1α-OH-D_3. The new vitamin D_2 derivative should prove very useful, however, for detailed studies of the causes for the well-known, but poorly understood, discrimination of chicken and other bird species against vitamin D_2.

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Decrease in Free Cystine Content of Cultured Cystinotic Fibroblasts by Ascorbic Acid

Abstract. *The 100-fold increase in free cystine content characteristic of cultured skin fibroblasts from patients with nephropathic cystinosis was decreased more than 50 percent by addition of* L-*ascorbic acid to the culture medium at concentrations of 0.29 to 2.9 millimolar. Fresh ascorbic acid must be added to the culture medium daily to produce a progressive decrease of the free cystine content of the cells over a 3-day period. Upon removal of ascorbic acid from the medium, the free cystine content returns to its initial value.*

Nephropathic cystinosis is a metabolic disease that is inherited in an autosomal recessive manner and is characterized biochemically by a high intracellular content of free (nonprotein) cystine. As a result, cystine crystals deposit in the conjunctiva, bone marrow, lymph nodes, peripheral leukocytes, and many internal organs (*1*). Affected children have impairment of both renal tubular and glomerular functions, which leads to end-stage renal disease with uremia within the first decade of life.

The cystine is thought to accumulate in lysosomes (*2*), but the primary defect that leads to cystine storage remains unknown. This disease has recently been diagnosed in a pregnant woman, on the basis of an increased content of free cystine in cultured amniotic fluid cells (*3*). The pregnancy was terminated, and we studied the cystinotic fetal organs and found that most organs contained 50 to 100 times more free cystine than similar organs from normal controls (*3*). Only the adrenal gland of the cystinotic fetus had a free cystine content in the normal range. Since the fetal adrenal gland is very rich in ascorbic acid (*4*), we tested the effect of ascorbic acid on cultured skin fibroblasts from patients with nephropathic cystinosis. Such fibroblasts contain 100 times more free cystine than fibroblasts from controls (*5*).

When growth medium containing 0.57 m*M* L-ascorbic acid was added to cultured cystinotic skin fibroblasts every 24 hours, the cystine content declined steadily for 3 days (Fig. 1A), reaching less than 50 percent of the initial value. If the daily medium changes contained freshly prepared ascorbic acid, the cystine concentration of these cells remained at this low level. When ascorbic acid was omitted from the medium, the cystine content of the cells returned to the initial value within 3 days. In cultured fibroblasts from five patients with nephropathic cystinosis and one patient with the intermediate (late onset) type of cystinosis (*1*) this decline averaged 58.6 percent (range 48 to 78 percent) (Table 1). It was not possible to lower the free cystine content of cystinotic fibroblasts faster or to a greater extent by adding fresh ascorbic acid more frequently (every 12 hours) or by using higher concentrations. A similar decline in the free cystine content of these cells was observed after the addition of ascorbic acid in concentrations ranging from 0.29 to 2.9 m*M*; with 0.11 m*M*, however, the free cystine content declined only 25 percent.

Dithiothreitol [DTT, Cleland's reagent (*6*)], a powerful reducing agent, removes cystine from cystinotic fibroblasts, presumably by reducing cystine to cysteine (*7*). When DTT (1 m*M*) was added every 2 hours to cystinotic fibroblasts in a cystine-free medium, the intracellular cystine pool was depleted by more than 90 percent in 8 hours (Fig. 1B). If medium containing 133 μ*M* cystine was then added, the cells regained their initial intracellular free cystine content in 24 to 48 hours. In the presence of 0.57 m*M* ascorbic acid, the rate of reaccumulation decreased about 50 percent, and the eventual free cystine content of the cells was less than half that in the control cells (Fig. 1B).

The growth rate and morphological appearance by phase microscopy of both control and cystinotic fibroblasts were unchanged in the presence or absence of 0.57 m*M* ascorbic acid. This concentration of ascorbic acid caused no significant change in the *p*H of the growth medium (± 0.01 *p*H unit). Raising the concentration of ascorbic acid to 2.9 m*M* inhibited the growth of both control (*8*) and cystinotic fibroblasts. Of the intracellular amino acids other than cystine, only the concentrations of proline, hydroxyproline, lysine, and hydroxylysine were changed in 0.57 m*M* ascorbic acid (*9*). The protein content per cell was unaltered by concentrations of ascorbic acid ranging from 0.29 to 1.16 m*M*.

The mechanism by which ascorbic acid lowers the free cystine content of

cystinotic fibroblasts is not known. The most obvious explanation is that ascorbic acid might act as a reducing agent. However, judging from the reported oxidation-reduction potentials, ascorbic acid should not be able to reduce cystine to cysteine directly (*10*).

The fact that dehydroascorbic acid, the first oxidation product of ascorbic acid, also removes free cystine from cystinotic cells (Table 1) is not helpful, since dehydroascorbic acid is quickly reduced to ascorbic acid in mammalian cells (*11*).

