Reversal by Copper of the Lathyrogenic Action of D-Penicillamine* (33359)

H. R. KEISER, R. I. HENKIN, AND M. KARE (Introduced by A. Sjoerdsma)
National Heart Institute, Bethesda, Maryland 20014; and Monell Chemical Senses Center, Philadelphia, Pennsylvania 19103

D-Penicillamine (D-Pen) or β, β-dimethylcysteine has been shown to produce an increase in soluble collagen in the skin of man and animals (1, 2). The complete picture of osteolathyrism with stunted growth, deformed bones, and increased urinary hydroxyproline (HOPro) has not been reported previously. The mechanism of action of D-Pen on collagen is not understood fully. Since D-Pen has been used to treat patients with Wilson's disease, cystinuria, rheumatoid arthritis, and scleroderma, further knowledge of its action would be desirable. In a previous study of 73 patients receiving D-Pen, we found that 23 (32%) had developed a marked loss of taste acuity; treatment of 4 of these patients with supplemental oral copper returned taste acuity to normal in each case (3). A similar taste loss was produced in each of 56 rats treated with D-Pen; they were also grossly stunted and had defects of skin, bone, and cartilage. Treatment of 8 of these rats with large supplemental doses of oral copper returned taste acuity to normal in each rat and also reversed the gross changes of skin, bone, and cartilage to normal (4). Since D-Pen is a chelator of copper, we evaluated the effects of D-Pen with and without oral copper supplementation on skin collagen and on the activities of skin proline hydroxylase and skin monoamine oxidase, metal-containing enzymes involved in collagen synthesis and collagen cross-linking.

Methods. Forty male weanling Holtzman rats were divided into groups of eight animals each and fed a diet of ground Purina rat chow containing 15% dextrose. Food and fluid intake of each animal were measured daily and each animal was weighed every third day. The first group served as controls. The second, third, and fourth groups were given D-Penicillamine[1], 10 g/kg, mixed in their food, for the entire study. Since the average daily food intake was 20 g/animal/day and the animals weighed about 100 g at the start of the experiment, the dose of D-Pen was about 2 g/kg of body weight. From the twenty-eighth day of the study copper sulfate was added to the diets of groups 3 and 4; group 3 was given 50 mg of copper/kg of diet, a low dose, while group 4 was given 2.5 g of copper/kg of diet, a high dose. Group 5 was given the diet with D-Pen for the first 28 days of the study after which time they were fed the diet alone. During the fifty-second and last day of the study only water was given to each animal and 24-hr urines were collected. On day 53 the animals were anesthetized with ether, had a polyethylene catheter placed in the inferior vena cava, were injected with 100 units of heparin, and then exsanguinated via the catheter. Blood was centrifuged immediately, the plasma was removed and frozen.

Dorsal skin was shaved free of hair, removed from each animal, and either processed immediately or frozen for subsequent analyses. A collagen profile was determined by a modification of methods previously reported from this laboratory (5). The subdermis was dissected away, and the dermis was minced finely with a scalpel. One sample of about 25 mg was weighed before and after drying at 110° under vacuum over phosphorous pentoxide for at least 18 hr to obtain the percentage water content. This dried sample was then hydrolyzed overnight in 6 *N* hydrochloric acid (HCl). The solution was filtered and an aliquot was assayed for HOPro to yield a measurement of total collagen content expressed as μg of HOPro/mg of dry weight of dermis. A separate full thickness sample of

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[1] Supplied through the courtesy of Merck and Co., Inc., West Point, Pa.

