Biochimica et Biophysica Acta, 643 (1981) 398—406
Elsevier/North-Holland Biomedical Press

BBA 79206

THE UPTAKE OF AMINES BY POLYMORPHONUCLEAR LEUKOCYTES

BEVERLY H. DULIS * and IRWIN B. WILSON

Department of Chemistry, University of Colorado, Campus Box 215, Boulder, CO 80309 (U.S.A.)

(Received August 6th, 1980)
(Revised manuscript received December 18th, 1980)

Key words: Muscarinic ligand; β-Adrenergic ligand, Amine uptake; (Polymorphonuclear leukocyte)

Summary

Muscarinic and β-adrenergic ligands associate with polymorphonuclear leukocytes to show high-affinity, saturable accumulation. This association can be distinguished from specific receptor binding by its temperature dependence, sensitivity to pH, requirement of an energy source, inhibition by ionophores, and inhibition by a variety of permeable basic amines.

Our results suggest that these amines accumulate in acidic lysosomes which are plentiful in these cells. This permeable amine effect can be inhibited without affecting specific receptor binding.

Introduction

We have recently been studying the uptake of the muscarinic ligand 3-quinuclidinyl benzilate [1] and the β-adrenergic ligand dihydroalprenolol by live human polymorphonuclear leukocytes. This uptake resembles specific receptor binding in that the uptake is saturable at low ligand concentrations, is reversible and can be displaced by other pharmacological ligands with a degree of the anticipated specificity.

We present evidence here that a large portion of the retention of these ligands is due to a nonspecific accumulation of permeable basic amines, which is distinguishable from receptor binding.

* To whom correspondence should be addressed.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Experimental procedures

Materials. [^{3}H]Quinuclidinyl benzilate, 16 Ci/mmol, and (−)-[^{3}H]dihydroalprenolol, 30 Ci/mmol, were purchased from Amersham. Oxotremorine, pilocarpine and CCCP were purchased from Aldrich Chemical Co., and acridine orange from Fisher. Hanks buffered salt solution was prepared according to the GIBCO catalogue and contained: 138 mM NaCl; 5.4 mM KCl; 0.34 mM Na_2HPO_4; 0.44 mM KH_2PO_4; 0.41 mM $MgSO_4$; 0.49 mM $MgCl_2 \cdot 6H_2O$; 1.3 mM $CaCl_2$; 5.5 mM glucose; and 4.2 mM $NaHCO_3$, at pH 7.4. All other reagents were from Sigma Chemical Company.

Cell isolation. Cells were isolated by the method of Boyum [2], and contaminating erythrocytes subjected to hypotonic lysis. The cells, largely polymorphonuclear leukocytes with some mononuclear cell and erythrocyte ghost contamination, were suspended in Hanks balanced salt solution. Polymorphonuclear leukocytes were always more than 90% of the leukocytes, and usually more than 97%. More than 95% of the polymorphonuclear leukocytes were neutrophils. Viability was always greater than 95% and usually greater than 99%.

Binding of [^{3}H]Quinuclidinyl benzilate and [^{3}H]dihydroalprenolol to cell preparations. Cell preparations were diluted to contain $(1.5—8) \cdot 10^6$ cells in 1.0 ml total incubation volume. The buffer used was Hanks balanced salt solution supplemented with 25 mM Hepes (pH 7.4), except as noted. In the adrenergic work, the buffer included 0.8 mM ascorbic acid and 0.3 mM catechol to inhibit catecholamine metabolism. Assays were started by the addition of tritiated ligand and continued for 30 min at 25°C, except as noted. Polystyrene culture tubes, which do not bind either the labeled ligands or the cells, were used for all incubations. All solutions were prepared before use.

The assays were terminated by addition of 3 ml ice-cold buffer followed by rapid filtration on Whatman GF/C filters using vacuum filtration. The filters were washed four times with 2 ml ice-cold buffer for a total filtration time of about 10 s. Filters were allowed to solubilize overnight in a toluene-based scintillation mixture before counting with a tritium efficiency of 35%.

Cell viability in the presence of the amines used for inhibition studies was checked by cellular ability to exclude trypan blue, retention of the cytoplasmic enzyme lactate dehydrogenase, and reversibility of the observed inhibition. In no case was there evidence of irreversible toxicity of the compounds studied. Similarly, no irreversible changes in the cells' ability to take up amines were observed after incubations at different temperatures.

