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CYSTINE DEPLETION BY WR-1065 IN CYSTINOTIC CELLS

MECHANISM OF ACTION

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Abstract—Cystinotic leucocytes and skin fibroblasts incubated with the aminothiol *N*-(2′-mercaptoethyl)-1,3-propanediamine (WR-1065) exhibited substantial intralysosomal cystine depletion within 2 hr. WR-2721, the thiol phosphorylated derivative of WR-1065, did not lower cystinotic leucocyte cystine in 1 hr but depleted cystinotic fibroblasts of cystine after 21 hr. Concentrations of cysteamine (β-mercaptoethylamine) equimolar with those of WR-1065 depleted cystine more rapidly than did WR-1065, but the extent of cystine depletion by WR-1065 approached that for cysteamine when longer periods of incubation or higher concentrations were used. Cystine depletion by WR-1065 was slower for leucocyte lysosomal granular fractions than for whole leucocytes. L-[^{35}S]Cystine-labeled fibroblasts exposed to WR-1065 exhibited new compounds not seen when cells were incubated without WR-1065: WR-1065-cysteine, cysteamine-cysteine and cysteamine-glutathione mixed disulfides. L-[^{35}S]Cystine-loaded lysosome-rich granular fractions from cystinotic leucocytes incubated with WR-1065 formed WR-1065-cysteine mixed disulfide but no cysteamine-cysteine mixed disulfide. We suggest that WR-2721 is dephosphorylated intracellularly to the free thiol, WR-1065, which subsequently is converted to cysteamine by an unknown route. Intracellular cysteamine then enters the lysosome and reacts with free cystine to form cysteamine-cysteine mixed disulfide and cysteine which move into the cytosol and the incubation medium where they participate in further interchange reactions with free thiols present there, namely WR-1065 and glutathione.

Cystinosis is an autosomal recessive genetic disease characterized by the accumulation of cystine in lysosomes of many body tissues [1]. Since the presence of 50- to 100-fold excess cystine in cystinotic cells is thought to be detrimental to many cellular functions, therapy has been directed toward depleting intracellular cystine. At present, cysteamine is the most effective agent for accomplishing this goal [1] and is currently being used in a national collaborative study for the treatment of cystinosis [2, 3]. However, since the dose of cysteamine is limited by adverse side effects [4], a search for equally effective but less toxic cystine depleting agents continues. Another possible thiolytic agent is the amino thiol *N*-(2′-mercaptoethyl)-1,3-propanediamine (WR-1065) or its thiol phosphate ester WR-2721. These compounds are used clinically as radioprotective and chemoprotective agents [5, 6] and as mucolytic agents in cystic fibrosis [7] and have been suggested as urinary cystine depleting agents in cystinuria [8]. WR-2721 is dephosphorylated in the stomach by high acidity or intracellularly by enzymatic hydrolysis to yield the free thiol WR-1065 which can act as a free radical scavenger or in thiol disulfide exchange reactions [8]. WR-2721 has shown little toxicity in man and is well absorbed and tolerated by patients [9, 10]. In this paper we examined the effect of WR-1065 on cystine stores in cystinotic cells with the view of assessing its possible clinical application. A mechanism of action of WR-1065 is proposed.

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MATERIALS AND METHODS

WR-1065 and WR-2721 were supplied by Dr. Larry Fleckenstein of the Department of Experimental Therapeutics of the Walter Reed Institute of Research, Washington, DC. L-Cystine, L-cysteine, cysteamine, sulfosalicylic acid, *N*-ethylmaleimide, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), dithiothreitol and L-cystine dimethyl ester were purchased from the Sigma Chemical Co., St. Louis, MO. Aquasol and L-[^{35}S]cystine (434 Ci/mmole) were from the New England Nuclear Corp., Boston, MA. Phosphate-buffered (10 mM) saline, pH 7.4 (PBS), and Eagle's minimum essential medium supplemented with nonessential amino acids were supplied by the NIH Media Preparation Department. Heat-inactivated fetal bovine serum and Hanks' balanced salt solution (Ca^{2+} and Mg^{2+} free) were from GIBCO, Grand Island, NY. All reagents were of the highest purity available.

