The effect of chloroquine on the metabolism of [^{35}S]cystine in normal and cystinotic human skin fibroblasts

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The present study concerns the effect of the lysosomotropic drug chloroquine on the uptake and metabolism of [^{35}S]cystine *in vitro* by normal human fibroblasts and those from patients suffering from the lysosomal storage disease cystinosis. When the cells were cultured with [^{35}S]cystine for periods in excess of 4 h, it was found that chloroquine considerably increased (up to 30-fold) the labelling of the intracellular cystine pool in cystinotic cells, with no increase or a much smaller increase in normal cells. For this effect chloroquine had an optimum concentration of $20\text{ }\mu\text{M}$, with a small effect still being noticeable at $1\text{ }\mu\text{M}$. A quinoline analogue, 4-(dimethylaminoethylamino)-7-iodoquinoline, had a similar effect to chloroquine. However, NH_4Cl at concentrations of between $100\text{ }\mu\text{M}$ and 50 mM showed either no effect (at the lower concentrations) or a depression of intracellular cystine labelling (at the higher concentrations). The differences between the effects of the quinolines on cystinotic acid normal cells were not due to differences in total cell uptake of drug.

Cystinosis is an autosomal recessive lysosomal storage disease in which the accumulated material is cystine. It is one of the few lysosomal storage diseases in which the basic biochemical lesion is unknown. The source of the intralysosomal cystine in cystinotic cells and its normal method of egress from the lysosomes have been the subject of much speculation. Knowledge of the proteolytic role (Gordon, 1973) and the membrane-permeability properties (Ehrenreich & Cohn, 1969; Schulman & Bradley, 1970) of lysosomes has led to the assumptions that the intralysosomal catabolism of endogenous or exogenous proteins provides the source of the accumulated cystine and that cystine is normally reduced to cysteine before it diffuses through the lysosomal membrane into the cytosol. Some evidence for the former assumption exists (Thoene *et al.*, 1977; Thoene & Lemons, 1980), but none for the latter.

In an attempt to aid the elucidation of the mechanism by which cystine is stored in the lysosomes of cystinotic cells, the effect of chloroquine, a drug that is known to disturb various aspects of the functioning of the lysosomal system, including protein catabolism, on the metabolism of exogenous [^{35}S]cystine by normal and cystinotic human skin fibroblasts has been investigated.

A small portion of this paper has been the subject of a previous short communication (Danpure & Fyfe, 1980).

Experimental

Materials

Chloroquine was obtained from Sigma. 4-(Dimethylaminoethylamino)-7- ^{125}I iodoquinoline and its stable counterpart were gifts from Dr. R. W. Goulding (M.R.C. Cyclotron Unit, Hammersmith Hospital, London W12 0HS, U.K.). [^{35}S]Cystine hydrochloride was obtained from The Radiochemical Centre, Amersham. All other chemicals were purchased from BDH and were of AnalaR grade where available.

Methods

Human skin fibroblasts from normal volunteers and cystinotic patients (passages 10–16) were grown to confluent monolayers in 75 cm^2 Falcon plastic tissue-culture flasks containing antibiotic-free Ham's F10 medium supplemented with 15% (v/v) foetal calf serum (Flow Laboratories) (total volume 15 ml). The cells were maintained at 37°C and gassed with air/ CO_2 (19:1). To each flask was added 5–25 μCi of [^{35}S]cystine to give a final concentration of $150\text{ }\mu\text{M}$. At the end of the incubation, the cells were washed *in situ* four times with phosphate-buffered saline (Dulbecco's formula, Ca^{2+} + Mg^{2+} -free), scraped off in 5 ml of phosphate-buffered saline containing 100 mM-N-ethylmaleimide and sonicated using a MSE Soniprep 150 sonicator (10 s, $10\text{ }\mu\text{M}$ amplitude). The samples were freeze-dried and the

residues were taken up in 200 μ l of water. Samples were taken for liquid-scintillation counting and protein determination (Lowry *et al.*, 1951) and the remainder was made 5% with respect to sulphosalicylic acid. The proteinaceous precipitate was spun down and 50 μ l of the protein-free supernatant was loaded on to a cartridge, without sucking, and analysed by using a Technicon TSM amino acid autoanalyser (Technicon Publication no. TAI-0233-00, 1971) with Technicon Chromobeads (type C3) as the cation-exchange resin. After passage down the column the stream was split equally. Half was monitored for ninhydrin-positive material, and from the other half 72 2.5 min fractions (500–600 μ l) were collected (total program running time 3 h) and analysed for ^{35}S by liquid-scintillation counting. Peaks were identified by using standards of cystine, cysteine-*N*-ethylmaleimide adduct, oxidized glutathione, reduced glutathione-*N*-ethylmaleimide adduct, cysteine-glutathione mixed disulphide and methionine.

