CHLOROQUINE ACCUMULATION IN ISOLATED RAT LIVER LYSOSOMES

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1. Introduction

Since hydrolytic processes in lysosomes are catalysed by hydrolases with an acid pH optimum, the intralysosomal pH must be low enough to allow the enzymes to function efficiently [1]. Indeed, qualitative observations on lysosomes in vivo [2–6] indicate that the intralysosomal pH is 3–6. Furthermore, studies with isolated lysosomes have shown that the pH within the organelles is lower than that of the suspending medium when the pH of the latter is > 5, and that the pH difference across the lysosomal membrane becomes greater as the pH of the medium is increased [7–9]. In the in vitro studies the intralysosomal pH was estimated by measuring the distribution of the lipid-soluble weak base methylamine between the lysosomes and the medium [7–9].

Weak bases are accumulated within the lysosomes in the intact cell [10]. Homewood et al. [11] have suggested that the antimalarial action of chloroquine is due to its being accumulated in the lysosomes of the malarial parasite. This leads to an increase in the intralysosomal pH, to an inhibition of the lysosomal hydrolysis of haemoglobin, and, consequently, to arrested growth of the parasite [11].

According to Homewood et al. [11] the extent of chloroquine accumulation is governed by the pH difference between the intra- and extralysosomal spaces and the buffering capacity of the lysosomes. De Duve et al. [10], however, consider that the amount of chloroquine accumulated in lysosomes, as estimated for instance by Wibo and Poole [12] in cultured fibroblasts, is too great to be accounted for solely by this mechanism, and suggest that an active pumping of protons into the lysosomes must occur (cf. [13]).

In this paper, we show that chloroquine is accumulated by isolated rat liver lysosomes, that the accumulation is accompanied by an increase in intralysosomal pH, and that the amount accumulated can be fully accounted for the protons made available by the increase in intralysosomal pH and the buffering capacity of the lysosomes.

2. Materials and methods

2.1. *Isolation of lysosomes*

Lysosomes were isolated from the livers of rats injected with Triton WR 1339 4 days prior to isolation by the flotation method of Trouet [14] as described by Kussendrager et al. [15].

2.2. *Measurement of accumulation of [^{14}C]chloroquine and [^{14}C]methylamine*

The incubations with radioactive compounds were carried out at 25°C as described previously [10], separation of the lysosomes from the medium being achieved by centrifugation–filtration through silicone oil. The reaction mixture (final volume, 1 ml) contained 5 mM morpholino-ethane sulphonic acid, 5 mM morpholino-propane sulphonic acid, sufficient Tris to bring the pH to the indicated value, 250 mM mannitol, ^3H_2O, lysosomes (about 1 mg protein) and either [^{14}C]methylamine, [^{14}C]chloroquine or [^{14}C]sucrose. The concentration of [^{14}C]methylamine or [^{14}C]chloroquine in the lysosomes was calculated after correction for adherent water, and

the intralysosomal pH was calculated from the distribution of [^{14}C]methylamine as described previously [8].

2.3. *Measurement of the buffering capacity of the lysosomes*

The lysosomes (about 20 mg protein) were suspended in an isotonic medium (130 mM KCl; final volume, 3 ml) to which no buffer was added, and the pH was adjusted to about 7.5. The pH of the suspension was monitored continuously with a Radiometer GK 2321C electrode attached to a digital pH meter (Philips PW 9405). Successive additions of small volumes of $H_2C_2O_4$, each containing 0.1 μg equiv. H^+, were made, and the pH decrease after each addition was measured. The buffering capacity (β) of the lysosomal suspension at any particular pH is defined as follows:

$$\beta = \frac{\Delta H^+}{\Delta pH \cdot (\text{mg lysosomal protein})}$$

where ΔH^+ = amount of protons added and ΔpH = the subsequent decrease in pH of the medium. β has the dimensions of μg equiv. H^+/pH unit per mg protein.

2.4. *Materials*

[^{14}C]Chloroquine, [^{14}C]methylamine, [^{14}C]sucrose and ^3H_2O were obtained from The Radiochemical Centre, Amersham, United Kingdom and Triton WR 1339 from Rohm and Haas, Philadelphia, USA. Nigericin was a gift from Dr W. Pettinga, Eli Lilly and Co., Indianapolis, USA.

3. Results

When isolated rat liver lysosomes are incubated with chloroquine it accumulates in the sucrose-impermeable space of the organelles, and the extent of the accumulation is dependent on the concentration of chloroquine in the medium. The results of a typical experiment are presented in fig.1 in the form of a Scatchard plot. The absence of a break in the line indicates that only one type of 'binding site' for chloroquine is present in the lysosomes. The 'dissociation constant' (K_d) can be calculated from the slope of the line and the maximum concentration of chloroquine that can be found in the lysosomes (S_m) from the intercept of the line on the abscissa.