Rat brain and heart homogenates. The heart and brain of a male Wistar rat were homogenized by mincing and grinding in a glass-glass tissue homogenizer in cold phosphate buffer, and by disrupting in 0.32 M sucrose using a glass-Teflon homogenizer, respectively. After centrifugation for 10 min at $1000 \times g$, the supernatants were assayed for muscarinic binding activity by the method of Yamamura and Snyder [3]. Incubations contained 390 μg heart protein or 460 μg brain protein in 2 ml of 50 mM sodium phosphate/potassium phosphate (pH 7.4), and were carried out for 40 min at 25°C, except as noted. Nonspecific binding was determined by incubating with [^{3}H]quinuclidinyl benzilate and 1.0 μM atropine sulfate or 100 μM oxotremorine. Specific muscarinic binding

was defined as the difference in quinuclidinyl benzilate bound in the presence or absence of atropine or oxotremorine.

Protein was determined by the method of Lowry et al. [4], using bovine serum albumin as a standard.

Results

Preliminary binding studies. As reported earlier [1], [^{3}H]quinuclidinyl benzilate is taken up by live polymorphonuclear leukocytes. The binding curve was saturable and showed a high affinity. The uptake was inhibited by other muscarinic ligands and was reversible. The equilibrium constant at 25°C calculated from the kinetic constants ($K_d = k_{off}/k_{on}$) agreed within a factor of 3 with the value determined by equilibrium measurements. The retention of quinuclidinyl benzilate was a linear function of cell density over a 10-fold range, and fit the Hill equation with $n_H = 1$. These findings were incorrectly interpreted as indications of binding by muscarinic receptors.

Similar results have been observed with these cells using the β-adrenergic ligand [^{3}H]dihydroalprenolol.

As further specificity studies started to reveal that much of the ligand retention was unrelated to receptor binding, it became necessary to be able to distinguish the two kinds of retention. For studies using [^{3}H]quinuclidinyl benzilate, conditions and compounds that inhibited retention by live cells were tested for effects on specific muscarinic binding in rat heart and brain homogenates. For studies using [^{3}H]dihydroalprenolol, factors affecting ligand retention were studied in the presence and absence of chloroquine, which inhibits dihydroalprenolol uptake without affecting β-adrenergic receptor binding [5].

Effect of amines: lipophilicity. Several ammonia derivatives were tested for their ability to inhibit the uptake of [^{3}H]quinuclidinyl benzilate by polymorphonuclear leukocytes. Increasing methylation from ammonia to methylamine, dimethylamine and trimethylamine does not affect the observed inhibition. Each of these amines at 1 mM competes for 78 ± 2% ($n = 2$) of [^{3}H]quinuclidinyl benzilate uptake in live cells, but does not affect specific muscarinic binding in heart or brain homogenates (data not shown). Quinuclidinyl benzilate accumulation in the presence of 1 mM concentration of the quaternary amines tetramethylammonium and phenyltrimethylammonium, however, is not statistically different from the control (10 ± 6% and 15 ± 10% competition, respectively, with $n = 2$). The quaternary muscarinic ligands acetylcholine, β-methyl carbamylcholine, carbamylcholine and *N*-methyl atropinium also failed to inhibit more than a few percent of total [^{3}H]quinuclidinyl benzilate retention by live cells, even though they displace this ligand to the same extent as atropine and oxotremorine in heart and brain homogenates. The importance of lipophilicity in determining the potency of larger amines is demonstrated in Table I. 1-Naphthyl-*N*-ethylenediamine is approximately 10-times more potent than the phenyl analog. Also, increasing hydroxylation of phenylethylamine to tyramine, dopamine and norepinephrine decreases inhibition. The *N*-isopropyl derivative of norepinephrine, isoproterenol, is less polar and competes for 21% more amine retention than does the parent compound, while epinephrine (not shown) inhibits the same percentage of retention as norepinephrine. Epine-

TABLE I

INHIBITION OF [^{3}H]QUINUCLIDINYL BENZILATE UPTAKE BY PHENYL AND NAPHTHYL AMINES

Cells were incubated with 1.5 nM [^{3}H]quinuclidinyl benzilate and the indicated amine, and ligand retention determined as described under Experimental procedures.