Glutathione-cysteine mixed disulfide was prepared by the method of Erickson and Erickson [11]. A solution of WR-1065-cysteine mixed disulfide suitable for use as a reference marker in high-voltage electrophoresis was prepared by mixing a 2-fold

molar excess of WR-1065 with cystine and a trace amount of [^{35}S]cystine in 10 mM potassium phosphate buffer, pH 7.3. The resulting mixed disulfide standard was identified following high-voltage electrophoresis by ninhydrin staining and radioactivity. Cysteamine-cysteine mixed disulfide was prepared as described by Gahl *et al.* [12].

Polymorphonuclear-rich leucocytes, prepared by dextran sedimentation and hypotonic lysis of erythrocytes [13], were isolated from freshly-drawn blood of cystinotic patients, age 5–11 years, who were receiving 40–70 mg/kg/day of oral cysteamine in four divided doses. In some experiments, leucocyte cystine levels were increased by a 30-min exposure at 37° to 0.25 mM L-cystine dimethyl ester [14]. Whole cells were suspended in Hanks' balanced salt solution containing 10 mM sodium phosphate, pH 7.4, and various concentrations of WR-2721, WR-1065 or cysteamine. After 1 hr at 37°, aliquots were removed, centrifuged at 300 *g* for 5 min, and assayed for cystine/mg cell protein as described below.

For studies on cystinotic granular fractions, cystine-loaded leucocytes were disrupted by brief (10 sec) sonication (model W140 Cell Disrupter, Heat Systems Ultrasonics, Inc., Plainview, NJ) using a microtip, and a lysosome-rich granular fraction was prepared by differential centrifugation [14]. The cystine-loaded granular fraction was suspended in 0.25 M sucrose-10 mM sodium HEPES, pH 7.0, containing different concentrations of WR-2721, WR-1065, or cysteamine. After 0 and 60 min at 37°, aliquots were removed and assayed for cystine/mg protein.

In experiments whose results are now shown, cystinotic granular fractions loaded with L-[^{35}S]cystine were prepared as above by exposure of whole cells to L-[^{35}S]cystine dimethyl ester (0.5 Ci/mmole) and were incubated at 37° in sucrose-HEPES with and without WR-1065. Aliquots were removed at 0 and 60 min and centrifuged, and supernatant fraction and pellet were analyzed for ^{35}S-containing compounds by high-voltage electrophoresis as described below.

Skin biopsies from cystinotic children obtained after informed parental consent were used as the source of fibroblasts. Cells were cultured in Eagle's minimum essential medium containing nonessential amino acids, 2 mM glutamine, and 10% fetal bovine serum and incubated at 37° in 5% CO_2 humidified atmosphere. Studies of the effects of WR-1065, WR-2721 and cysteamine on the cystine content of cystinotic fibroblasts were carried out in the absence of serum. Cells were harvested by trypsinization (0.25% trypsin in PBS), washed in PBS, and centrifuged. The cell pellet was treated with 0.3 ml of 5 mM *N*-ethylmaleimide to inactive free thiol groups and protein removed by acidification with 0.1 ml of 12% sulfosalicylic acid.

Protein-free leucocyte and fibroblast extracts were analyzed for cystine by the cystine binding protein assay of Oshima *et al.* [15]; sulfosalicylic acid precipitated protein was determined by the method of Lowry *et al.* [16] after solubilization in 0.5 N sodium hydroxide.