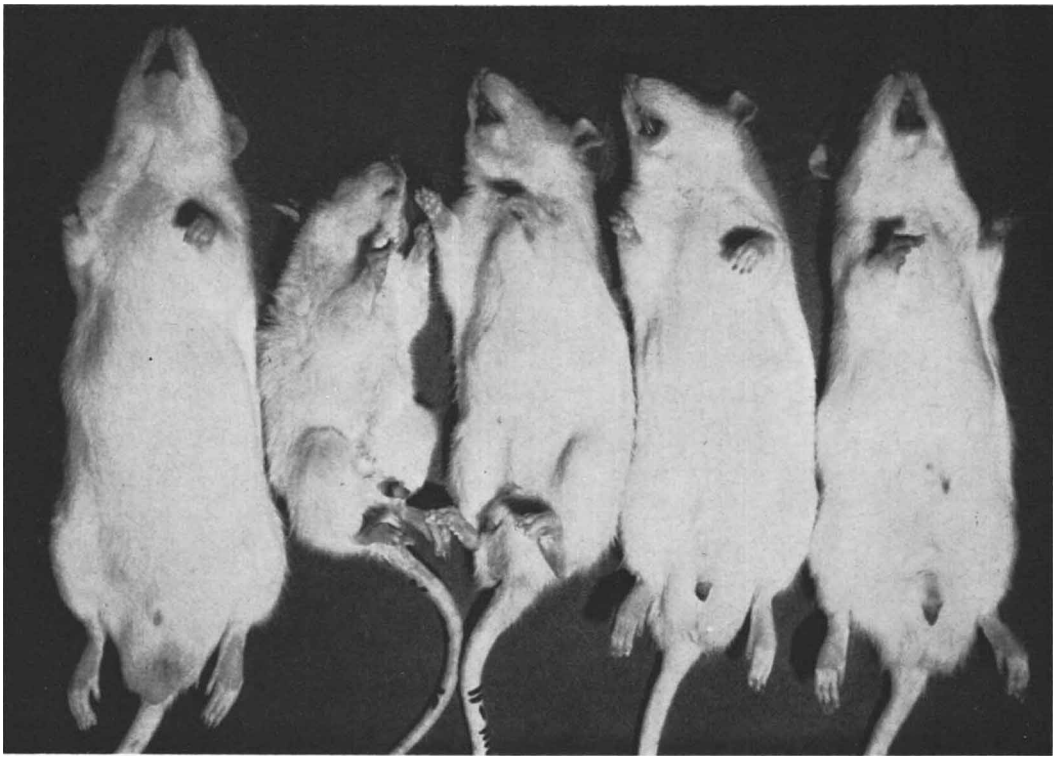

FIG. 1. Photograph of a representative animal from each group; L to R: control, D-Pen, D-Pen + lo Copper, D-Pen + hi Copper, D-Pen/off.

fresh dermis was taken with a skin punch, 2 mm in diameter. The HOPro was determined and expressed as μg of HOPro/mm^2 surface area of skin. A third sample of skin, approximately 100 mg in weight, was extracted twice with 10 ml of 0.15 *N* sodium chloride, pH 7.5, at 4° for 24 hr with constant stirring. The extracts were combined, centrifuged at 30,000*g* for 20 min and filtered through Whatman no. 1 filter paper. An aliquot of the extract was analyzed for HOPro content. This was related to the total HOPro content to yield the percentage of collagen soluble in neutral salt solution. Urinary creatinine was determined by a modification of the method of Jaffe (6) and HOPro in urine and tissue was measured by the method of Prockop and Udenfriend (7).

The monoamine oxidase (MAO) activity of fresh skin minces was measured by methods previously reported from this laboratory using benzylamine-1-^{14}C as substrate (8). Results are expressed as mμmoles of benzylamine oxidized per mg of wet weight of minced dermis per hour of incubation. Proline hydroxylase activity of fresh skin minces was assayed by the method of Hutton *et al.* (9); results are expressed as dpm of tritiated water released per mg of wet weight of minced dermis per hour of incubation. Radioactivity was measured in a Packard liquid scintillation spectrometer model 3375 with automatic external standardization. Copper was assayed in a Perkin-Elmer atomic absorption spectrophotometer model 303 with a direct concentration readout model DCR1 by a method to be reported in detail elsewhere.