The ^{35}S was assayed in a Philips PW4700 liquid-scintillation counter. The scintillation fluid used consisted of toluene, containing 125 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 10 g of 2,5-diphenyloxazole in 2.5 litres of toluene/Triton X-100 (2:1, v/v). The sample and scintillation fluid were mixed in the ratio 1:9 (v/v). Chloroquine was assayed by the method of Wibo & Poole (1974), and the ^{125}I from 4-(dimethylaminoethylamino)-7-[^{125}I]-iodoquinoline was measured by γ -spectrometry in a Wallac G.T.L. 300-1000 scintillation spectrometer.

Results

The technique used in the present study for determining the distribution of ^{35}S in acid-soluble fractions of fibroblasts was able to distinguish a variety of sulphur metabolites, including cystine, cysteine (as *N*-ethylmaleimide adducts), reduced glutathione (as an *N*-ethylmaleimide adduct), oxidized glutathione, cysteine-glutathione mixed disulphide, methionine, acidic metabolites such as cysteic acid, taurine etc. (as a group) and certain uncharacterized metabolites (Fig. 1).

The distribution of ^{35}S between the major intracellular pools of cystinotic and normal fibroblasts when cultured for 7 days in the presence or absence of chloroquine (20 μM) and when labelled with [^{35}S]cystine for 1 or 7 days is shown in Tables 1 and 2 respectively. The presence of chloroquine caused a large increase in the labelling of the cystine pool, this effect being considerably larger in the cystinotic cells than in the normal cells. The magnitude of the effect varied considerably and depended on the particular cell line used and the conditions of labelling. The addition of chloroquine increased the proportion of the total cell label in the cystine pool from 8 to 20%

Fig. 1. Specimen profile of the distribution of ^{35}S obtained from the automated amino acid analysis (see the Experimental section) of a sulphosalicylic acid supernatant from cystinotic fibroblasts labelled with [^{35}S]cystine in the presence of 20 μM -chloroquine.

The fractions are labelled in reverse from the start of the LiOH wash, which is arbitrarily nominated fraction 0. Key: GS, glutathionyl moieties; CyS, cysteinyl moieties; Met, methionine; LiOH, start of lithium hydroxide wash; NEM, *N*-ethylmaleimide adducts.

(13 to 27% of the non-protein label) in cystinotic cells and 0.8 to 2.2% (1.4 to 4.2%) in normal cells when labelled for 1 day, and 1.2 to 11% (7 to 30%) in cystinotic cells and 0.1 to 0.6 (0.4 to 2.5%) in normal cells when labelled for 7 days. No consistent changes in the labelling of any of the other pools were found as a result of the addition of chloroquine.

The difference between the 1- and 7-day labelling was largely confined to the proportion of the label that was associated with protein, which was 25–50% after 1 day and 60–95% after 7 days labelling. In addition, the proportional effect of chloroquine on the increase in intracellular cystine radioactivity was greater after 7 days labelling (i.e. 8–28 fold for cystinotic cells) than after only 1 day labelling (i.e. 1.5–4-fold for cystinotic cells), probably owing to the higher percentage labelling of the cystine pool in the cystinotic controls after 1 day.

When cystinotic cells were labelled with [^{35}S]cystine during the 24 h immediately before harvesting, the period of incubation of the cells with chloroquine, at least between 1 and 7 days, had no effect on the magnitude of the increase in intracellular cystine labelling (Table 3). On the other hand, when the cells were labelled with [^{35}S]cystine

Table 1. *Effect of chloroquine on the distribution of ³⁵S after 24 h labelling*

Cystinotic cells (two lines, 1C and 5C) and normal cells (two lines, 5N and 6N) were cultured for 7 days in the presence or absence of 20 μ M-chloroquine and labelled with [³⁵S]cystine for the final 24 h.