Cystinotic fibroblasts were radioactively labeled by incubation with 20 μCi/ml L-[^{35}S]cystine in medium for 24 hr. The labeled cells were washed five times with PBS and further incubated with 1 mM WR-1065 in serum-free medium. Spent media (1 ml) collected at 2 and 21 hr were acidified with 0.1 ml of 30% sulfosalicylic acid. Cells were harvested, and the acid precipitated protein was quantitated as described above. Supernatant fractions (20 μl) from sulfosalicylic acid treated fibroblasts or leucocyte granular fractions were spotted on Whatman 3 MM chromatography paper and submitted to high-voltage electrophoresis along with labeled and unlabeled standards in a Gilson model D Electrophorator at 4 kV for 45 min in 7.4% formic acid, pH 1.9. After drying, the paper was stained with 0.25% ninhydrin in acetone to locate the standards, cut into 1-cm sections, and counted in Aquasol in a Beckman LS-250 Scintillation Counter. For data presentation, radioactivity in the ^{35}S-containing metabolites was summed and presented in a bar graph.

Table 1. Cystine content of cystinotic leucocytes incubated with thiolytic agents*

Expt. no.	Treatment	(nmoles ½ Cystine/mg cell protein)
1	Control	1.4
	WR-1065, 0.1 mM	0.4
	WR-1065, 0.7 mM	0.4
2	Control†	20.0
	WR-1065, 2.0 mM	5.4
	WR-1065, 10.0 mM	3.4
	Cysteamine, 2.0 mM	2.0
	Cysteamine, 10.0 mM	1.8
3	Control†	14.0
	WR-1065, 1.0 mM	5.8
	Cysteamine, 1.0 mM	1.3
	WR-2721, 1.0 mM	14.1

* One-hour incubation at 37° in Hank's balanced salt solution, 10 mM sodium phosphate, pH 7.4.

† Isolated leucocytes were loaded with cystine by incubation with L-cystine dimethyl ester [12].

Table 2. Cystine content of cystinotic leucocyte lysosome-rich granular fractions incubated with thiolytic agents*

Expt. no.	Treatment	(nmoles ½ Cystine/mg protein)
1	Control	24.3
	WR-1065, 0.1 mM	19.3
	Cysteamine, 0.1 mM	11.8
2	Control	113.5
	WR-1065, 0.3 mM	46.8
3	Control	65.9
	WR-1065, 0.2 mM	38.9
	WR-2721, 2.0 mM	57.3

* Granular fractions from cystine-loaded cystinotic leucocytes were incubated for 1 hr at 37° in 0.25 M sucrose-10 mM HEPES, pH 7.0, with or without the indicated thiolytic agents.

RESULTS

Whole cystinotic leucocytes incubated at 37° for 1 hr with WR-1065 (0.1 and 0.7 mM) lost a substantial portion of their endogenous cystine (Table 1, Expt. 1). Cystinotic leucocytes preloaded with cystine by incubation with L-cystine dimethyl ester and then treated for 1 hr with various concentrations of WR-1065 also lost intracellular cystine (Table 1, Expts. 2 and 3). In particular, 1 mM WR-1065 depleted whole cells of 59% of cystine found in untreated control cells (Table 1, Expt. 3), and in the same experiment cysteamine at 1 mM effected a 91% decrease in cell cystine. At 2 mM, WR-1065 and cysteamine decreased cellular cystine by 73% and 90% respectively (Table 1, Expt. 2). WR-2721 at 1 mM did not alter cystine content (Table 1, Expt. 3).

Crude lysosome-rich granular fractions prepared from cystine-loaded leucocytes were also exposed to WR-1065, WR-2721 or cysteamine (Table 2). After 1 hr, 0.2 or 0.3 mM WR-1065 lowered intralysosomal cystine levels approximately 50% (Table 2, Expts. 2 and 3), while 0.1 mM WR-1065 or cysteamine lowered cystine levels by 20% and 50% respectively (Table 2, Expt. 1). A 20-fold higher concentration of WR-2721 (2 mM) lowered cystine levels of the preloaded granular fraction only 13% (Table 2, Expt. 3). In general, the leucocyte experiments demonstrated that WR-1065 could deplete cystine from intact leucocytes and, to a lesser extent, from isolated granular fractions, while WR-2721 was essentially incapable of depleting stored cystine in these short-term experiments.

