Cystine Depletion of Cystinotic Cells by Aminothiols

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Nephropathic cystinosis is an inherited error of cystine metabolism characterized by autosomal recessive transmission, free (nonprotein) cystine accumulation within lysosomes, renal tubular dysfunction, and progressive glomerular insufficiency leading to end-stage renal failure in the first decade of life. In spite of extensive investigations (Schulman & Bradley 1973, Schneider & Seegmiller 1972), the primary defect leading to cystine accumulation and renal failure is unknown.

Penicillamine has been evaluated as a specific therapy for the inborn error of metabolism, cystinosis. It is known to be effective in the inherited disease, cystinuria (Crawhall et al. 1963). The original observations on the effect of penicillamine therapy in cystinosis (Clayton & Patrick 1961) were encouraging, based on an increased sense of well-being in patients while receiving the drug, and a fall in serum pyruvate concentration. Unfortunately, further studies demonstrated that penicillamine treatment was without merit in this condition (Crawhall et al. 1968, Hambraeus & Broberger 1967). It is certainly easy to be misled in evaluating treatment for chronic diseases in which there is no specific objective parameter which can be followed.

In 1967 it was learned that many tissues from patients with cystinosis which contained no cystine crystals nonetheless contained abnormally high amounts of nonprotein free cystine (Schneider, Bradley & Seegmiller 1967; Schneider, Rosenbloom et al. 1967). This provided for the first time an objective measurement which could be used to ascertain whether any particular treatment was effective in depleting cystinotic cells of their abnormal stores of nonprotein cystine. At this point, we were disappointed to find that the white blood cell cystine content of a patient who had been receiving D-penicillamine for three years was in the same abnormally high range as we observed in cystinotic patients not receiving this drug. Furthermore, no penicillamine could be detected in white cells prepared for amino-acid analysis when blood was obtained from this patient 1 h after the oral ingestion of 1 g of D-penicillamine (Schneider & Seegmiller, unpublished observation).

Further work on D-penicillamine was deferred until the studies described below, which were done as part of a larger study on the effect of aminothiols on the cystine content of cystinotic fibroblasts (Thoene et al. 1976). The accumulation of free cystine in skin fibroblasts cultured from cystinotic patients has provided a convenient in vitro test system for the evaluation of methods of lowering the intracellular nonprotein cystine content of these cells. Effective methods include growth in a low cystine medium (Schulman & Bradley 1971), treatment with dithiothreitol (DTT) (Goldman et al. 1970, Aaron et al. 1971), and treatment with ascorbic acid (Kroll & Schneider 1974). None of these methods has been proved effective clinically, although DTT treatment has been shown to lower leukocyte nonprotein cystine content in vivo (Goldman et al. 1974). One technical advance which made such studies much easier was the development of an isotope dilution assay for cystine, utilizing a specific cystine binding protein (Oshima et al. 1974).

We found that both cysteamine and cystamine are extremely effective in depleting cystinotic fibroblasts of free cystine (Thoene et al. 1976). Fig 1 demonstrates the rapid depletion of free cystine from cystinotic fibroblasts by both cysteamine and cystamine, the much less effective removal of cystine from these cells by dithiothreitol, and the lack of effect of D-penicillamine. In a separate series of experiments numerous compounds were tested for their ability both to reduce cystine in a simple chemical reaction and to remove cystine from cystinotic fibroblasts (Table 1). As shown in Table 1, cysteamine is able both to reduce cystine to cysteine in a simple chemical reaction and to remove cystine from cystinotic fibroblasts.

On the contrary, although the disulphide cystamine is also able to remove cystine from cystinotic fibroblasts, it is of course unable to reduce cystine to cysteine. The reason for this discrepancy appears to be that, in living cells, intracellular reduced glutathione is able to reduce the cystamine to cysteamine, which is then able to deplete these cells of free cystine. It is of particular interest here that, although

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Fig 1 The depletion of intracellular nonprotein cystine from cystinotic fibroblasts. This figure combines the results of two experiments in different cystinotic cell lines. The initial nonprotein cystine content for open symbols was 1.5 nmol/10⁶ cells. Initial value for closed symbols was 2.8 nmol/10⁶ cells. Cystine-deficient medium was used for all experiments.