Results. Grossly the members of the five groups of rats were easily distinguishable from each other (Fig. 1). The animals treated with D-Pen alone (Group 2) were less than one-half the size of control animals (Group 1), had scruffy-looking coats and were very inactive. They dragged their hind legs behind them, had deformed snouts from contact with their food cups, and were appar-

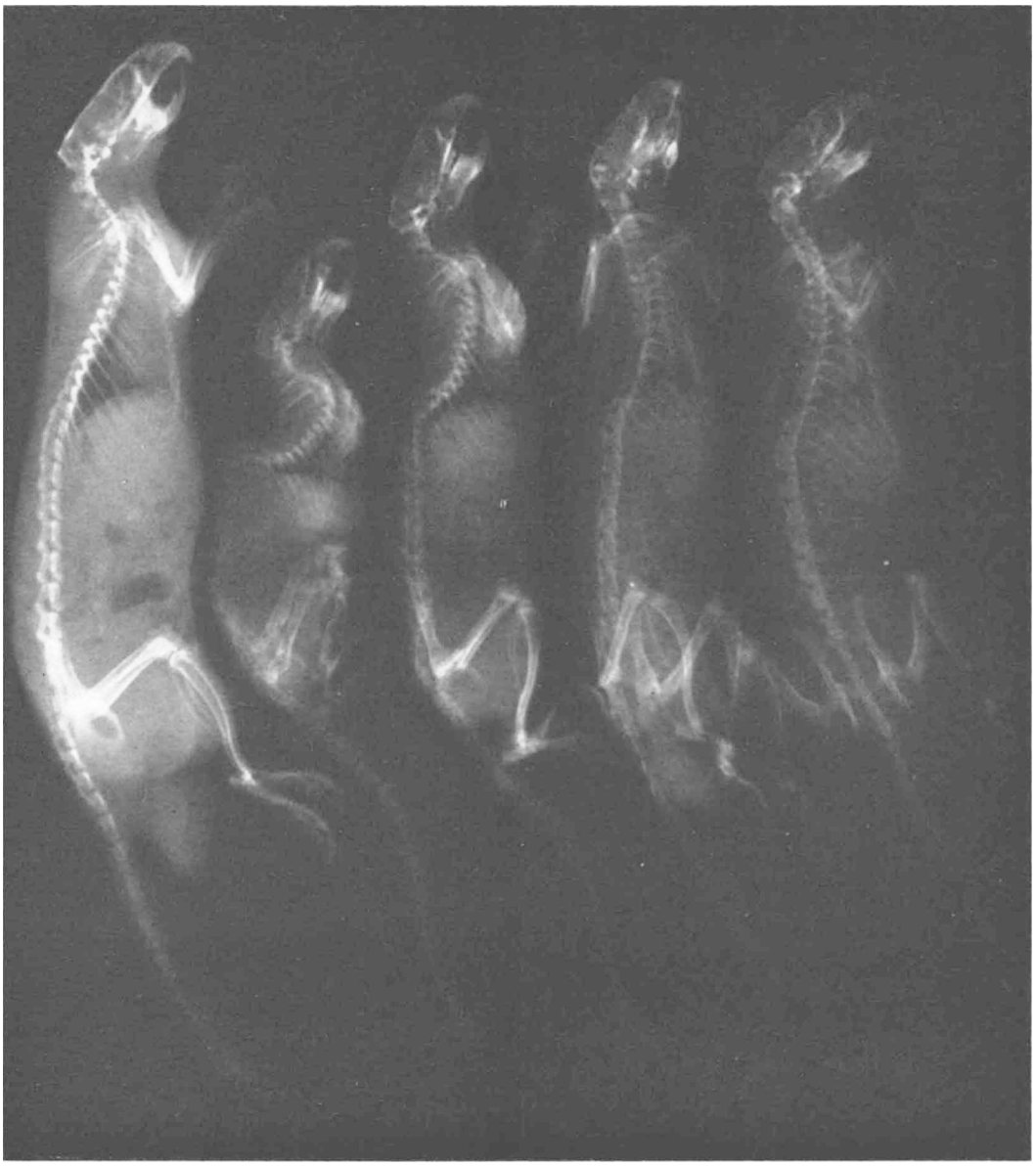

FIG. 2. Roentgenogram of a representative animal from each group; same animal and same order as shown in Fig. 1.

ently in pain even when gently handled. The entire skin covering of the tail pulled free in several animals if they were picked up by their tail, and the skin covering the pressure points over the extremities was macerated and bleeding due to contact with their metal cages. Each animal had a persistent hemorrhagic priapism. One animal treated with D-Pen alone and one with D-Pen plus low copper (Groups 2 and 3) died before the end of the study. Roentgenograms comparing the animals from each group (Fig. 2) showed the marked changes in body size and revealed deformed bones, kyphoscoliosis and subperiosteal new bone formation in the animals treated with D-Pen alone. These changes