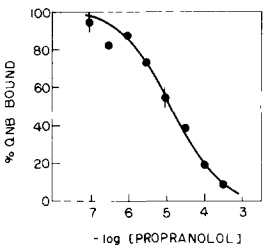
Amine	Concentration (mM)	% total uptake ± S.D. (n = 2)
1-Naphthyl-*N*-ethylenediamine	0.01	57 ± 1
	0.1	16 ± 5
Phenyl-*N*-ethylenediamine	0.1	49 ± 1
	1.0	12 ± 3
β-Phenylethylamine	0.1	49 ± 3
	1.0	11 ± 0
Tyramine	1.0	26 ± 3
Dopamine	1.0	75 ± 1
Norepinephrine	1.0	83 ± 1
Isoproterenol	1.0	62 ± 2

phrine and norepinephrine at 100 μM do not compete for amine retention, and can therefore be used in adrenergic studies to distinguish specific from nonspecific ligand retention.

The lipophilic amines propranolol and chloroquine were also used to compete for amine uptake by polymorphonuclear leukocytes. Chloroquine has been shown to specifically displace nonreceptor amine retention in these cells without affecting β-adrenergic receptor binding [5], and was therefore checked for possible effects on muscarinic binding. In brain homogenates, chloroquine shows competitive inhibition of [^{3}H]quinuclidinyl benzilate binding (data not shown), with K_i = 0.4 μM (n = 4, r = 0.998), and should thus be considered a muscarinic ligand.

Competition by propranolol for [^{3}H]quinuclidinyl benzilate retention is shown in Fig. 1. The curve was drawn using the Hill equation with n_H = 0.7 and I_{50} = 13 μM. Propranolol displacement of [^{3}H]dihydroalprenolol retention in these cells therefore includes both specific receptor competition [5] and the quantitatively more important permeable amine uptake. Similar problems of specificity in propranolol binding competition are evident in other leukocytes as well (Dulis, B., unpublished results), and are diminished but not abolished by homogenization.

Effect of amines-basicity. In order to investigate the role of amine protonation in determining inhibitory potency, lipophilic amines with different pK_a values were tested for inhibition of quinuclidinyl benzilate in these cells (Table II). The failure of 1-naphthylamine to affect uptake is readily understood because its basicity is far too low for this compound to become protonated in the lysosomes and it will not raise the pH of the lysosome. The small effect observed with acridine suggests that the pH of the lysosomes is not very much lower than 5.6, the pK_a of this compound. The other related compounds with higher basicity do inhibit quinuclidinyl benzilate retention.



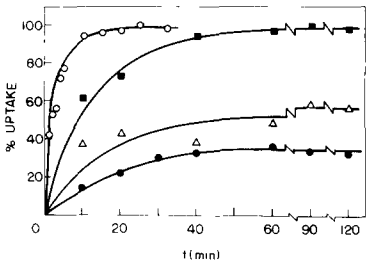


Fig. 1. Propranolol inhibition of [^{3}H]quinuclidinyl benzilate retention. (±)-Propranolol can be used to reduce total quinuclidinyl benzilate retention by live polymorphonuclear leukocytes. Data are plotted as mean values ± S.D. (n = 2), with error bars either shown or included within the symbol. Ligand retention was determined as described under Experimental procedures, using 1.1 nM ligand. Fitting the data to the Hill equation gives n_H = 0.7 and I_{50} = 13 μM for propranolol.

Fig. 2. Temperature effects on amine accumulation. Incubations were carried out with 10 nM [^{3}H]quinuclidinyl benzilate at 0°C (●), 15°C (△), 18°C (■), and 25°C (○) for the indicated times, as described under Experimental procedures.

We found that pilocarpine (pK_a = 6.8) and oxotremorine (pK_a = 7.9) [8] are equally effective in inhibiting uptake of both quinuclidinyl benzilate and dihydroalprenolol. The effectiveness of pilocarpine indicates that a compartment with a pH very much less than 6.8 is involved in the retention of amines.

Temperature effects. Although the specific binding of quinuclidinyl benzilate by rat brain homogenates reaches the same equilibrium at 2°C and 35°C (data not shown), the uptake of quinuclidinyl benzilate by live polymorphonuclear leukocytes increases both in rate and extent at higher temperature (Fig. 2). Approximately half of the retention is lost when the temperature drops from 18°C to 15°C.