Confluent cystinotic skin fibroblasts were treated with WR-1065, WR-2721 or cysteamine at various concentrations in serum-free medium and were harvested at 2 and at 21 or 24 hr as outlined in Materials and Methods (Table 3). WR-1065 depleted cystine

Table 3. Cystine content of cystinotic fibroblasts incubated with thiolytic agents

Expt. no.	Treatment	Incubation time	(nmoles ½ Cystine/mg cell protein)
1	Control	2 hr	6.3
	WR-1065, 0.01 mM		5.7
	0.1 mM		5.1
	1.0 mM		4.4
	Control	24 hr	6.6
	WR-1065, 0.01 mM		5.3
	0.1 mM		2.6
	1.0 mM		1.0
2	Control	2 hr	15.3
	WR-1065, 0.1 mM		12.3
	1.0 mM		10.7
	Cysteamine, 0.1 mM		1.9
	1.0 mM		0.5
	Control	21 hr	9.8
	WR-1065, 0.1 mM		2.6
	1.0 mM		1.3
	Cysteamine, 0.1 mM		0.4
	1.0 mM		0.3
3	Control	21 hr	13.2
	WR-1065, 0.03 mM		11.3
	0.1 mM		9.7
	0.3 mM		1.2
	1.0 mM		0.9
	3.0 mM		0.9
	WR-2721, 1.0 mM		2.5

in a dose- and time-dependent manner but more slowly than cysteamine. At 2 hr, 0.1 mM and 1 mM WR-1065 decreased cell cystine levels an average of 20% and 30%, respectively, while 0.1 mM and 1 mM cysteamine decreased cellular cystine 88% and 97%, respectively (Table 3, Expts. 1 and 2). At 21 and 24 hr, 0.1 mM WR-1065 effected variable depletion of cystinotic cell cystine averaging 62% in the three experiments compared to a 95% decrease for 0.1 mM cysteamine. After exposure of fibroblasts to 1 mM WR-1065 for 21 hr, cystine-depleting effects (average 92%) approached those of cysteamine (average 97%). At a concentration of 1 mM, WR-2721 was also effective in depleting cystinotic cystine levels after 21 hr (81%) but less so than WR-1065 (Table 3, Expt. 3). No toxic effects were seen in these cells by morphologic criteria.

To examine the mechanism of cystine depletion by WR-1065, cystinotic skin fibroblasts labeled with L-[^{35}S]cystine for 24 hr were thoroughly washed and incubated with 1 mM WR-1065 in serum-free medium. Media and cells were collected at 2 and 21 hr and analyzed for radioactive products as described in Materials and Methods. After exposure of fibroblasts to WR-1065 (Fig. 1A), cellular radioactivity in the cystine peak was high at 2 hr (27% of total cpm) and was reduced 87% by 21 hr, consistent with nonradioactive experiments (Table 3). A radioactive peak of relatively high mobility, containing 6% of total cpm at 2 hr which decreased 80% from 2 to 21 hr, was identified as the mixed disulfide of WR-1065-cysteine by comparison with standards. An appreciable amount of radioactivity was seen in glutathione (reduced plus oxidized) which also decreased greatly from 2 to 21 hr. Detectable amounts of radioactivity were seen in *N*-ethylmaleimide-cysteine, cysteamine-glutathione mixed disulfide and cysteamine-cysteine mixed disulfide, all of which decreased with time. In the media (Fig. 1B), comparatively little radioactivity appeared in the cystine peak, and this increased only 36% from 2 to 21 hr. Most of the radioactivity in the media was seen in the WR-1065-cysteine mixed disulfide peak which comprised 50% of the total radioactivity at 21 hr. A substantial amount of radioactivity also appeared as cysteamine-cysteine mixed disulfide; this peak increased with time to 10% of the total radioactivity at 21 hr. A trace amount of cysteamine-glutathione mixed disulfide was also detected which increased in the media with time. As a control, cystinotic fibroblasts labeled with L-[^{35}S]cystine were incubated in serum-free medium for 21 hr in the absence of WR-1065 and analyzed by high-voltage electrophoresis. Radioactive glutathione and cystine were seen in cells and media but no mixed disulfides containing L-[^{35}S]cysteine were detected, in particular cysteamine-cysteine (Fig. 2).