10⁻³ $\times$ ³⁵S (c.p.m./mg of protein equivalent)

Cell type	Cell line	Chloroquine	Total	Protein	Acidic	Glutathione	Cystine
Cystinotic	1C	—	1266	510	105	355	142
		+	1957	499	120	443	570
	5C	—	1373	341	209	654	72
		+	1058	334	105	397	106
Normal	5N	—	1005	435	106	398	12.5
		+	794	359	84	276	22.6
	6N	—	976	451	94	393	2.6
		+	914	468	66	324	13.8

Table 2. *Effect of chloroquine on the distribution of ³⁵S after 7 days labelling*

Cystinotic cells (three lines, 1C, 2C and 5C) and normal cells (three lines, 4N, 5N and 6N) were cultured for 7 days with [³⁵S]cystine in the presence or absence of 20 μ M-chloroquine.

10⁻³ $\times$ ³⁵S (c.p.m./mg of protein equivalent)

Cell type	Cell line	Chloroquine	Total	Protein	Acidic	Glutathione	Cystine
Cystinotic	1C	—	696	581	18.2	74.9	7.8
		+	795	499	30.7	70.3	121.4
	2C	—	786	712	18.5	32.7	2.1
		+	1023	730	46.9	68.3	60.1
	5C	—	544	460	12.9	48.0	9.6
		+	735	452	33.7	113.0	83.7
Normal	4N	—	1249	1064	49.4	91.0	0.9
		+	1392	1134	72.4	91.6	9.0
	5N	—	732	581	22.1	116.0	0.9
		+	643	469	31.0	115.0	5.6
	6N	—	679	614	10.7	43.0	0
		+	659	516	20.8	101.0	1.3

Table 3. *Effect of time of incubation with chloroquine on the distribution of ³⁵S after 24 h labelling*

Cystinotic cells (line 1C) were incubated for various periods of time in the presence or absence of 20 μ M-chloroquine and labelled with [³⁵S]cystine for the final 24 h.

Time of incubation (days) (+ chloroquine)	Chloroquine	10 ⁻³ $\times$ ³⁵ S (c.p.m./mg of protein equivalent)					Cystine (as % of total)
		Total	Protein	Acidic	Glutathione	Cystine	
1	—	653	349	48.6	152	67.4	10.3
	+	813	349	47.4	182	175	21.5
2	—	1025	472	50.9	330	109	10.6
	+	1113	508	62.9	293	162	14.6
3	—	545	308	30.4	115	49.8	8.5
	+	889	299	33.7	116	321	34.1
6	—	817	362	50.7	279	91.2	11.0
	+	869	214	63.1	299	242	26.4
7	—	1266	510	105	355	142	11.2
	+	1957	499	120	443	570	29.1

for 7 days and chloroquine was added for the final 1–48 h there was a marked time dependency with respect to the chloroquine-induced increase in the labelling of the intracellular cystine pool (Fig. 2).

Fig. 3 shows the uptake of ³⁵S into the various metabolic pools over various periods of labelling from 1 to 24 h in the absence of chloroquine or when chloroquine was added at the same time as the

[^{35}S]cystine or when the cells were preincubated in chloroquine for 1 week beforehand. The labelling of protein was largely unaffected by the presence of chloroquine, except a slight depression when incubated with chloroquine for 1 week. The labelling of glutathione and the acidic metabolites was rather erratic and there was no clear effect of chloroquine. However, whether chloroquine was added for 1

Fig. 2. Effect of the time of incubation with chloroquine ($20\mu\text{M}$) on the labelling of the cystine pool (●) and reduced glutathione pool (○) in cystinotic cells (line 1C)

The cells were labelled for 7 days with [^{35}S]cystine and chloroquine was added during the final 1–48 h. For further details see the text.

week before the label or at the same time, the effect on the labelling of the intracellular cystine pool was marked. Chloroquine decreased the labelling of the cystine pool at short time intervals (1–2 h), but elevated it at longer ones (24 h). The plateau in the cystine labelling found after about 4 h without chloroquine was absent in its presence.

