Biochemical Pharmacology, Vol. 29, pp. 2969–2974.

0006-2952/80/1101-2969 $02.00/0

Ca REPLACEMENT BY CATIONIC AMPHIPHILIC DRUGS FROM LIPID MONOLAYERS

H. LÜLLMANN, H. PLÖSCH and A. ZIEGLER
Department of Pharmacology, Hospitalstrasse 4–6, 23 Kiel, Federal Republic of Germany

(*Received* 21 *May* 1980; *accepted* 26 *June* 1980)

Abstract—Using ^{45}Ca, the binding of Ca to phospholipid monolayers was determined by means of an endwindow Geiger counter. Phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol accumulated Ca dependent upon the pH of the aqueous subphase, whereas no binding of Ca to phosphatidylcholine monolayers could be detected. The presence of cationic amphiphilic drugs decreased the Ca binding in a dose-dependent manner. The potencies of 30 drugs for replacing Ca are given in ID_{50} values. From the results it is concluded that a protonated nitrogen is necessary for competition with Ca for binding sites at lipid monolayers; the potency for replacing Ca is, however, determined by the hydrophobicity of the molecule. It is believed that the procedure applied provides information on the interaction of amphiphilic drugs with biomembranes.

Cationic amphiphilic compounds possess the tendency to interact with polar lipids. As many drugs belong to this group of agents, the interaction seems to be of significance from at least two pharmacological aspects:

(1) the drug-induced lipid storage disease;

(2) drug accumulation at water–lipid interphases of cellular membranes, the consequence of which might be nonspecific alterations of membrane functions.

A simple way to investigate drug–lipid interaction is to use lipid monolayers. Changes of surface pressure [1–4], surface potential [5–7] or of binding of calcium by monolayers [2, 3, 8–10] have been measured to characterize the drug–lipid interaction. In the present study a number of cationic amphiphilic drugs were investigated with respect to their affinities towards different phospholipids. Various agents from therapeutically different groups were employed, such as local anaesthetics, β-blockers, psychotropic drugs and anti-anginal drugs. The selection of compounds was made particularly according to whether or not they (a) can exert unspecific cardiodepressive effects, and (b) can induce lysosomal lipid storage [11–13]. The results suggest that the replacement of calcium from lipid monolayers is a useful parameter for measuring the potency of cationic amphiphilic drugs at interacting with polar lipids under physiological conditions.

METHODS

The radiation of an isotope can be measured on an indefinite thin layer if the radiation is of low energy, that is when only disintegrations occurring at the surface are detected, whereas those below the surface are lost by absorption. ^{45}Ca fulfills this requirement and thus provides the possibility for observation of concentration changes occurring at the surface of an aqueous solution by lipid monolayers. Therefore, we determined the radioactivity of an aqueous ^{45}Ca-solution before and after spreading polar lipids on the surface. The difference was taken as a measure of Ca binding by the lipid. The determinations were repeated in the presence of different concentrations of various drugs to establish dose–response curves indicating the ability of the drugs to replace calcium from the lipid layer.

The aqueous phase consisted of a 2 mM TES*-histidine buffer adjusted to the desired pH (range 5.0–10.0) by NaOH and HCl. The Ca concentration in all experiments amounted to 1.2×10^{-5}M. Tracer amounts of ^{45}Ca were added to give a specific activity of about 14 Ci/mole in the solution. The temperature was kept constant at 24°.

Four phospholipids were investigated with respect to Ca binding ability, i.e. phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI), all purchased from Sigma Chemical Co. (München, F.R.G.). The lipids were dissolved in chloroform and placed on the aqueous surface to yield a surface pressure of 10–12 mN m^{-1}.

The ^{45}Ca radiation was determined by a methane flow tube with an endwindow (0.9 mg/cm^2, diameter 6.7 cm) directly placed above the surface. A gas-tight sample exchanger warranted the exact position of the planchettes below the tube (Frieseke & Hoepfner, Erlangen, F.R.G.; electronic equipment from Berthold, Wildbad, F.R.G.). Under the given conditions, the yield amounted to about 7000 cpm from the aqueous solution, and increased to a maximum of about 14,000 when a lipid monolayer was spread on the surface. Thus, about 7000 cpm were available for measuring the calcium-replacing effects of the drugs.

To estimate the absolute amount of calcium present in the surface layer, the system was standardized by samples of known specific activities [9]. The

*N-tris (hydroxymethyl)-methyl-2-amino-ethanesulfonic acid.

