, Stuttgart (1975).
2. G. J. Kaloyanides and E. Pastoriza-Munoz, *Kidney Int.* **18**, 571 (1980).
3. G. Aubert-Tulkens, F. van Hoof and P. Tulkens, *Lab. Invest.* **40**, 481 (1979).
4. M. Just, G. Erdmann and E. Habermann, *Naunyn-Schmiedeberg's Archs Pharmac.* **300**, 57 (1977).
5. S. Lodhi, N. D. Weiner, J. Mechigian and J. Schacht, *Biochem. Pharmac.* **29**, 597 (1980).
6. S. Lodhi, N. D. Weiner and J. Schacht, *Biochim. biophys. Acta* **426**, 781 (1976).
7. H. Lüllmann, H. Plösch and A. Ziegler, *Biochem. Pharmac.* **29**, 2969 (1980).
8. E. Rojas and J. M. Tobias, *Biochim. biophys. Acta* **94**, 394 (1965).
9. T. Seimiya and S. Ohki, *Biochim. biophys. Acta* **298**, 546 (1973).
10. J. T. Dodge, C. Mitchell and D. J. Hanahan, *Archs Biochem.* **100**, 119 (1962).
11. D. J. Hanahan and J. E. Ekholm, in *Methods of Enzymology*, Vol. 31: *Biomembranes* (Eds S. Fleischer and L. Packer), Part A, p. 168. Academic Press, London (1974).
12. H. Lüllmann and T. Peters, in *Recent Advances in Studies on Cardiac Structure and Metabolism*, Vol. 9: *The Sarcolemma* (Eds P. E. Roy and N. S. Dhalla), p. 311. University Park Press, Baltimore (1976).
13. A. Zachowski, L. Lelievre, J. Aubry, D. Charlemagne and A. Paraf, *Proc. natn. Acad. Sci. U.S.A.* **74**, 633 (1977).
14. O. Scharff and B. Foder, *Biochim. biophys. Acta* **509**, 67 (1978).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
16. K. Imai, *J. biol. Chem.* **249**, 7607 (1974).
17. B. Larsson and H. Tjälve, *Biochem. Pharmac.* **28**, 1181 (1979).
18. B. Larsson, M. Nielsson and H. Tjälve, *Acta pharmac. tox.* **48**, 269 (1981).
19. M. Just and E. Habermann, *Naunyn-Schmiedeberg's Archs Pharmac.* **300**, 67 (1977).
20. R. M. C. Dawson and A. D. Bangham, *Biochem. J.* **72**, 493 (1959).
21. G. H. de Haas, N. M. Postema, W. Nienwenhuizen and L. L. M. van Deenen, *Biochim. biophys. Acta* **159**, 118 (1968).
22. K. Y. Hostetler and L. B. Hall, *Proc. natn. Acad. Sci. U.S.A.* **79**, 1663 (1982).
23. H. Lüllmann, R. Lüllmann-Rauch and O. Wassermann, *Biochem. Pharmac.* **27**, 1103 (1978).
24. R. Lüllmann-Rauch, in *Lysosomes in Applied Biology and Therapeutics* (Eds J. T. Dingle, P. J. Jacques and I. H. Shaw), Vol. 6, p. 49. North-Holland, Amsterdam (1979).
25. P. D. Williams, P. D. Holohan and C. R. Ross, *Toxic. appl. Pharmac.* **61**, 243 (1981).