Fig. 4 shows the effect of different concentrations of chloroquine ($1\text{--}100\mu\text{M}$) on the metabolism of [^{35}S]cystine by normal and cystinotic fibroblasts. Even at $1\mu\text{M}$ there was a slight increase in the labelling of the cystine pool in cystinotic cells. This labelling increased to reach an optimum at $10\text{--}20\mu\text{M}$ and then declined at higher concentrations. At the highest chloroquine concentration ($100\mu\text{M}$) there were major changes in the distribution of label, most notably a sharp decline in the labelling of the reduced-glutathione pool.

The effect of chloroquine on the metabolism of exogenous [^{35}S]cystine in cystinotic cells has been compared with the effect of NH_4Cl , another inhibitor of protein catabolism. The latter showed little or no effect on the distribution of ^{35}S , except at concentrations of 10 mM and above (Table 4). This effect was manifested by a decrease in the labelling of all the intracellular pools studied, including cystine.

4 - (Dimethylaminoethylamino) - 7 - iodoquinoline ($20\mu\text{M}$), which is an iodinated analogue of chloroquine, caused a similar alteration to the intracellular distribution of label to that caused by $20\mu\text{M}$ -chloroquine (Table 5).

When cystinotic and normal cells were labelled for 1 week with [^{35}S]cystine in the presence or absence of $20\mu\text{M}$ -chloroquine and then chased with non-

Fig. 3. Changing intracellular distribution of ^{35}S with time when chloroquine ($20\mu\text{M}$) and [^{35}S]cystine were added simultaneously (●) or when [^{35}S]cystine was added alone (○) to cystinotic cells (line 1C)

(a) Protein; (b) reduced glutathione; (c) cystine. Apart from the protein labelling being decreased to 50% after 24 h, the curves obtained when the chloroquine was added 1 week beforehand were essentially identical with those obtained when chloroquine and [^{35}S]cystine were added simultaneously (●).

Fig. 4. Effect of various concentrations of chloroquine on the labelling of the intracellular cystine pool when cystinotic cells (line 1C, ●) and normal cells (line 5N, ○) were cultured in chloroquine for 3 days and labelled with [35S]cystine for the final 24 h

(a) 35S in the intracellular cystine pool. (b) Percentage of sulphosalicylic acid-supernatant 35S in the intracellular cystine pool. (c) Ratio of 35S in the intracellular cystine pool to that in the intracellular reduced glutathione pool.

Table 4. Effect of NH4Cl on the distribution of 35S after 24 h labelling
Cystinotic cells (line 1C) were cultured for 3 days with various concentrations of NH4Cl and labelled with [35S]-cystine for the final 24 h.

Fraction	[NH4Cl] (mm)	...	10 ⁻³ × 35S (c.p.m./mg of protein equivalent)				
			0	0.1	1	10	50
Total			1825	1851	1822	1156	630
Protein			679	570	666	612	131
Acidic			103	102	95	47	63
Glutathione			503	557	469	256	170
Cystine			382	432	321	140	172

Table 5. Comparison of the effect of chloroquine and 4-(dimethylaminoethylamino)-7-iodoquinoline (IQ-13), on the distribution of 35S

Cystinotic cells (line 1C) were cultured for 3 days with 20 μM -drug and labelled with [35S]cystine for the final 24 h.

Fraction	Drug	...	10 ⁻³ × 35S (c.p.m./mg of protein equivalent)		
			None	IQ-13	Chloroquine
Total			607	841	1271
Protein			342	188	333
Acidic			32	45	56
Glutathione			137	130	161
Cystine			55	350	551

radioactive medium for up to 3 days, again in the presence or absence of chloroquine, it was found that radioactivity was preferentially lost from the low-molecular-weight pools rather than from pro-

tein (Table 6). In addition, in cystinotic cells label was lost much more rapidly from reduced glutathione than from cystine. The presence of chloroquine apparently delayed the loss of label from the

Table 6. Effect of a chase, with or without chloroquine, on the distribution of ^{35}S after 7 days labelling with or without chloroquine

Cystinotic cells (line 1C) and normal cells (line 5N) were labelled with [^{35}S]cystine for 7 days in the presence or absence of $20\mu\text{M}$ -chloroquine. The cells were then washed *in situ* and chased with unlabelled medium with or without $20\mu\text{M}$ -chloroquine for up to 3 days.