At 37°C the uptake follows a different time course, reaching a maximum, and then declining to a lower steady value. This time course also occurs with dihydroalprenolol (Fig. 3). However, in the presence of chloroquine, both total

TABLE II

INHIBITION OF AMINE UPTAKE AS A FUNCTION OF pK_a

Cells were incubated with 1.6 nM [^{3}H]quinuclidinyl benzilate with various amines, as described under Experimental procedures. Very similar values were determined using 1.6 nM [^{3}H]dihydroalprenolol.

Amine	Concentration (μM)	pK_a *	% total uptake ± SD (n = 2)
Acridine	10	5.6	97 ± 2
	100		85 ± 3
Acridine orange	10	10.4	80 ± 6
	100		21 ± 6
1-Naphthylamine	10	3.9	100 ± 6
	100		106 ± 2
1-Naphthyl-*N*-ethylenediamine	10	10	56 ± 2
	100		17 ± 5

* Values from [6,7].

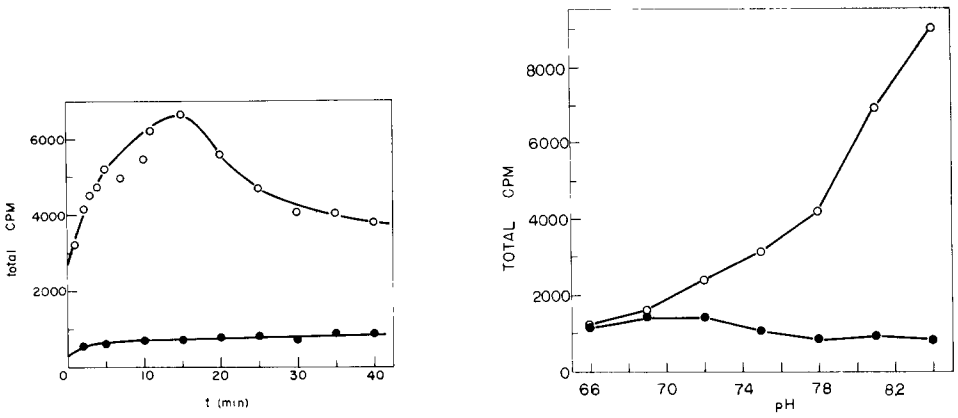


Fig. 3. Dihydroalprenolol uptake by cells at 37°C. Total ligand retention is shown in the absence (○) and presence (●) of 50 μM chloroquine using 1 nM [^{3}H]dihydroalprenolol and 4 · 10^6 cells. Propranolol at 1 μM displaced roughly 30% of the total counts after 30 min for both incubations.

Fig. 4. Effect of pH on [^{3}H]dihydroalprenolol retention. Cells were incubated in 24 mM NaP_i, 137 mM NaCl, and 5.4 mM KCl with 1.6 nM [^{3}H]dihydroalprenolol at the indicated pH values, as described under Experimental procedures. Total retention is shown in the absence (○) and presence (●) of 50 μM chloroquine. Duplicate determinations agreed within 5%.

and specific β-adrenergic binding follow simple association kinetics (Fig. 3) [5]. This unusual time course at 37°C (for both quinuclidinyl benzilate and dihydroalprenolol) must be a property of the amine accumulation system in the cells. The maximum accumulation and decline is reached earlier at higher ligand concentrations, occurring within 1 min at a ligand concentration of 10 nM.

Effect of ionophores. Two ionophores, the potassium ionophore valinomycin and the protonophore CCCP, were studied for possible effects on amine uptake (Table III). The protonophore inhibited amine retention, while valinomycin at 1 μM had little or no effect. Together, valinomycin and CCCP acted synergistically, giving a marked decrease in amine accumulation.

Effects of inhibitors of energy metabolism. The effect of inhibitors of energy metabolism on quinuclidinyl benzilate retention is shown in Table IV. The glycolytic inhibitor 2-deoxyglucose is the only inhibitor showing a definite effect.

TABLE III

EFFECTS OF VALINOMYCIN AND CCCP ON [^{3}H]QUINUCLIDINYL BENZILATE ACCUMULATION

Valinomycin and CCCP were added to incubations containing 5.8 mM potassium, and total tritium uptake was determined as described under Experimental procedures. Results are from two separate experiments.