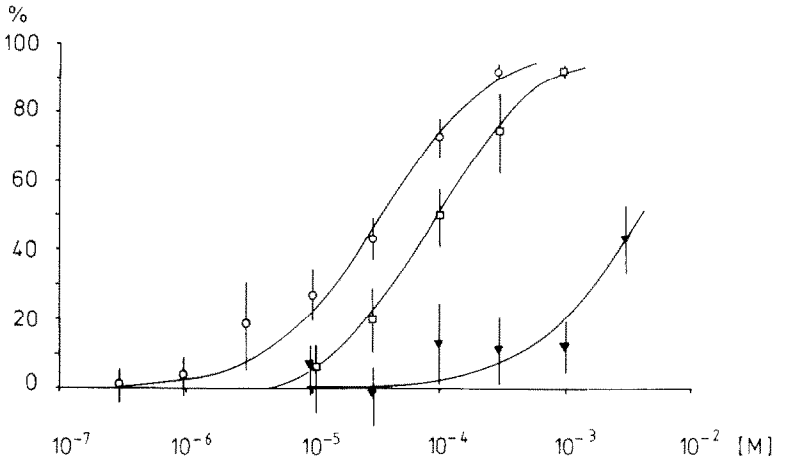

Fig. 1. Displacement of calcium from phosphatidylserine monolayers by dibucaine (—○—), tetracaine (—□—) and procaine (—▼—). Ordinate: Percentage of ^{45}Ca displaced, 100% refers to complete displacement; abscissa: molar concentrations of drugs. Symbols represent means ± S.E. (N = 6).

amount of calcium bound to the lipid layer could, therefore, be expressed as moles Ca/moles lipid. The drug effects are given as ID_{50} values obtained from the dose–response curves indicating the concentration which displaces 50 per cent of the calcium bound by the lipid layer. Since all dose–response curves obtained were parallel, as demonstrated for three local anaesthetics in Fig. 1, ID_{50} values were sufficient to characterize the affinity of the drugs in question.

RESULTS

pH dependence of Ca *binding.* The Ca accumulation by lipid monolayers as a function of pH of the aqueous phase is depicted in Fig. 2. PC was not included because Ca binding could not be detected over the entire pH range. The Ca binding of PE, PI and PS increased with increasing pH. The lowest Ca binding capacity was found with PE, increasing from about 0.03 to 0.2 moles Ca/moles lipid in the pH range. PI reached a plateau at a pH 6.5 amounting to 0.3 moles Ca/mole PI. The pH curve obtained with PS suggests the presence of two steps at 6.5 and 9.0, respectively. At physiological pH the binding capacity of PS was about 0.28 moles Ca/moles at a Ca concentration of 1.2×10^{-5}M in the subphase.

Drug effects on Ca *binding at pH 7.5.* In Figs. 3a and b, the ID_{50} value of drugs belonging to certain groups are depicted. As a rule, the highest drug concentrations were required with PS monolayers and the lowest drug concentrations were needed with PE monolayers to replace equal amounts of Ca; PI took an intermediate position. Among the local anaesthetics, procaine was the compound with the

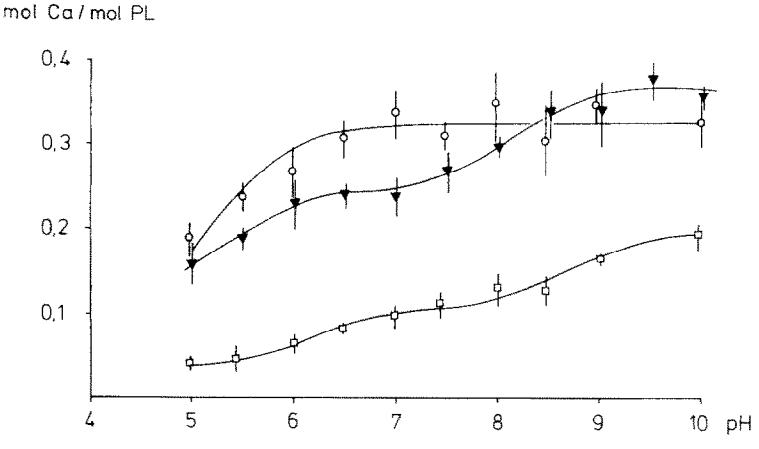

Fig. 2. Binding of calcium by phospholipid (PL) monolayers dependent upon the pH of the aqueous phase (abscissa). Ordinate: bound ^{45}Ca expressed as molar ratio. Phosphatidylethanolamine (—□—), phosphatidylserine (—▲—) and phosphatidylinositol (—○—). Symbols represent means ± S.E. (N = 6).