TABLE I. Effects of D-Pen with and without Copper Supplementation on Body Weight, Urinary Creatinine and Hydroxyproline Excretion.[a]

Group	Body wt. (g)	Urine creatinine (mg/day)	Urine hydroxyproline (μg/day)	Urine hydroxyproline (μg/mg of creatinine)
1. Control	298 ± 7[b]	12.6 ± .4[b]	849 ± 44	68 ± 4[b]
2. D-Pen	131 ± 8	6.0 ± .4	690 ± 76	117 ±15
3. D-Pen + lo Cu	160 ± 8[c]	7.3 ± .5	558 ± 94	90 ± 8
4. D-Pen + hi Cu	197 ± 7[b]	8.2 ± .5[b]	774 ± 73	95 ± 8
5. D-Pen/Off	224 ± 7[b]	10.2 ± .4[b]	1100 ± 63[b]	109 ± 8

[a] Data reported as mean ± SEM.
[b] $p < .01$ with respect to D-Pen group.
[c] $p < .05$ with respect to D-Pen group.

were less marked in the animals given copper and in those in whom the D-Pen was stopped.

The final body weight of the animals treated with D-Pen alone was 44% of that of the control animals (Table I). There was a progressive increase in weight after copper supplementation of the diet; animals given high copper supplements weighed 50% more than the animals treated with D-Pen alone. Urinary creatinine excretion paralleled the final body weight. Total urinary HOPro excretion was decreased in those animals treated with D-Pen with or without copper supplementation by a maximum of only 33% from control levels. However when urinary HOPro is expressed per unit of creatinine, to take differences in body size into account, then the excretion of the group treated with D-Pen alone was increased 72% over that of the control animals. Copper administration reduced these abnormally high levels of HOPro excretion by 50% (Table I).

The dermal collagen profile of each group indicates that the water content of the dermis of each of the D-Pen treated groups was higher than the control animals (Table II). There was considerable variability and no consistent pattern of change noted after copper supplementation. The total collagen content of the dermis per mg of dry weight was reduced in each of the D-Pen treated groups, but the variability was again large. More importantly the collagen content expressed per unit of skin area was reduced by 40% after treatment with D-Pen alone and was not altered after copper supplementation. The most striking chemical difference and the most important change which illustrates the lathyritic action of D-Pen is the 14-fold increase in the percentage of neutral salt soluble collagen in the D-Pen treated animals

TABLE II. Effects of D-Pen with and without Copper Supplementation on Dermal Collagen Profiles.[a]

Group	Water content (% wet wt.)	Collagen content (μg of HOPro/mg of dry wt.)	Collagen content (μg of HOPro/mm²)	Salt soluble collagen (% total collagen)
1. Control	57 ± 1[b]	105 ± 4	37 ± 2[b]	0.5 ± 0.1[b]
2. D-Pen	62 ± 1	96 ± 4	22 ± 2	7.2 ± 1.3
3. D-Pen + lo Cu	59 ± 2	86 ± 1[c]	24 ± 2	6.3 ± 0.7
4. D-Pen + hi Cu	63 ± 1	76 ± 6[c]	22 ± 1	2.9 ± 0.3[b]
5. D-Pen/Off	62 ± 1	76 ± 3[b]	22 ± 1	0.8 ± 0.1[b]

[a] Data reported as mean ± SEM.
[b] $p < .01$ with respect to D-Pen group.
[c] $p < .05$ with respect to D-Pen group.

TABLE III. Effects of D-Pen with and without Copper Supplementation on Dermal Enzymes, Plasma Copper and Urinary Copper.[a]

Group	MAO (mμmoles/mg of wet wt./hr)	Proline hydroxylase (dpm/mg of wet wt./hr)	Plasma copper (μg/100 ml)	Urine copper (μg/mg of creatinine)
1. Control	.83 ± .05[b]	274 ± 20[b]	116 ± 6[b]	.53 ± 0.12[b]
2. D-Pen	.47 ± .04	85 ± 22	23 ± 4	1.31 ± 0.15
3. D-Pen + lo Cu	.57 ± .04	163 ± 24[c]	40 ± 8	1.91 ± 0.24
4. D-Pen + hi Cu	.63 ± .04[c]	300 ± 11[b]	84 ± 2[b]	7.02 ± 1.48[b]
5. D-Pen/Off	.91 ± .06[b]	293 ± 15[b]	103 ± 7[b]	.77 ± 0.08[b]