To further identify the mixed disulfides seen in the fibroblast experiment depicted in Fig. 1, an aliquot of the sulfosalicylic acid treated medium from the 21-hr incubation with WR-1065 was further treated with an equal volume of 20 mM dithiothreitol in 10 mM potassium phosphate buffer, pH 7.3, and the radioactive products were separated on high-voltage electrophoresis. The radioactivity in the mixed disulfide peaks of glutathione-cysteine, cysteamine-cysteine, and WR-1065-cysteine and in cystine itself had disappeared following dithiothreitol treatment

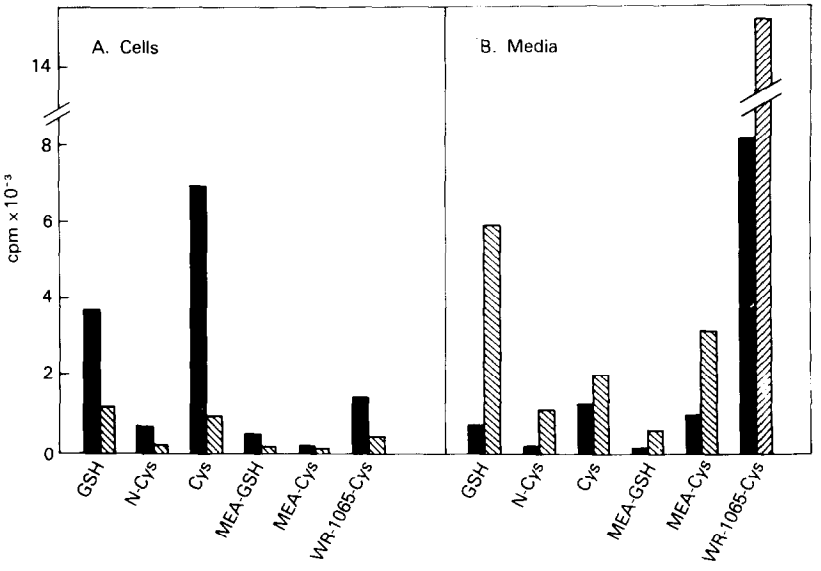

Fig. 1. High-voltage electrophoretic separation of labeled compounds found in sulfosalicylic acid extracts of cystinotic fibroblasts (A) and incubation media (B). Cells were prelabeled with L-[^{35}S]cystine and exposed to 1 mM WR-1065 for 2 hr (■) and 21 hr (▨). Compounds are shown in the order of their electrophoretic migration from left to right. The glutathione data represent the sum of radioactivities found in the reduced, oxidized and glutathione-cysteine mixed disulfide forms. Abbreviations: GSH, glutathione; *N*-Cys, *N*-ethylmaleimide-cysteine; Cys, cystine; MEA-GSH, cysteamine-glutathione mixed disulfide; MEA-Cys, cysteamine-cysteine mixed disulfide; and WR-1065-Cys, WR-1065-cysteine mixed disulfide.

and subsequent reaction with *N*-ethylmaleimide; this radioactivity was accounted for in the *N*-ethylmaleimide-cysteine peak (Table 4).

In cystinotic leucocyte lysosomes loaded with L-[^{35}S]cystine and exposed to WR-1065 for 1 hr, WR-1065-cysteine mixed disulfide was detected inside and outside lysosomes but no cysteamine-cysteine mixed disulfide was seen either in the organelles or in the incubation medium when analyzed by high-voltage electrophoresis (data not shown).