				10 ⁻³ × ³⁵ S (c.p.m./mg of protein equivalent)					
		Chase (days)	...	0		1		3	
		Chloroquine	...	—	+	—	+	—	+
Cystinotic cells	Fraction								
	Total			6.96	7.95	4.56	4.97	3.86	4.89
	Protein			5.81	4.99	4.30	3.99	3.73	4.08
	Acidic			0.18	0.31	0.07	0.15	0.02	0.15
	Glutathione			0.75	0.70	0.08	0.10	0.01	0.07
Normal cells	Cystine			0.08	1.21	0.03	0.32	0.01	0.27
	Total			7.32	6.43	3.75 ^a	3.73	2.96	3.14
	Protein			5.81	4.69	3.44	3.40	2.88	2.99
	Acidic			0.22	0.31	0.11	0.13	—	—
	Glutathione			1.16	1.15	0.18	0.11	—	—
	Cystine			0.01	0.06	0.00	0.01	—	—

Table 7. Effect of a chase, with or without chloroquine, on the distribution of ^{35}S after 7 days labelling without chloroquine

Cystinotic cells (line 1C) were labelled with [^{35}S]cystine for 7 days in the absence of chloroquine. The cells were then washed *in situ* and chased with unlabelled medium with or without $20\mu\text{M}$ -chloroquine for up to 24 h.

		$10^{-3} \times {}^{35}\text{S}$ (c.p.m./mg of protein equivalent)									
Chase (h) ...		0	1		2		4		24		
Fraction	Chloroquine ...		—	+	—	+	—	+	—	+	
Total		576	595	568	537	507	521	492	417	501	
Protein		497	534	500	474	466	478	457	390	464	
Acidic		19.5	13.4	15.0	13.8	11.1	12.0	9.9	8.6	11.6	
Glutathione		24.6	20.0	20.4	23.6	5.6	16.2	8.3	11.4	9.0	
Cystine		16.7	17.0	23.7	15.7	16.4	7.1	11.7	3.1	9.7

Fig. 5. Uptake of chloroquine (a) and 4-(dimethylaminoethylamino)-7-[^{125}I]iodoquinoline (IQ-13) (b) by cystinotic cells (line 1C, ●) and normal cells (line 5N, ○) when incubated with $20\mu\text{M}$ drug for various times up to 18 h. Chloroquine was determined fluorimetrically and the iodoquinoline was determined by γ -counting of ^{125}I .

already greater labelled cystine pool in cystinotic cells, so that after 3 days of chase there was still 22% of the cystine label remaining in the chloroquine-treated cells but only about 10% in the untreated cells.

When cystinotic cells were labelled for 7 days with [^{35}S]cystine in the absence of chloroquine and then chased for up to 24 h in the presence or absence of $20\mu\text{M}$ -chloroquine, it was found again that proportionally more radioactivity was lost from the

non-protein pools than from protein (Table 7). Chloroquine inhibited the loss of label during the chase from protein (after 24 h) and cystine (from 1 h onwards), but increased the loss of label from glutathione (from 2 h onwards).

The uptake of chloroquine and 4-(dimethylaminoethylamino)-7-[¹²⁵I]iodoquinoline by normal and cystinotic fibroblasts was measured over a period of 18 h. The results (Fig. 5) showed that these quinolines were taken up very rapidly, with no significant difference between the two cell types. Chloroquine concentration (as determined by fluorescence) reached a peak by 3 h and accounted for 50–60% of the drug added. Thereafter the cellular chloroquine fluorescence decreased, so that by 18 h there was only about 60% of the peak value remaining associated with the cells. The uptake of 4-(dimethylaminoethylamino)-7-[¹²⁵I]iodoquinoline reached a plateau between 1 and 4 h and thereafter increased slowly, so that by 18 h 20–30% of the total drug had been taken up.

Discussion

As demonstrated by previous workers (States *et al.*, 1974; Oshima *et al.*, 1976), incubating cystinotic fibroblasts with [³⁵S]cystine leads to a much greater incorporation of label into the intracellular cystine pool than in normal cells, and probably simply reflects the relative sizes of the respective cystine pools.

Under almost all of the experimental conditions used (except when the cells were labelled for 2 h or less), 20 μ M-chloroquine was found to increase the labelling of the intracellular, presumably intralysosomal (Patrick & Lake, 1968; Schulman *et al.*, 1969), cystine pool, especially in cystinotic cells.