Table 1
Residual cystine after reaction in fibroblasts and in a cell-free chemical reaction

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Fraction of control Fibroblasts</th>
<th>Simple chemical reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Cysteamine</td>
<td>HS-CH₂-CH₂-NH₂</td>
<td>0.03</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cystamine</td>
<td>S-CH₂-CH₂-NH₂</td>
<td>0.03</td>
<td>0.93</td>
</tr>
<tr>
<td>Dimethylaminoethanethiol</td>
<td>HS-CH₂-CH₂-NH₃CH₃</td>
<td>0.08</td>
<td>-</td>
</tr>
<tr>
<td>Bis(2-aminoethyl)sulfide</td>
<td>S-CH₂-CH₂-NH₂</td>
<td>1.06</td>
<td>1.23</td>
</tr>
<tr>
<td>Ethanethiol</td>
<td>HS-CH₂-CH₃</td>
<td>1.06</td>
<td>-</td>
</tr>
<tr>
<td>Mercaptoethanol</td>
<td>HS-CH₂-CH₂-OH</td>
<td>1.00</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Penicillamine</td>
<td>HS-CH₂-CH₂-OH</td>
<td>1.10</td>
<td>0.03</td>
</tr>
<tr>
<td>1-Propanethiol</td>
<td>HS-CH₃-CH₂-OH</td>
<td>0.88</td>
<td>-</td>
</tr>
<tr>
<td>1,3 Propanedithiol</td>
<td>HS-CH₃-CH₂-CH₂-SH</td>
<td>0.52</td>
<td>-</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>HS-CH₂-CH₂-CH₂-SH</td>
<td>0.35</td>
<td>-</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>H₂N-CH₂-CH₂-OH</td>
<td>1.13</td>
<td>1.15</td>
</tr>
</tbody>
</table>

*Compounds were added to cystine-deficient medium to a final concentration of 1 mM, and cells harvested after 1 h incubation at 37°C. Table 1 combines results of two experiments in two different cystinotic cell lines. Control value for DTT and cystamine, 7.15 nmol/mg cell protein; control value, 3.73 nmol/mg cell protein for other compounds.

Cell-free chemical reaction between L-cystine and 1 mM concentration of reagents at pH 7.4 and 10 mM potassium phosphate buffer at 37°C. Incubation time was 30 min. The reaction was stopped by addition of 5 mM NEM. The remaining cystine was determined by cystine-binding protein assay. Table 1 combines results of two experiments. The control value was 1.53 μM cystine for cystamine, and 3.5 μM cystine for all others.
Penicillamine is very effective in reducing cystine in the simple chemical reaction, it is completely unable to remove cystine from cystinotic fibroblasts. The reason for this is not entirely clear. The most reasonable explanation is that penicillamine is probably not able to enter the lysosome. In fact it is possible that penicillamine is not even able to enter the cell. Unfortunately, these experiments were not designed to answer these questions specifically. Perhaps it enters these spaces in an altered form which is unable to reduce cystine. Nonetheless it does seem clear that penicillamine does not gain access to lysosomes in a form which is able to reduce cystine.

The mechanism by which cysteamine depletes the cystinotic cell of free cystine is shown diagrammatically in Fig 2. Obviously no compound could function in such a model unless it were able to enter the lysosome.

![Diagram of cysteamine and cysteine reactions](image)

Fig 2 Proposed model for nonprotein cystine depletion from cystinotic fibroblasts produced by cysteamine.

Cysteamine (mol wt 77) enters the lysosomal pool and reacts with cystine (mol wt 240) to form cysteine (mol wt 120) and the mixed disulphide of cysteamine and cysteine (mol wt 196). Further reaction between cysteamine and the mixed disulphide leads to formation of cysteine and the disulphide, cystamine (mol wt 152). All reaction products have a mol wt of less than 200 daltons, and thus should freely diffuse from the lysosome. Cystamine is reduced to cysteine in the cytoplasm by reaction with glutathione (GSH). Cysteine enters normal biosynthetic pathways.