DISCUSSION

Experiments presented here indicate that WR-1065 lowers the cystine content of cystinotic cells after intracellular conversion to cysteamine, a cystine-depleting agent currently used clinically to deplete tissue cystine levels of cystinotic children. The action of WR-1065 in lowering cystine levels of human leucocytes and cultured fibroblasts was slower than that of cysteamine but appeared nearly as effective at longer times or at higher concentrations. This was particularly apparent in the experiments with fibroblasts which permitted prolonged incubation periods. Incubations of leucocyte lysosomal granular fractions with WR-1065 did not produce greater cystine depletion than that noted in whole leucocytes or fibroblasts. Furthermore, high-voltage electrophoretic analysis of protein-free extracts of L-[^{35}S] cystine-labeled fibroblasts exposed to WR-1065 showed the expected appearance of WR-1065-cysteine mixed disulfide but an unanticipated appearance of the mixed disulfides cysteamine-cysteine and cysteamine-glutathione. Comparable analysis of leucocyte granular fractions treated with WR-1065 showed the presence of WR-1065-cysteine mixed disulfide but no cysteamine-cysteine mixed disulfide, and depletion of cystine from this fraction was slower than for whole leucocytes treated with WR-1065. These observations suggest that, although WR-1065 itself has some cystine-depleting effects, prior conversion of WR-1065 to cysteamine in the cytosol enhances the cystine-depleting action of this agent in whole cystinotic cells. Cysteamine thus formed enters the lysosome and reacts with cystine to form cysteamine-cysteine mixed disulfide and cysteine, both of which can freely exit the lysosome and the cell [2]. Although little of the above products was seen within cells, disulfides would not be expected to accumulate there due to the reducing conditions prevailing in the cytosol.

The appearance of cysteamine-cysteine and cysteamine-glutathione mixed disulfides following treatment of cystinotic fibroblasts with WR-1065 was unexpected since these compounds were not seen in the media or in cell supernatant fractions of untreated cells. WR-1065 can be thought of as comprised of two components, cysteamine and propylamine. The catabolism of WR-1065 to cysteamine has not been reported previously. Cysteamine may form by reactions analogous to those known to occur in the breakdown of the aliphatic polyamines, that is, Wr-1065 may be oxidatively deaminated by a diamine oxidase to an aldehyde which undergoes β-elimination. Human fibroblasts in culture contain a diamine oxidase that acts on putrescine [17] and may recognize WR-1065 as a substrate. It is important to