Valinomycin (μM)	CCCP (μM)	% total uptake ± S.D. (n = 2)
1.0	—	100 ± 4
—	1.0	89 ± 4
1.0	1.0	49 ± 8
1.0	—	94 ± 1
—	10	48 ± 1
1.0	10	17 ± 2

TABLE IV

EFFECT OF INHIBITORS OF ENERGY METABOLISM ON AMINE UPTAKE

Approx. $4 \cdot 10^6$ cells were incubated with 2–5 nM [^{3}H]quinuclidinyl benzilate and total uptake was determined as described under Experimental procedures.

Inhibitor	% total uptake + S.D. (n = 2)
2-Deoxyglucose (5.5 mM) *	45 ± 2
Cyanide (1 mM)	101 ± 4
Fluoride (1 mM)	105 ± 5
Azide (1 mM)	97 ± 3
Iodoacetic acid (1 mM)	84 ± 1
Dinitrophenol (0.1 mM)	84 ± 3

* In the absence of added glucose; lack of glucose is not inhibitory.

pH effects. We studied the effect of the extracellular pH on [^{3}H]dihydroalprenolol retention in the presence and absence of 50 μM chloroquine in 25 mM sodium phosphate, 137 mM NaCl and 5.4 mM KCl buffer. The uptake of dihydroalprenolol shows a marked dependence upon external pH (Fig. 4), whereas in the presence of chloroquine, specific receptor binding of dihydroalprenolol is small and relatively constant.

Discussion

The uptake of [^{3}H]dihydroalprenolol and [^{3}H]quinuclidinyl benzilate can be offset to varying degrees by a variety of basic amines. This observation indicates that the displacing amines are also taken up by the cell and in such a way that they are competitive with the labeled amines. This general basic permeable amine phenomenon can be explained readily by the existence of compartmentalized regions of the cell that have a lower pH than the external medium, with the labeled ligands functioning as lysosomotropic agents [9]. Polymorphonuclear leukocytes are, in fact, especially rich in acidic lysosomes. At any pH there is an equilibrium between protonated and unprotonated amine and the unprotonated form can freely permeate the cell. Thus, there will be a tendency for the unprotonated amine to reach the same concentration in the lysosome and the external medium. In contrast, the concentration of protonated amine inside the lysosome will be much higher than in the external medium if the pH difference is maintained, and will tend to approach:

$$\frac{[BH^+]_{lys}}{[BH^+]_{ext}} = \frac{[H^+]_{lys}}{[H^+]_{ext}} = \frac{10^{-4.7}}{10^{-7.4}} = 500$$

taking pH = 4.7 for the lysosome pH [10]. Here $[BH^+]_{lys}$ is the concentration of protonated amine in the lysosomes and $[BH^+]_{ext}$ is the concentration in the external medium. If the amine is fairly basic, this ratio will also apply to the total amine concentration. If the lysosomal volume is 1% of the cell volume, for a cell of 7.5 μm radius and an external amine concentration of 20 nM, the cell could accumulate more than 10^5 molecules. This would correspond to 10^5 apparent binding sites per cell if the uptake were attributed (falsely) to receptor binding.

The mechanism of displacement by other amines is clear enough. The displacing amine can easily raise the pH of the lysosome and diminish trapping of the labeled amine. In fact, it has been shown (using the pH dependent fluorescence of fluorescein isothiocyanate-dextran) that ammonia, methylamine and chloroquine can raise the pH of the lysosomes of macrophages by 1.5 pH units at concentrations that inhibit quinuclidinyl benzilate uptake [10].

The mechanism of saturation involves an accumulation that tends to approach 500-fold, as discussed above. However, the entering unprotonated amine will tend to raise the pH if the lysosome is not well buffered, and thereby lower the accumulation. It also seems probable that the accumulation should be limited by leakage of protonated amine (along with an anion or in exchange for an external cation). Since the driving force for the uptake of amines is the acidity of the lysosomes, and since this depends upon an energy yielding process, the metabolic state of the cells must influence the uptake of permeable amines. The number of lysosomes and the permeability are also involved. Evidently, the steady state of amine accumulation is a complicated process that depends upon a number of factors, including cell types and the metabolic status of the cell.