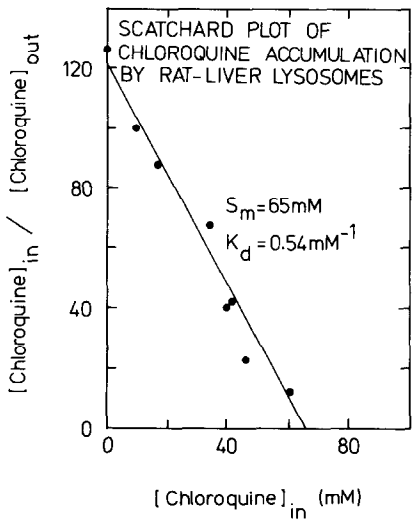

Fig.1. Scatchard plot of chloroquine accumulation in rat liver lysosomes. For conditions, see Materials and methods. The lysosomes were incubated with 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 mM [^{14}C]chloroquine.

The effect of chloroquine on the intralysosomal pH, calculated from the distribution of trace amounts of [^{14}C]methylamine, is shown in fig.2. Addition of chloroquine led to an increase in intralysosomal pH, the magnitude of which was dependent on the concentration of chloroquine added.

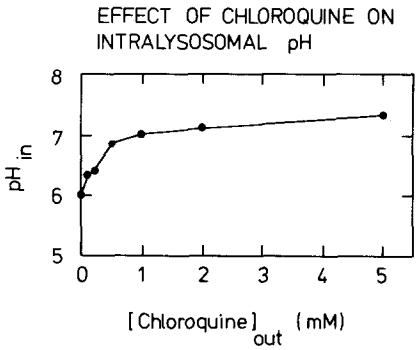

Fig.2. Effect of chloroquine on the intralysosomal pH. For conditions, see Materials and methods. The lysosomes were incubated with 0.1, 0.2, 0.5, 1.0, 2.0 and 50 mM unlabelled chloroquine. The intralysosomal pH was calculated from the distribution of [^{14}C]methylamine.

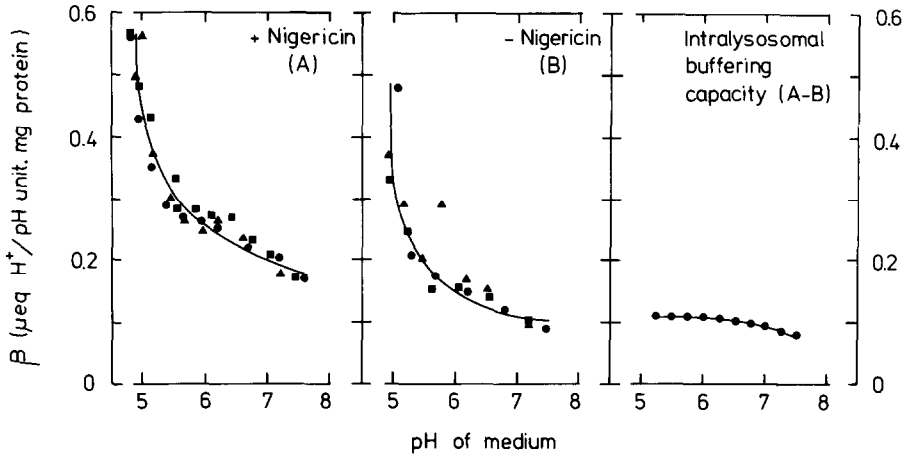

Fig.3. Buffering capacity of isolated rat liver lysosomes in a KCl-containing medium in the absence and presence of nigericin, and the intralysosomal buffering capacity. (A) nigericin present. (B) no nigericin (C) intralysosomal buffering capacity (A–B). Each symbol represents a separate titration.

In order to be able to calculate the amounts of protons made available by the changes in intralysosomal pH observed, the buffering capacity of the lysosomes was calculated. Fig.3 shows the results of a typical experiment. Intact lysosomes were suspended in a K^+-containing medium in the absence or presence of nigericin, and titrated at about 20°C with $H_2C_2O_4$. Nigericin brings about a K^+–H^+ exchange across membranes [16], so that in the presence of the ionophore and K^+, the buffering capacity both inside and on the outside of the lysosomal membrane was measured. This is shown in fig.3A. The buffering capacity on the outside of the lysosomes could be estimated from the titration in the absence of nigericin and is shown in fig.3B. The difference between these two curves represents the buffering capacity of the lysosomal matrix, which as fig.3C shows, is rather constant between pH 5 and 7.5 (β = 0.075–0.11 μg equiv. H^+/pH unit per mg protein). In lysed lysosomes, the buffering capacity was found to be identical to that measured in the presence of nigericin + K^+ (not shown).

Table 1 summarizes the results of 6 experiments, in 4 of which the buffering capacity in lysed lysosomes was measured and in 3 the accumulation of chloroquine in intact lysosomes.