[a] Data reported as mean ± SEM.
[b] $p < .01$ with respect to D-Pen group.
[c] $p < .05$ with respect to D-Pen group.

compared to that of controls. When low and high copper supplements were given, the abnormally high levels of soluble collagen were reduced by 13 and 60%, respectively. In the group in which D-Pen was stopped the percentage of soluble collagen was not statistically different from control animals.

The dermal MAO activity was decreased by 43% in the D-Pen treated animals (Table III). Low and high copper supplementation reduced the decrease to 31 and 24%, respectively. In the animals in whom D-Pen was stopped the MAO activity was not significantly different from that of control animals. Dermal proline hydroxylase activity was decreased by 69% in the D-Pen treated animals while low copper supplementation reduced the decrease to 40%. High copper supplements or stopping the drug returned this enzyme activity to normal levels.

Plasma copper was reduced 80% in the D-Pen treated animals (Table III). Low and high copper supplementation raised serum copper levels progressively; the serum copper of the group given high copper supplementation was not statistically different from that of the control group.

Discussion. The administration of D-penicillamine to weanling rats produces classic signs of osteolathyrism. To our knowledge this is the first time D-Pen has been shown to produce such gross changes of osteolathyrism with stunting of growth and increases in urinary HOPro excretion. Increased levels of soluble collagen in skin of rats and humans receiving D-Pen have been demonstrated previously (1, 2). The multiple abnormalities observed in the animals of this study are probably related to a number of factors including the large dose of D-Pen used (about 2 g/kg of body wt.), its administration to weanling animals, and the long duration (52 days) of the study. Weanlings have been shown previously to be more susceptible to lathyrogenic agents than adult animals (10) and this effect is both dose and time related.

The measurement of HOPro or collagen per unit weight of dermis is a concentration figure which varies little despite wide variation in skin thickness. Collagen content expressed per unit of skin area yields a measure of the "collagen thickness" of the skin. Thus while there was no significant difference in total collagen content per mg of dermis between the animals treated with D-Pen alone and with control animals (Groups 1 and 2) there was a highly significant difference in collagen content per unit of skin area. This latter difference was readily apparent in that the skin of the D-Pen treated animals was friable and thin compared to that of the controls.

The specific action of D-Pen in producing lathyrism is still open to discussion. Nimni presents evidence that D-Pen forms a reversible complex with aldehyde groups derived from polypeptide-bound lysine just prior to the cross-linking step (11). This leads to the accumulation of large amounts of aldehyde-rich soluble collagen which will cross-link normally when the D-Pen is removed by dialysis. This is in contrast to β-aminopropioni-

trile (BAPN) which appears to produce lathyrism by blocking the conversion of the epsilon-amino groups of polypeptide-bound lysine to the aldehyde (12). However, as shown in this paper, D-Pen also inhibits proline hydroxylase activity which converts polypeptide-bound proline to HOPro. This is a necessary step in the synthesis of the collagen monomer, or alpha chain, and precedes cross-linking. This enzyme has been shown to be dependent upon ferrous ion in the chick embryo (9) but its metal requirement in rat skin is unknown. The present studies suggest that it is copper dependent. The D-Pen also inhibits tissue MAO activity which may be involved in the conversion of the epsilon-amino group of polypeptide-bound lysine to the aldehyde. Whether the crude MAO activity measured in this study in dermal minces reflects the activity of such a cross-linking enzyme is unknown. However our studies suggest that whatever its action the enzyme is copper dependent. Monoamine oxidases of other tissues have been studied extensively and many have been shown to contain copper (13, 14). The inhibition of these enzymes by D-Pen seems to be related directly to its metal chelating action. Thus D-Pen may interfere at multiple sites and the degree of enzyme inhibition and the amount of aldehyde complex occurring may relate to effective local levels of the drug.