Chloroquine is a well-known lysosomotropic agent (de Duve *et al.*, 1974). Its mechanism of uptake by and intracellular translocation within cultured cells has been studied by a number of workers (e.g. Polet, 1976; Di Donato *et al.*, 1977). Inside the lysosomes the drug is trapped by protonation and accumulates to concentrations several orders of magnitude greater than that in the surrounding medium (Wibo & Poole, 1974; Reijngoud & Tager, 1976). It is known to have a wide variety of effects. For example, at concentrations of 10–500 μ M it causes an elevation of intralysosomal pH (Reijngoud & Tager, 1976; Ohkuma & Poole, 1978), it specifically inhibits cathepsin B₁ (Wibo & Poole, 1974; Mego & Chung, 1979) and it stimulates release and inhibits uptake of lysosomal enzymes (Weismann *et al.*, 1975; Sando *et al.*, 1979; Willcox & Rattray, 1979). The overall manifestation of these properties of chloroquine is a generalized depletion of intracellular lysosomal enzyme activity, leading to an equally generalized inhibition

of catabolism such as that of mucopolysaccharides (Lie & Schofield, 1973) and protein (Wibo & Poole, 1974).

Many of these effects of chloroquine can also be found with other weak bases. For example NH₄Cl, but at 100 times the concentration, has some similar effects to chloroquine, but these are much more easily reversible (Ohkuma & Poole, 1978; Sando *et al.*, 1979; Seglen *et al.*, 1979).

Using 50–100 μ M-chloroquine and 10 mM-NH₄Cl as inhibitors of protein catabolism, Thoene *et al.* (1977) and Thoene & Lemons (1980) showed that these drugs inhibited the reaccumulation of cystine when cystinotic fibroblasts, which had been treated with mercaptoethanolamine to deplete them of cystine, were placed in cystine-free medium. They also showed that in such conditions the intracellular cystine pool only became labelled with ³⁵S during a chase when the protein pool was previously labelled. They interpreted their results as indicating that both endogenous and exogenous protein disulphides could be sources for the cystine in cystinotic cells. These data may appear to conflict with the results of the present study.

However, the cystine pool (presumably intralysosomal) in cystinotic cells can be labelled rapidly by exogenous cystine, a plateau being reached by 4 h. It is difficult to reconcile this with a view that protein catabolism is the major source of cystine in cystinotic cells. The apparently rapid influx and efflux of cysteinyl moieties into and out of the cystine pool in cystinotic cells, despite its vastly increased size compared with normal cells, indicates that anything that produces these cytoplasmic moieties (including protein catabolism) would act as a source. The proportional contribution presumably depends on the circumstances. For example, in the absence of exogenous cystine, protein catabolism may be the major source (Thoene *et al.*, 1977; Thoene & Lemons, 1980), whereas in the presence of exogenous cystine we have found that the latter is the most important source. This view is confirmed by Oshima *et al.* (1976), who showed that, although the cystine accumulates from an intracellular source, it can be labelled by exogenous [³⁵S]cystine, owing to the accessibility of the internally compartmentalized cystine pool to cytoplasmic cysteine by thiol/disulphide exchange.

It seems likely that the effects of chloroquine, at concentrations of 20 μ M or less, found in the present study are not associated with any effect that it may be having on protein catabolism, firstly because its stimulatory effect on the ³⁵S labelling of the intracellular cystine pool was observed down to a concentration of 1 μ M, which is much lower than is normally used for inhibiting protein catabolism, and secondly because its effects were quite different from those of NH₄Cl. Evidence was found for a depres-

sion in the labelling of the cystine pool with 10mM-NH₄Cl and very high concentrations of chloroquine (100 μM), possibly as a result of decreased protein catabolism, but in these cases there was also a decrease in the labelling of other pools such as reduced glutathione and protein.

Although 20 μM-chloroquine appeared to inhibit the labelling of the cystine pool when the cells were labelled for 2 h or less, this also was extremely unlikely to be a result of decreased protein catabolism, because at these short time intervals the protein pool was hardly labelled.