Although we do not have enough information to determine whether ascorbic acid decreases the free cystine content of cystinotic fibroblasts because it is a reducing agent, we do know that it acts differently from DTT in two respects. First, even very high concentrations of ascorbic acid cannot remove all the free cystine from these cells. Second, ascorbic acid lowers the free cystine content of these cells much more slowly than DTT.

Another explanation of the decrease in free cystine content after the addition of ascorbic acid is that it might alter transport of cystine into or out of the cystine pool in cystinotic fibroblasts. The cystine in these cells is known to be in a state of dynamic equilibrium, having a half-life of approximately 7 hours (*12*). Since ascorbic acid has been reported to increase the lability of lysosomal membranes (*13*), it might be expected to increase the efflux of cystine from the abnormal cystine pool in these cells. To test this possibility, we grew cystinotic fibroblasts for 24 hours in medium containing [^{35}S]cystine. After replacement with nonradioactive complete growth medium, ^{35}S efflux was unchanged in the presence or absence of 0.57 m*M* ascorbic acid over a 24-hour period. In contrast, during the first hour 1 m*M* DDT caused the release of 250 percent more ^{35}S than control cells or cells treated with ascorbic acid. The experiments outlined in Fig. 1B are compatible with a slowed entry of cystine into the cystine pool. However, there are other interpretations of the data. Ascorbic acid might alter the final concentration of free cystine by changing the intracellular distribution of cystine, affecting a particular enzyme or pathway, such as its known ability to stimulate the hexose monophosphate shunt in human leukocytes (*14*) or in some other unknown way.

Our study demonstrates another method of removing free cystine from cultured cystinotic fibroblasts. Previous biochemical methods have utilized either DDT (*7*) or growth of cystinotic cells in a cystine depleted medium (*15*). The hybridization of cystinotic fibroblasts with noncystinotic cells also resulted in a marked lowering of the intracellular content of free cystine (*16*). DTT (1.0 m*M*) can remove almost all the free cystine from cystinotic cells. Some promising information concerning use of this drug to treat patients has been reported (*7*), but the potential toxicity of this compound prohibits its clinical use except under strict investigational control. The fact that cystinotic cells can be depleted of cystine by growth in a cystine-free medium suggested that treatment with a cystine-free diet might be useful. In practice, however, such a diet has proved ineffective (*17*). Since ascorbic acid consistently lowers the free cystine content of cultured cystinotic fibroblasts and appears to be safe for human use, a controlled therapeutic trial with high doses of this drug seems warranted in this fatal disease. It will not be possible to attain plasma ascorbic acid concentrations as high as we have used in culture media. The highest plasma concentration that can be reached is about 0.15 m*M*, but other tissues (including the kidney) will attain ascorbic acid concentrations as high or higher than those used in our experiments (*18*).

Although the use of ascorbic acid was suggested because the cystinotic fetal adrenal gland had a normal content of free cystine, there is no direct evidence that ascorbic acid caused the low free cystine content of this gland (*19*).

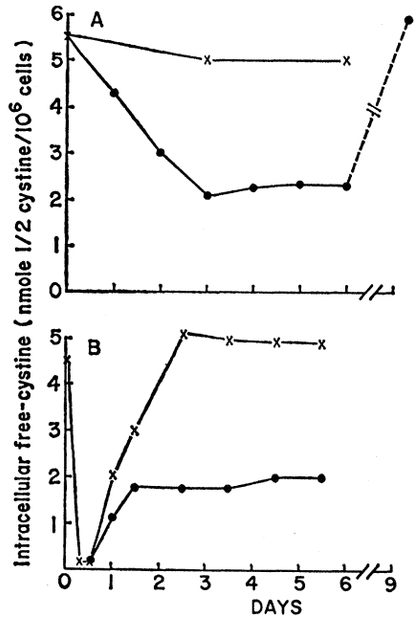

Fig. 1. (A) Effect of L-ascorbic acid on the intracellular content of free (nonprotein) cystine in cystinotic skin fibroblasts. (B) Effect on the reentry of cystine into these cells after depletion of free cystine by the combined use of dithiothreitol (DTT) and cystine-free medium. Cystinotic fibroblasts were grown nearly to confluency in Falcon petri dishes (100 mm) in a humidified incubator continuously flushed with a mixture of 10 percent CO_2 and 90 percent air (*20*) in modified Ham's F12 medium (*21*) with 10 percent fetal bovine serum. Control cells (x - x). Ascorbic acid treated cells (• - •). Intracellular free cystine was assayed by ion-exchange chromatography (*20*). For (A), 0.57 m*M* L-ascorbic acid was added at day 0 to this medium which was subsequently changed daily, with fresh ascorbic acid added each day through day 5. From day 6 through day 8 (- - - - -) ascorbic acid was omitted although the medium was changed daily. (B) Cystine-free medium with dialyzed 10 percent fetal bovine serum containing freshly dissolved 1 m*M* DTT was added every 2 hours for 8 hours. The cells were kept in the cystine-free medium for 4 hours longer and then medium containing 133 μM L-cystine was added either with or without 0.57 m*M* L-ascorbic acid. Medium was changed at 12 and 24 hours, and then daily as described in (A).