The lathyritic and biochemical changes observed were either partially or completely reversed by dietary copper supplementation even while D-Pen administration continued. When copper was added to the diets the animals ate better, began to grow again and appeared to be much improved. The effects of copper supplementation would have been more striking if copper had been added at the very beginning of the study instead of after the development of lathyrism. The possible combination of D-Pen with copper in the diet or in the gastrointestinal tract of animals so treated might result in a spurious decrease in the D-Pen dosage administered. This possibility is unlikely in view of measurements made of D-Pen and of its disulfide and mixed disulfide metabolic products in the urine as sulfonic acid derivatives using an automatic amino acid analyzer. Equal amounts of D-Pen and its metabolites were found in the urine of animals treated with D-Pen alone and with D-Pen plus large amounts of copper. Pyridoxine deficiency secondary to D-Pen administration is not part of the mechanism of its lathyrogenic action. In a subsequent series of experiments to be reported later pyridoxine supplementation added to the diet (400 mg/kg of diet) of those animals treated with D-Pen did not alter any of the observed lathyritic changes. In the same series of additional experiments animals fed the same diet as the controls but an amount similar to that consumed by the D-Pen treated animals were similar to control animals fed *ad libitum* except their body weight was 33% less and they showed a 20% decrease in collagen content per unit of skin area.

Since copper reverses many of the lathyritic changes in both skin and bone it must play an important role in mechanisms of collagen synthesis. Further confirmation of the role of copper in collagen synthesis can be found in the literature where lathyritic changes have been noted in pigs, chickens, and rabbits raised on copper deficient diets (15–17).

Summary. Administration of D-Penicillamine to weanling rats as 1% of the diet was found to produce marked osteolathyrism, with concomitant increases in urinary hydroxyproline and salt-soluble dermal collagen and decreases in dermal monoamine oxidase and proline hydroxylase activities. Supplementation of the diet with copper either partially or completely reversed the osteolathyritic and biochemical changes. Thus, an important role for copper in collagen synthesis is suggested.

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The Detection and Characterization of Phagocytic Cells in Established Human Cell Lines Synthesizing Immunoglobulins (33360)

J. K. KAMMERMEYER, R. K. ROOT, D. P. STITES, P. R. GLADE, AND L. N. CHESSIN
(Introduced by S. M. Wolff)

Laboratory of Clinical Investigations, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

Long-term suspension cultures derived from the peripheral blood of patients with infectious mononucleosis have been established and characterized in our laboratory (1). The cells in these continuous cultures have light microscopic, histochemical, and ultrastructural features which are characteristically lymphoid (1); and they are capable of immunoglobulin synthesis *in vitro* (2). Preliminary screening studies indicated, however, that some of the cells in these established lines as well as in continuous lines derived from patients with other lymphoproliferative disorders were capable of phagocytosis, a function usually attributed only to monocytes and macrophages. The present investigations were undertaken to quantitate and further characterize the phagocytic cells in these cultures in an effort to discern, if possible, their origin and significance. As part of this characterization the ability of these cells to release leukocytic pyrogen after phagocytosis was also examined, since this is a function previously known to be associated only with granulocytes and monocytes (3).

Materials and Methods. Cell Lines. Seventeen human, long-term cell lines were used. These included AL-1 (derived from a patient with Burkitt's tumor) (4), LK1D (derived from a patient with lymphoblastic leukemia) (5), IM-1 (derived from a patient with lymphosarcoma) (6), and 14 cell lines isolated from patients with heterophile-positive infectious mononucleosis as shown in Table I. These cell lines were maintained in suspension culture containing RPMI 1640 medium (7), with 20% heat-inactivated fetal calf serum (Flow Laboratories, Rockville, Maryland) and were regularly transferred into fresh medium twice weekly.

Phagocytic study. Phagocytic activity was investigated by incubating cell suspensions with both heat-killed *Staphylococcus epidermidis* and polystyrene particles. Cultures of *S. epidermidis* grown in trypticase soy broth at 37° were washed three times in normal saline, heat-killed by immersion in a boiling water bath for 30 min, counted, and then resuspended in normal saline at a concentration of 2×10^9 organisms/ml. The polystyrene particles (1.3-μ diam, Dow Chemical Co., Midland, Michigan) were suspended in normal saline at a concentration of 6×10^8 particles/ml.