Interpretation of the chase experiments is difficult. It would appear that chloroquine stimulated intracellular redistribution of ³⁵S in favour of the cystine pool rather than decreasing the efflux of label from this pool. To elucidate any direct role that chloroquine might have on the efflux of ³⁵S from the cystine pool, chase experiments would have to be done after short-term labelling to minimize labelling of other pools, especially protein. Under these conditions States *et al.* (1974) have shown that label is lost from the intracellular cystine pool into the medium extremely rapidly, that from cystinotics being somewhat slower than that of normal cells.

The much greater effect of chloroquine on cystinotic cells compared with normal cells cannot be explained by any differences in uptake of the drug by the whole cell. Using [¹⁴C]chloroquine, Di Donato *et al.* (1977) also showed the drug to be taken up very rapidly by fibroblasts, reaching a plateau after 1–6 h depending on the concentration.

Okhuma & Poole (1978) showed that in macrophages 100 μM-chloroquine brought about a rapid elevation of intralysosomal pH. This decrease in proton concentration might be expected to have considerable effects on the processes likely to be responsible for the reduction of intralysosomal cystine. For example, if its reduction was a consequence of a thiol/disulphide exchange reaction (e.g. with membrane or other protein thiols), then, as reactions of this kind are dependent on the S⁻ anion rather than the SH group, pH would be expected to play a major role in determining its rate. The consequences of this are unclear and would depend

on many things, for example the rate of re-reduction of lysosomal-membrane disulphides, perhaps by cytosolic reduced glutathione. However, the effects of chloroquine found in the present study may be properties of quinolines, as similar effects were found with 4-(dimethylaminoethylamino)-7-iodoquinoline, rather than of weak bases in general. If these effects of chloroquine were due simply to elevation of intralysosomal pH, then other weak bases such as NH₄Cl would be expected to show similar effects, and this was not the case.

It has been shown that polyanions, such as poly(D-glutamic acid), and 10mM-NH₄Cl inhibit the fusion between phagosomes and lysosomes in mouse macrophages (Hart & Young, 1979; Gordon *et al.*, 1980), whereas 20 μM-chloroquine stimulates this fusion and in fact reverses the inhibition caused by polyanions (Hart & Young, 1978, 1979). If this occurred with endocytic vesicles in general, it would provide a possible explanation for the apparently divergent effects of chloroquine and NH₄Cl found in the present study. The mechanism of cellular uptake and intracellular translocation of cystine is unclear, but it may involve vesicular processes, owing to its high molecular weight and therefore low membrane permeability compared with other amino acids. Presumably in normal circumstances vesicular cystine would be rapidly reduced, diffuse through the vesicle membrane and be metabolized into glutathione, proteins etc. Chloroquine-induced diversion of cystine from this pathway by fusion of the cystine-containing vesicles with lysosomes, which in cystinosis may possess a malfunctioning disulphide-reduction system, could explain the apparent synergism between the effect of chloroquine and the disease process of cystinosis found in the present study. There is no direct evidence of the vesicular transport of cystine. However, circumstantial evidence that most of the labelled cystine in protein is released from the cell as cystine on catabolism of the protein (Thoene & Lemons, 1980) and that the cystine stored in cystinosis is not directly available for metabolic need (Schneider *et al.*, 1967), despite its distinct turnover (Schulman *et al.*, 1971; States *et al.*, 1974), indicate that cystine is released from

Fig. 6. Suggested reaction between chloroquine and membrane or other thiols

the cell via a mechanism that protects it from the enzymes of the cytosol (i.e. vesicular).

A further possible mechanism of action of chloroquine in cystinosis is that, by analogy with the reactions of compounds structurally similar to chloroquine, such as meprazine (Wild & Young, 1965), it may react with thiols (Fig. 6) either in the plasma, vesicular or lysosomal membranes or elsewhere. If these thiols were important in the reduction of intralysosomal cystine, whether in the form of protein disulphides (Griffiths & Lloyd, 1979) or not, then the effect of chloroquine on the labelling of the intracellular, presumably intralysosomal, cystine pool, especially in cystinosis, would be understandable.

Further investigations into the mechanism of action of drugs like chloroquine, and indeed those with potential reverse effects, for example polyanions and, if transport of cystine is vesicular in nature, the effect of cytoskeletal poisons in cystinosis, might prove fruitful in illuminating the basic defect in one of the few remaining unsolved lysosomal storage diseases.

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