Table 1. Decline of the intracellular free cystine in cultured skin fibroblasts from five patients with nephropathic cystinosis and one patient with intermediate (late onset) type of cystinosis (D.S.) after treatment with L-ascorbic acid, dehydroascorbic acid, and isoascorbic acid. Cultured fibroblasts from normal skin have a free cystine content of < 0.1 nmole of half-cystine per milligram of protein.

Cystinotic fibroblasts	Half-cystine (nmole/mg protein)		
	Initial	3 days	6 days
0.57 mM L-ascorbic acid			
K.M.	16.2	6.8	6.0
Y.D.	15.8	7.2	6.9
R.B.	9.7	4.9	5.0
M.M.	7.6	3.8	3.6
M.C.	5.6	3.0	3.0
D.S.	11.3	4.8	4.8
0.57 mM dehydroascorbic acid			
M266	9.3	4.5	4.5
0.57 mM isoascorbic acid			
M266	9.3	3.9	3.9

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Peromyscus: Effect of Early Pairing on Reproduction

Abstract. *Sibling mating in prairie deer mice* (Peromyscus maniculatus bairdi) *results in poor reproductive performance. Siblings experimentally paired before puberty exhibit delayed reproduction when adult. A behavioral mechanism is involved in this reproductive delay, since prepubertal familiarity also delays reproduction in nonsibling pairs. Such a reproductive delay may act to reduce inbreeding depression and regulate population growth.*

In natural populations of rodents the panmictic breeding unit is small, and there is genetic isolation between local populations, or demes (*1*). In *Peromyscus maniculatus*, a North American species, deme size is further limited because juveniles travel in sibling groups and disperse only short distances from their parents' home range (*2*). Such conditions may result in a high incidence of inbreeding. Since close inbreeding can lead to inbreeding depression, a serious reduction of various components of fitness (*3*), many species possess some mechanism to reduce the probability of inbreeding. Pregnancy block (*4*) may serve this function in mice but at the cost of energy and time expended in mating and the initial stages of pregnancy. A genetic mechanism in which males mature later than females would reduce inbreeding without this waste of time, energy, and gametes (*5*). However, in *P. maniculatus* such a mechanism would have minimal effect since the difference in age of maturation is at most a few days. A behavioral phenomenon which reduces inbreeding by inhibiting consanguineous mating is the incest taboo in man (*6*). To the best of my knowledge, a functional incest taboo has not been reported in any rodent species.

In the study reported here sibling pairs of *Peromyscus maniculatus bairdi* exhibited delayed breeding (*7*). This delay in breeding apparently results because a nonsexual relationship formed before puberty interferes with the later establishment of a sexual relationship. Although the exact mechanism of this interference in *Peromyscus* is as yet unknown, there is evidence that in other genera behavioral factors are involved. For example, in male rats play behavior habits established before puberty interfere with adult copulatory behavior (*8*), and in humans childhood association interferes with the later establishment of a sexual relationship (*9*).

Four experimental groups of bisexual pairs of mice were used (*10*). The first two groups were paired at 21 days of age. Of these, one group consisted of sibling pairs ("early siblings," $N = 30$ pairs) and one group of nonsibling pairs ("early nonsiblings," $N = 30$ pairs). The other two groups were paired at 50 days of age and also consisted of sibling pairs ("late siblings," $N = 31$ pairs) and nonsibling pairs ("late nonsiblings," $N = 29$ pairs). All individuals mated late were maintained in unisexual sibling pairs until mated. The two ages for mating made it possible to compare pairs mated before sexual maturity with pairs mated after sexual maturity. In natural popualtions of *P. m. bairdi* sexual maturity in females has been reported as early as 35 days of age (*11*). In the study reported here, sexual maturity may have been attained as early as 37 days of age: this subspecies has a gestation period of 21 to 23 days (*5, 12*), and the youngest early-mated pair to produce a litter was 60 days old.

Whenever possible, experimental animals were chosen from litters that contained at least two males and two females. From each such litter one sibling pair was mated and the remaining male and female were mated with individuals from a similar litter. Thus, a litter was represented in both the sibling and nonsibling matings to reduce the effect of any difference in fertility between litters. Members of nonsibling pairs were from litters born not more than 2 days apart. All animals were from the second laboratory-reared generation descended from wild-caught stocks.

All mice were housed in clear plastic cages (15 by 30 by 15 cm) at 20° ± 1°C and 20 to 70 percent relative humidity; they were on a daily cycle of 15 hours light and 9 hours dark. Under these conditions, *P. m. bairdi* breeds throughout the year with a peak from summer through early fall (*13*). My experiment was begun in early fall and continued until fall of the following year.

After mating, females were examined for any indication of pregnancy at least once a week. Pregnant females were examined twice a day until the litter was born. The litter was not disturbed until 24 hours after its birth, when the number, sex, and average weight of