Although our experiment with metabolic inhibitors did not provide proof for the role of metabolic energy in amine uptake, there can be no doubt that it is required to maintain a pH gradient. Our cells were not 'starved' and probably had ample energy stored. The picture of amine diffusion into the cells and concentration in the lysosomes by protonation is supported by studies with HeLa cells [11]. The fluorescent dye, acridine orange, diffuses into starved HeLa cells at a low temperature but it does not concentrate in the lysosomes until the temperature is raised and an energy source is provided.

Our experiments showing that quaternary and weakly basic amines do not displace quinuclidinyl benzilate strongly supports the general idea of accumulation by permeation and protonation since quaternary amines will not readily pass into the cell and weak bases cannot readily increase the pH of lysosomes. These ideas are also strongly supported by the experiment showing that the uptake of dihydroalprenolol increases sharply as the pH of the external medium is raised. The maximum uptake should depend markedly upon the difference in pH between the lysosome and the external medium, and the rate of uptake should depend on the concentration of unprotonated amine in the medium. Both of these factors are increased at higher pH.

The model of amine accumulation in response to a pH difference is finally supported by our studies with ionophores. Valinomycin allows K^+ to travel down a concentration gradient (reviewed in [12]), and CCCP similarly promotes proton transport [13]. The protonophore alone inhibits amine accumulation, but the combination with valinomycin leads to synergistic inhibition. This synergism suggests that the two agents together allow the exchange of potassium ions and protons. The proton gradient is more easily disrupted with the combination of ionophores.

The amines chloroquine and propranolol are particularly potent inhibitors of amine accumulation in these cells. Chloroquine has proved to be a useful inhibitor of amine uptake in the study of β-adrenergic receptors [5], but cannot be used to distinguish between nonspecific and specific receptor binding of quinu-

clidinyl benzilate because of its muscarinic character. Similarly, propranolol can be a particularly misleading inhibitor of dihydroalprenolol retention, because it can display both specific receptor binding and permeable amine uptake with similar potency. β-Adrenergic receptor identification by propranolol competition is only reliable in leukocytes when nonspecific amine retention is inhibited, using, for example, chloroquine [5] or another basic amine, such as phentolamine [14].

The studies reported here demonstrate that cells with acidic vesicles such as lysosomes can take up relatively large amounts of basic permeable amines. This amine uptake is probably general to all cells but is especially prominent in polymorphonuclear leukocytes because they contain a large number of lysosomes.

Acknowledgement

This work was supported by the National Institutes of Health grant NS-07156-13A1.

References

1 Dulis, B.H., Gordon, M.A. and Wilson, I.B. (1979) Mol. Pharmacol. 15, 28—34
2 Boyum, A. (1967) Scand. J. Clin. Lab. Invest. 97 (suppl.) 77—89
3 Yamamura, H.I. and Snyder, S.H. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 1725—1729
4 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
5 Dulis, B.H. and Wilson, I.B. (1980) J. Biol. Chem. 255, 1043—1048
6 Weast, R.C. (ed.) (1969) Handbook of Chemistry and Physics, 50th edn., pp. D115—116, The Chemical Rubber Co., Cleveland
7 Wittekind, D. (1966) in Fluorescence Techniques in Cell Biology (Thaer, A.A. and Sternetz, M., eds.), p. 96, Springer-Verlag, New York
8 Hanin, I., Jenden, D.J. and Cho, A.K. (1966) Mol. Pharmacol. 2, 352—359
9 De Duve, C., de Barsy, T., Poole, B., Trouet, A., Tulkens, P. and van Hoof, F. (1974) Biochem. Pharmacol. 23, 2495—2531
10 Ohkuma, S. and Poole, B. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 3327—3331
11 Robbins, E., Marcus, P.I. and Gonatas, N.K. (1964) J. Cell. Biol. 21, 49—62
12 Harold, F.M. (1972) Bacteriol. Rev. 36, 172—230
13 Hopfer, U., Lehninger, A.L. and Thompson, T.E. (1968) Proc. Natl. Acad. Sci. U.S.A. 59, 484—490
14 Aarons, R.D., Nies, A.S., Gal, J., Hegstrand, L.R. and Molinoff, P.B. (1980) J. Clin. Invest. 65, 949—957
15 Zurier, R.B., Weissman, G., Hoffstein, S., Kammerman, S. and Tai, H.H. (1974) J. Clin Invest. 53, 297—309
16 Ignarro, L.J., Lint, T.F. and George, W.J. (1974) J. Exp. Med. 139, 1395—1414