We have utilized cysteamine in one patient. Details have been reported by Thoene et al. (1976). To summarize this clinical experience, we were able to produce over 90% depletion of leucocyte free cystine content in this patient with oral cysteamine. Unfortunately, after two months of such treatment the patient had a seizure which could only be attributed to cysteamine treatment. This patient was in end-stage kidney failure, and we were unable to observe any improvement in the renal function during the two-month period. There was no measurable impairment of hematological findings or liver functions from this treatment. In retrospect, we feel this patient might have accumulated unusually high amounts of cysteamine in her body during this treatment and we are not prepared to make further attempts at cysteamine treatment until we have an appropriate way to monitor plasma cystamine and cysteamine concentrations.

Acknowledgment: Figs 1 and 2 and Table 1 are reproduced from Thoene et al. (1976) by kind permission.

REFERENCES


DISCUSSION

Dr I H Scheinberg (New York): In 1960 at the first meeting at which penicillamine was discussed, Sir Rudolph Peters said at the end of the meeting that perhaps we ought not to abandon his brainchild, BAL. He thought that perhaps with the very severely ill Wilson's patients that we saw even at that time BAL might be useful. He reasoned that being both of small molecular weight and uncharged, it was much more likely to be transported intracellularly than penicillamine, which carries four charges on the molecule.
at physiologic pH. Sir Rudolph thought that a combined therapy, which most of us have used over the years on the most severely ill patients, would allow BAL to get into the cell and literally take out copper and allow penicillamine – as he put it – to stand on guard extracellularly, intravascularly and, taking copper from albumin or other compound which had loosely-bound copper, promote excretion of copper into urine.

I suspect that the reason for Dr Schneider’s success with cysteamine, and his lack of success with penicillamine, is the same as that pointed out 16 years ago by Sir Rudolph: in general charged molecules do not cross intracellular barriers, whereas uncharged molecules are much more likely to do so.

In fact, one might wonder whether BAL might not be effective in the treatment of cystinosis for the same reason.

**Dr Schneider:** BAL has been tried.

**Dr J Czekalowski (Leeds):** Has histology been carried out on the kidneys in cystinosis?

**Dr Schneider:** I have not done so personally, but many people have.

**Dr Czekalowski:** Have crystals been seen in the tubular cells and glomeruli?

**Dr Schneider:** Yes, crystals have been seen in the cell. Unfortunately, the histology is extremely difficult to do because by the time these children are diagnosed they already have severe kidney damage. Consequently, the kidneys that are studied are scarred and fibrotic. But it appears that crystals are seen in some of the tubular cells and the glomeruli.

**Dr Czekalowski:** It was mentioned that kidney transplant may be of help later in these children – but for how long?

**Dr Schneider:** I do not advocate this as a cure because it is clearly not a cure. But we now know of several patients alive 10 years after transplantation. These children do as well with kidney transplants as any children do – meaning that some do very well, some do very poorly and most do so-so.

**Dr Stephens:** Does Dr Schneider know what the difference is between the two types of intracellular crystals that he demonstrated – the rectangular birefringent ones and the typical hexagonal cystine crystals?

**Dr Schneider:** It might simply relate to the angle from which they are being observed.

**SUMMING UP**

**Dr J C Crawhall (Montreal):** I will take this opportunity to try to sum up. I wanted to summarize because I did not know what Dr Stephens and Mr Purkiss would say in their papers. I can now tie in their comments with my own experience.

There are two parts to the summary: the first is the practical one of the management of cystinuria. Cystinuria is a condition which leads to considerable distress from renal colic, ureteric obstruction and recurrent surgery. Penicillamine is of value to those suffering these disorders. We want to try to establish whether the complications are dose-related. On the premise that they may be, we want to be able to use the minimum penicillamine dosage compatible with prevention of stone formation.

Secondly, the reason why we started to talk a little about cystinosis is to enlarge our ideas on thiol drugs and their mechanism of action. As Dr Schneider showed, there are a large number of possible substances of this sort. There may be a number of factors of importance: for instance, molecular size, charge or optical configuration – which has not been mentioned, One reason why penicillamine may have a limited effect in this condition is that the active transport mechanisms of cell membranes are geared to L-amino acids, not D-amino acids. Thus, there may be a specific mechanism for keeping the D-amino acids out.

Cysteamine carries quite a heavy charge, yet it seems able to get in to cells. The other compound, dithiothreitol, which carries no charge, is also extremely effective in this respect. It seems that there is probably a whole area of fundamental study that could be carried out, and about which we will probably hear more during the course of this symposium.