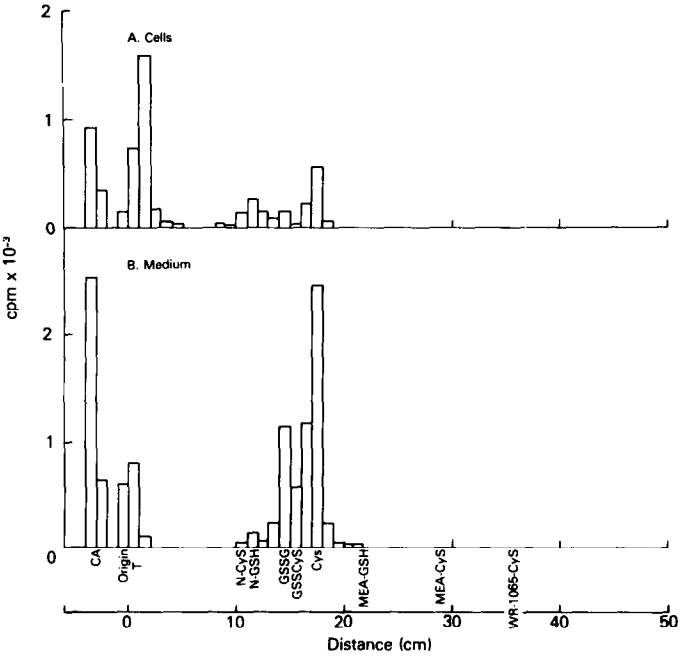

Fig. 2. L-[^{35}S]Cystine metabolites in cells and medium of 24-hr L-[^{35}S]cystine-labeled cystinotic fibroblasts allowed to efflux into cystine-free medium for 21 hr. Metabolites were separated by high-voltage electrophoresis as described in Materials and Methods. Abbreviations for standards used to identify radioactive peaks are as indicated in the legend of Fig. 1. Additional abbreviations: GSSG, oxidized glutathione; GSSCyS, glutathione-cysteine mixed disulfide; CA, cysteic acid; and T, taurine.

Table 4. Identification of mixed disulfides in experiment of Fig. 1 by treatment with dithiothreitol and separation of products by high voltage electrophoresis

Compounds	Counts/min in original medium	Counts/min in dithiothreitol-treated medium*
Medium	25,188	24,524
Cysteic acid	9,257	8,834
Origin	176	98
Taurine	736	1,032
N-Ethylmaleimide-glutathione	256	1,233
N-Ethylmaleimide-cysteine	150	7,770
Oxidized glutathione	183	38
Glutathione-cysteine mixed disulfide	90	38
Cystine	980	63
Cysteamine-cysteine mixed disulfide	593	26
WR-1065-cysteine mixed disulfide	6,337	18

* An aliquot of the sulfosalicylic acid treated medium from 21-hr incubation of fibroblasts with WR-1065 (see Fig. 1B) was neutralized with 10 N KOH and incubated for 30 min with 20 mM dithiothreitol in 10 mM potassium phosphate buffer, pH 7.3. *N*-Ethylmaleimide was then added to give a final concentration of 3 mM in a volume two times the original aliquot. The original medium (20 λ) and the treated medium (40 λ) were spotted on 3 MM paper for high voltage electrophoresis as described in Materials and Methods.

note that the use of serum-free medium in the present experiments precluded the action of medium fetal bovine serum diamine oxidase on WR-1065, which could have led to misinterpretation of the data.

The ineffectiveness of WR-2721 in depleting cystinotic leucocyte cystine as compared with its effective action toward cystinotic fibroblasts is probably due to a requirement for enzymatic dephosphorylation to the free thiol. The 21-hr incubation of fibroblasts may have been sufficient for this intracellular activation to occur. It has been reported that radioprotection afforded by WR-2721 is much more effective *in vivo* than *in vitro* [18]. The ability of WR-2721 to protect cultured mouse cells against irradiation was nearly the same as that of cysteamine when the phosphorylated compound was first activated by a mouse liver extract [19].

WR-1065 appears to be another example of a compound whose cystine-depleting effects in cystinotic cells can be attributed to formation of cysteamine. Pantethine is also a cystine-depleting agent with activity *in vitro* [20] and its activity can be attributed to enzymatic hydrolysis to cysteamine [21, 22]. In the pantethine experiments [22], only cysteamine-cysteine mixed disulfide effluxed to the medium. In contrast, in the present experiments, the appearance of a large fraction of the radioactivity in the medium as WR-1065-cysteine mixed disulfide is probably due to considerable extracellular interchange between the exiting cysteamine-cysteine disulfide and medium WR-1065. As noted previously, the pathway for conversion of WR-1065 to cysteamine is unknown; however, we suggest that WR-1065 and pantethine may each act as carriers of the more reactive cysteamine moiety to the cell interior and that the known radioprotective and chemoprotective activity of these compounds may be attributable in part to their conversion to cysteamine.

In the clinical use of WR-1065 and WR-2721 as radioprotective, chemoprotective and mucolytic agents and of pantethine as a hypolipidemic agent, the reported side effects have been minimal [9, 10*]. These observations as well as those reported in this paper suggest that these compounds may be useful as adjuncts or alternatives to cysteamine in the treatment of cystinosis.

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