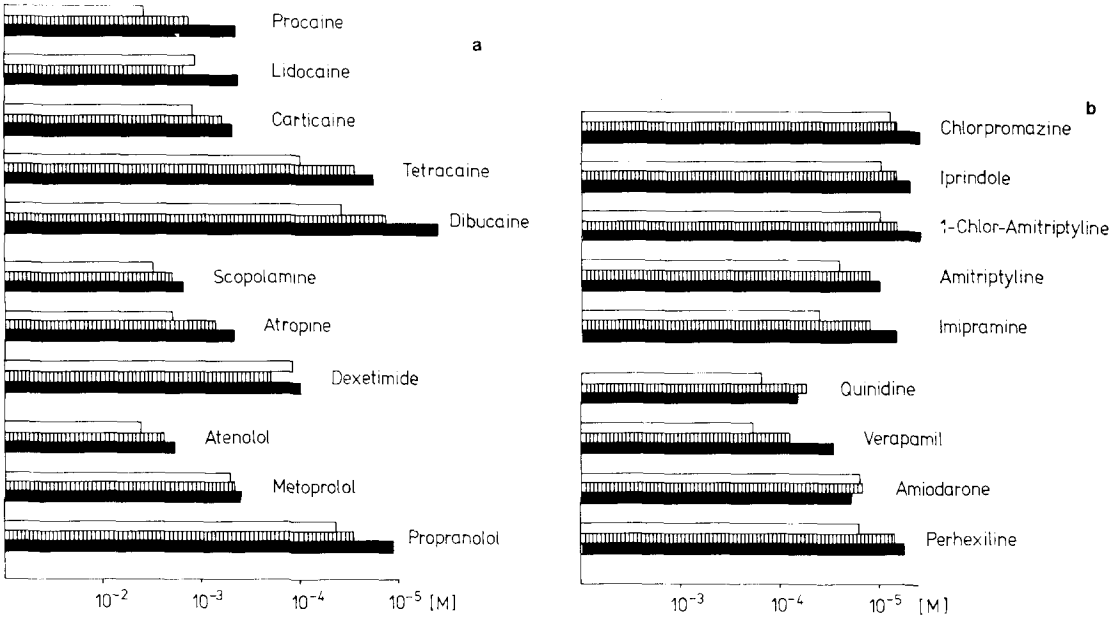

Fig. 3. Concentration of drugs replacing 50 per cent calcium (ID_{50}) bound to phospholipid monolayers. The length of the horizontal columns indicates the affinity of the drugs (abscissa) to phosphatidylserine (open columns), phosphatidylinositol (hatched columns) and phosphatidylethanolamine (dark columns). The drugs are listed according to the group to which they belong: local anaesthetics, cholinolytics, β-blockers, psychotropic drugs and cardiac drugs.

lowest affinity and dibucaine that with the highest affinity, the difference between the two drugs amounting to about 100-fold. A similar difference was found between the two β-blocking agents atenolol and propranolol, metoprolol taking an intermediate position (Fig. 3a). Only slight differences were obtained in the group of psychotropic drugs, the ID_{50} values of which lay in the range of 3×10^{-5} and 3×10^{-6}M for chlorpromazine, iprindol, 1-chlor-amitriptyline, amitriptyline and imipramine. Among the cardiac drugs, perhexeline possessed a remarkable affinity to PI and PE (ID_{50} of $6\text{–}7 \times 10^{-6}$M), amiodarone, verapamil and quinidine displayed lower affinities (Fig. 3b).