In table 2, a comparison has been made between the amount of chloroquine accumulated and the amount of protons liberated, as calculated from the concomitant increase in pH and the buffering capacity. The calculation procedure was as follows. In one set of incubations, the accumulation of [^{14}C]chloroquine was measured. In parallel experiments with unlabelled chloroquine, the accumulation of methylamine was measured, from which the 'pH_{in}' was calculated. It should be stressed that the accumulation of methylamine or chloroquine in lysosomes in the salt-poor medium used represents not only accumulation within the lysosomal matrix, but also adsorption due to the presence of negatively charged groups on the outside of the lysosomal membrane [19]. The experiments were deliberately carried out in a salt-poor medium in order to avoid competition between cations and chloroquine or methylamine for the negatively charged groups on the outside of the membrane. For this reason, then, the total buffering capacity of lysed lysosomes was measured, in order to assess the role of negatively charged groups within and on the outside of the lysosomes in the accumulation.

The mean value of the ratio protons liberated: chloroquine accumulated was 2.4 (table 2, column 5), which is only a little higher than the expected value of 2, assuming that an electroneutral exchange of divalently-charged chloroquine for protons or binding of two protons to one uncharged chloroquine molecule occurs.

Table 1
Summary of experiments concerning chloroquine accumulation in isolated rat liver lysosomes and the buffering capacity of the organelles

Expt.	Lysosomal protein (mg/ml)	Sucrose-impermeable space (μl/mg protein)	S_m[a] (mM)	K_d[b] (mM^{-1})	β[c] (μequiv. H^+/pH unit·mg protein)
1	0.9	2.7	75	0.41	–
2	0.8	4.2	52	0.61	–
3	0.7	5.8	65	0.54	0.55
4	0.9	–	–	–	0.34
5	1.1	2.3	–	–	0.46
6	1.4	–	–	–	0.22
		Mean	64	0.52	0.39

For experimental details, see text and legends to figs.1 and 2.

[a] S_M = maximal accumulation of chloroquine in sucrose-impermeable space, determined from Scatchard plots (cf. fig.1).

[b] K_d = 'dissociation constant' for chloroquine, determined from Scatchard plots (cf. fig.1).

[c] β = mean buffering capacity of lysed lysosomes between pH 6 and 7.5.

Table 2
Calculation of the ratio protons liberated: chloroquine accumulated in isolated rat liver lysosomes

[Chloroquine] in sucrose-impermeable space (mM) (A)	ΔpH_{in}[a]	$\beta \times \Delta pH_{in}$[b] (μequiv· H^+/mg protein)	Concentration liberated protons in sucrose-impermeable space (mM) (B)	B/A
10	0.34	0.187	32	3.2
17	0.38	0.205	36	2.1
34	0.87	0.478	82	2.4
41	1.00	0.550	96	2.3
46	1.12	0.615	106	2.3
60	1.33	0.732	125	2.1
			Mean	2.4

Data obtained from Expt 3 of table 1, in which the sucrose-impermeable space was 5.8 μl/mg protein and the total buffer capacity 0.55 μequiv. H^+/pH unit·mg protein. The pH_{in} values for this experiment are shown in fig.2.

[a] ΔpH_{in} is the difference between pH_{in} in the absence and presence of chloroquine (cf. fig.2).

[b] $\beta \times \Delta pH_{in}$ gives the amount of protons made available by the increase in pH in the addition of chloroquine.

[c] This column represents the concentrations of protons made availabe on addition of chloroquine, assuming that they remain within the sucrose-impermeable space.

4. Discussion

Wibo and Poole [12] have calculated that the concentration of chloroquine in the lysosomes of rat fibroblasts cultured in the presence of the drug can reach values of 75–100 mM. De Duve and co-workers [10,12] have proposed that an ATP-dependent proton pump must be present in the lysosomal membrane (cf. [13]) in order to explain this high extent of accumulation. They suggest that chloroquine enters the lysosome as the uncharged molecule together with anions (e.g. chloride), that it becomes protonated in the lysosomal matrix, and that protons are pumped into the lysosome to compensate for those lost by protonation of the drug.

The results presented in this paper show that isolated ratliver lysosomes can accumulate chloroquine to a maximum extent of about 64 mM in the absence of ATP, a value which is comparable to that calculated by Wibo and Poole [12] for rat fibroblast lysosomes in vivo. This accumulation can be fully accounted for by the proton reserve in the lysosomes and the decrease in intralysosomal pH. In agreement with Homewood et al. [11], we suggest that the lipid-soluble, uncharged chloroquine molecule enters the lysosome, where it becomes protonated, and that there is an electrostatic binding of protonated chloroquine to the negatively charged groups formed as a result of the protonation process. In addition, protonated chloroquine binds to negatively charged groups on the outside of the lysosomal membrane. Thus there is no reason to invoke an ATP-dependent proton pump in order to explain the accumulation of drugs like chloroquine in lysosomes. Moreover, in contrast to Mego et al. [13] we have not been able to obtain any evidence for an ATP-dependent proton pump in the lysosomal membrane [17].

Finally, the inhibition by chloroquine of hydrolytic activity in lysosomes (see [18]) could be due in part to the increase of the intralysosomal pH that occurs, and in part to a direct inhibition by the compound of certain lysosomal hydrolases.

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