All drugs investigated at pH 7.5 are summarized in Table 1, which presents the ID_{50} values of Ca replacement for PS, PI and PE, the logarithm of the octanol/water coefficient, and pK_a values.

pH dependence of drug effects. Most of the drugs under study possess pK_a values >7. For the sake of comparison we tested a few of them at pH 7.5 and 9.5. As shown in Fig. 4 for procaine (pK_a 8.9) and for dibucaine (pK_a 8.8), higher concentrations of the compounds were required to replace 50 per cent of the PS-bound Ca at pH 9.5 than at 7.5. This is in favour of the assumption that only the protonated form of the molecules is able to compete for the bound Ca. In order to obtain further support we

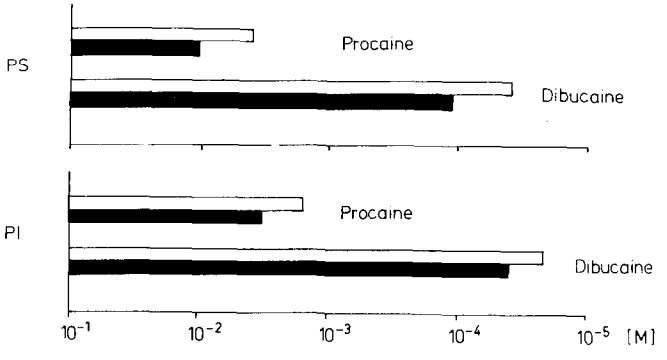

Fig. 4. Concentration of two local anaesthetics replacing 50 per cent of the bound calcium at two different pH values. Open columns: pH 7.5, dark columns: pH 9.5. Phosphatidylserine (PS) above, phosphatidylinositol (PI) below.

Table 1. Investigated drugs, ID_{50} to replace Ca from PS-, PI- and PE-monolayers; the respective partition coefficients and pK_a values

Compound	ID_{50} PS $\times 10^5$M	ID_{50} PI $\times 10^5$M	ID_{50} PE $\times 10^5$M	Partition coefficient (octanol/water) Log p	Ref.	pK_a	Ref.
1. Sodium chloride	3500	3700	—	−2.95	[17]	—	
2. 3-Amino-pyridine	3000	—	—	0.11	[17]	6	[20]
3. 2-Amino-pyridine	1200	—	—	0.2	†	6.95	[20]
4. Ammonia	1100	1000	—	−1.3	[17]	9.25	[24]
5. Atenolol	450	250	200	0.17	[19]	9.5	‡
6. Procaine	400	150	50	1.9	[17]	8.9	[6]
7. 4-Aminopyridine	300	—	—	0.28	[17]	9.25	[20]
8. l-Scopolamine	300	250	170	0.8	*	7.55	[20]
9. Atropine	200	70	50	1.76	[18]	10	[21]
10. Carticaine	120	65	50	2.41	[18]	9.5	‡
11. Lidocaine	250	200	80	2.52	*	7.9	‡
12. Metoprolol	90	50	45	2.34	[18]	9.5	‡
13. Phentermine	70	40	20	1.9	[19]	10.1	[19]
14. Quinine	4.5	6	8	1.78	[17]	8.34	[21]
15. Quinidine	5	6	7	1.78	†	8.77	[20]
16. Verapamil	12	8	3	2.51	[18]	9.2	‡
17. Dexetimide	12	20	10	3.55	[18]	8.7	‡
18. Tetracaine	10	1.1	2	3.73	[17]	8.24	[6]
19. Chlorphentermine	8	5	3	2.6	[19]	9.6	[19]
20. Propranolol	3	3	1.2	3.14	[19]	9.6	[19]
21. Dibucaine	3.5	1.4	0.4	4.3	[17]	8.83	[23]
22. Imipramine	4	1.3	0.7	4.62	[17]	9.5	[22]
23. Amitriptyline	2.5	1.3	1	4.92	[17]	9.4	[22]
Amiodarone	1.6	1.7	1.8	—	—	—	
24. Chloroquine	3	0.4	0.4	4.63	[17]	10.1/8.1	[20]
25. Perhexiline	1.5	0.7	0.6	5.0	*	10.5	‡
26. l-Chloramitryptiline	1	0.7	0.4	5.55	[19]	9.4	[19]
27. Iprindol	1	0.7	0.5	4.9	*	8.2	‡
28. Chlorpromazine	0.7	0.7	0.4	5.33	[17]	9.3	[22]
29. Mepacrine	0.5	0.22	0.22	6	*	10.2/7.7	[20]

* Calculated according to formula of Leo *et al.* [17].

† The distribution coefficient of quinidine is assumed to be equal to that of quinine and that of 2-aminopyridine to be equal to that of 3-aminopyridine.

‡ According to product information given by the manufacturer.

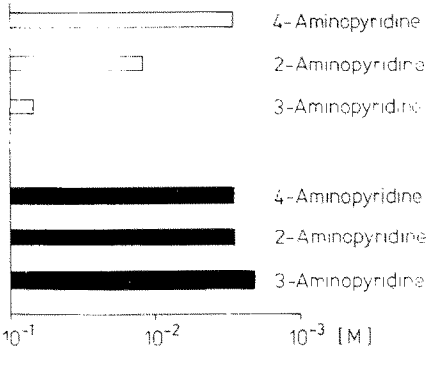

Fig. 5. Concentration of three isomeric aminopyridines possessing different pK_a replacing 50 per cent of Ca bound to phosphatidylserine monolayers at pH 7.5 (open columns). The dark columns resulted when corrected for the actual concentration of the protonated form. pK_a for 4-aminopyridine, 9.25; for 3-aminopyridine, 6.95; and for 2-aminopyridine, 6.0.

studied three closely related compounds with different pK_a values, i.e. 4-aminopyridine (pK_a 9.25), 3-aminopyridine (pK_a 6.95) and 2-aminopyridine (pK_a 6.0). At pH 7.5 the potency of the isomers for replacing Ca from PS ranged from 3×10^{-3}M to 7×10^{-2}M (Fig. 5). If the total concentration was corrected for the protonated species, the three isomers became equally potent, again suggesting that only the charged form is active with respect to replacement from polar lipids.

DISCUSSION

The actual amount of Ca which is bound to lipid monolayers depends on the Ca concentration of the subphase. This explains the differing data reported in the literature (10^{-4}M Ca, Ref. 2; 8×10^{-5}M Ca, Ref. 9; 10^{-7}M Ca, Ref. 3). The present results obtained with an intermediate concentration are in line with the published figures. Similarly, the potency of drugs for replacing Ca from lipid monolayers is essentially influenced by the actual Ca concentration, the apparent potency increasing with declining Ca

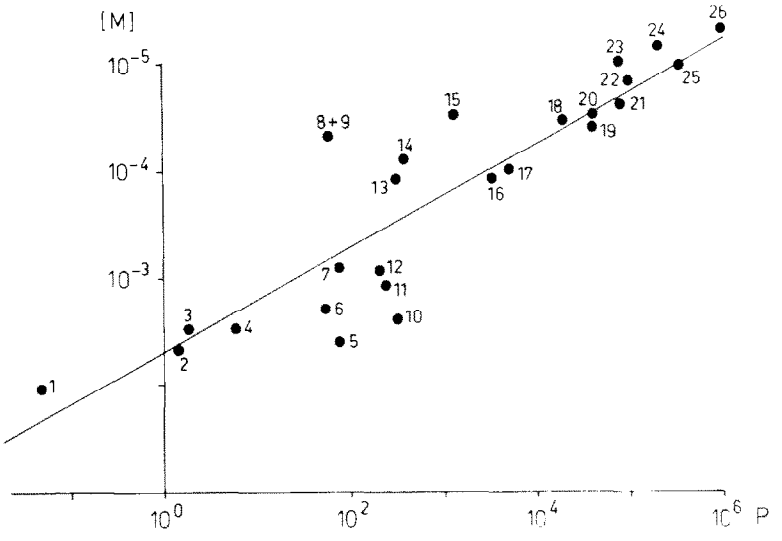

Fig. 6. Relationship between the ID_{50} values (ordinate) representing the efficiency for displacing Ca from PS-monolayers and the partition coefficients (octanol/water) for 26 compounds (abscissa). The line is the regression obtained when using log values for both parameters. The numbering corresponds to that given in Table 1.

concentrations [3, 14, 15]. This implies that using ID_{50} values of drugs as a measure of their ability to compete with Ca for binding sites at lipids is only valid for defined conditions. Irrespective of these limitations, the order found for different compounds is indicative of their relative affinities to the Ca binding sites at lipid monolayers.

Our results suggest that the protonation of the amine group is the prerequisite for an interaction with the Ca binding site in polar lipids; the affinity, however, is determined by the hydrophobicity of the drug molecules. This can be demonstrated by a plot of the log ID_{50} values obtained for PS vs the logarithm of the partition coefficient octanol/water of the free bases (Fig. 6). A reasonable correlation is obtained indicating that a 100-fold increase of the hydrophobicity leads to an almost 10-fold increase of the affinity. The same holds true for the two other lipids as can be taken from the regression lines:

for PS, $\log ID_{50} = -0.49 \log p - 2.32$, $r = 0.90$;

for PI, $\log ID_{50} = -0.48 \log p - 2.67$, $r = 0.93$;

for PE, $\log ID_{50} = -0.54 \log p - 2.60$, $r = 0.90$.

As mentioned in the introductory section, the cationic amphiphilic drugs are of particular interest with respect to two pharmacological side effects: the lipid storage disease occurring upon chronic treatment and the unspecific cardiodepressive effects. According to our experience, the ID_{50} values reported above, taken as an indicator for the affinity of drugs to phospholipids, correlate fairly well with the potency of the drugs for inducing a lysosomal accumulation of polar lipids; at least if the simple system of cultured cells is considered, a system which is devoid of superimposed processes such as metabolic alteration of drug molecules [16]. Furthermore, there is an obvious correlation between the ID_{50} values and the acute cardiodepressive action of a number of drugs investigated, such as local anaesthetics, β-blockers and psychotropic drugs.

In conclusion, the determination of ^{45}Ca replacement from phospholipid monolayers by cationic amphiphilic drugs provides a simple tool to estimate the affinity of the compounds to polar lipids. These estimates allow tentative predictions concerning the potencies of compounds for accumulating in biological membranes and thus exerting unspecific effects. Furthermore, tentative predictions can be made concerning the potency for inducing a lipid storage disease which is the consequence of a direct drug–lipid interaction within the lysosomes [12]. Finally, it should be mentioned that the experimental procedure does not require radioactively labelled drugs.

Acknowledgements—The work was supported by a grant of the Deutsche Forschungsgemeinschaft (Lu 31/24), Bonn-Bad Godesberg and by Bayer AG, Leverkusen.

REFERENCES

1. J. A. Clements and K. M. Wilson, *Proc. natn. Acad. Sci. U.S.A.* **48**, 1008 (1963).
2. E. Rojas and J. M. Tobias, *Biochim. biophys. Acta* **94**, 394 (1965).
3. H. Hauser and R. M. C. Dawson, *Europ. J. Pharmac.* **1**, 61 (1967).
4. H. Hauser and R. M. C. Dawson, *Biochem. J.* **109**, 909 (1968).
5. D. Papahadjopoulos, *Biochim. biophys. Acta* **163**, 240 (1968).
6. S. Ohki and R. Sauve, *Biochim. biophys. Acta* **511**, 377 (1978).
7. A. D. Bangham and W. Mason, *Br. J. Pharmac.* **66**, 259 (1979).
8. H. Hauser, D. Chapman and R. M. C. Dawson, *Biochim. biophys. Acta* **183**, 320 (1969).

9. T. Seimiya and S. Ohki, *Biochim. biophys. Acta* **298**, 546 (1973).
10. T. Seimiya, M. Ashida, T. Muamatsu, I. Hara and M. Hayash, *Chem. Phys. Lipids* **22**, 221 (1978).
11. H. Lüllmann, R. Lüllmann-Rauch and O. Wassermann, *Crit. Rev. Tox.* **4**, 185 (1975).
12. H. Lüllmann, R. Lüllmann-Rauch and O. Wassermann, *Biochem. Pharmac.* **27**, 1103 (1978).
13. 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).
14. M. P. Blaustein, *Biochim. biophys. Acta* **135**, 653 (1967).
15. S. Lodhi, N. D. Weiner and J. Schacht, *Biochim. biophys. Acta* **426**, 781 (1976).
16. D. Drenckhahn, L. Kleine and R. Lüllmann-Rauch, *Lab. Invest.* **35**, 116 (1976).
17. A. Leo, C. Hansch and D. Elkins, *Chem. Rev.* **71**, 525 (1972).
18. H. Lüllmann, P. B. M. W. M. Timmermans, G. M. Weikert and A. Ziegler, *J. med. Chem.* **23**, 560 (1980).
19. H. Lüllmann and M. Wehling, *Biochem. Pharmac.* **28**, 3409 (1979).
20. D. D. Perrin, *Dissociation Constants of Organic Bases in Aqueous Solution*. Butterworth, London (1965).
21. J. Clark and D. O. Perrin, *Q. Rev. Chem. Soc.* **18**, 295 (1964).
22. A. L. Green, *J. Pharm. Pharmac.* **19**, 10 (1967).
23. F. Merki, J. Büchi and X. Perlia, *Arzneimittelforsch.* **25**, 997 (1975).
24. H. Beyer, *Lehrbuch der Organischen Chemie*, Hirzel Verlag, Stuttgart (1973).