Inhibition by Penicillamine of DNA and Protein Synthesis by Human Bone Marrow[1] (36143)

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Penicillamine (β-mercaptovaline) is a sulfhydryl-containing amino acid which has been used in the treatment of Wilson's disease (1–3), cystinuria (4, 5), rheumatoid arthritis (6–8), other autoimmune diseases (9–11), macroglobulinemia (23), cryoglobulinemia (24), scleroderma (25, 26), and animal neoplasia (12–14). D-Penicillamine is formed during the catabolism of penicillin in man (15). The drug is commercially available in either the D or L, or mixed DL enantiomorphs. Because of the greater toxicity of the L form (16), D-penicillamine is more generally used.

Penicillamine has various biochemical effects, usually explained in terms of penicillamine inhibition of pyridoxine metabolism or penicillamine chelation of metals. B_6 deficiency may be produced (17); and D-penicillamine has been shown to inhibit at least 2 enzyme systems requiring pyridoxal-5-PO_4 as coenzyme: liver transaminases (20) and liver cysteine desulfhydrase (21). Though some mercapto compounds seem to exert a cellular protective effect against radiation-induced damage, penicillamine may enhance cell kill by radiation (22). Penicillamine may also reduce disulfide bonds with subsequent deaggregation of macroglobulin polymers (23). Depression of serum levels of IgG, IgA, and IgM immunoglobulins may occur during therapy with penicillamine in cryoglobulinemia (24). Penicillamine also may prevent collagen cross-linking (25) and this property has been exploited in the use of penicillamine in scleroderma (25, 26). Side effects of clinical usage include neuritis (18), transient eosinophilia, infrequent leukopenia (16), proteinuria (19), and gastrointestinal and skin sensitivity (6), diminished taste acuity (7), and thrombocytopenia (1).

The current experiments were designed to help delineate underlying mechanisms for some of the above effects of penicillamine in cellular metabolism. Penicillamine effect on DNA, RNA, and protein synthesis by human bone marrow cells *in vitro* was studied, as well as the effects of B_6 and certain metals in relation to penicillamine.

Methods. Twenty to 40 ml of bone marrow were aspirated from the posterior superior iliac spine directly into 10 ml of 0.06 *M* Tris buffered Hanks' balanced salt solution (THBSS) at pH 7.4, containing a mixture of 200 units/ml each of streptomycin and penicillin plus 10,000 units of heparin. The subjects were patients on either the general medical or hematology wards; all but one had either nutritional anemia, anemia of inflammatory or neo-plastic disease, blood loss anemia, or anemia due to liver disease.

Materials and general procedures. Incorporation of tritiated radionuclides and formate-^{14}C was used as a measure of DNA and RNA synthesis. Protein synthesis was evaluated by incorporation of tritiated amino acids. In every experiment, incorporation in the absence of penicillamine or other materials was the "100% value" control against which

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unknowns were compared. Thymidine (TdR-^{3}H) (sp act = 18.7 Ci/mmole; ICN Corp., Irvine, CA); serine (serine-^{14}C) (sp act = 48 mCi/mmole; Amersham/Searle, Chicago, IL); deoxyuridine (dU-^{3}H) (sp act = 5.59 Ci/mmole; Tracer Lab, Waltham, MA); uridine (U-^{3}H) (sp act = 21.7 Ci/mmole; ICN Corp.); leucine (Leu-^{3}H) (sp act = 5200 mCi/mmole; Tracer Lab); formic acid (formate-^{14}C) (sp act = 52 mCi/mmole; Amersham/Searle); were the radioactive compounds employed. The labeled materials were added to the bone marrow cultures 1 hr before termination of incubation in concentrations of 0.1 μCi/ml of culture for all except formate-^{14}C which was 10 times as concentrated. It was assumed that TdR-^{3}H was incorporated into DNA only, U-^{3}H into RNA, formate-^{14}C into both DNA and RNA, and Leu-^{3}H and serine-^{14}C were used as protein labels.

Neutral D-penicillamine and L-penicillamine HCl were purchased from Nutritional Biochemical Co., Cleveland, OH. Penicillamine disulfide was obtained from K & K Laboratories, Inc., Plainview, NY. Pyridoxine HCl was obtained from The Vitarine Co., New York, NY. Pyridoxal PO_4 was obtained from Sigma Chemical Co., St. Louis, MO. Mercaptoethanol, ascorbic acid, and amino acids were obtained from Sigma.

Short-term bone marrow culture technique. The procedure for maintaining bone marrow for short-term studies *in vitro* is essentially that employed previously in our laboratory (27, 28).

Upon arrival in the laboratory, the bone marrow suspension was filtered 6 times through a 50 mesh, 0.003 in. gauge wire screen. [Disposable new B-D siliconized vacutainers (Becton-Dickinson Co., New Brunswick, NJ) and disposable sterile pipettes were used and discarded. Any other material contacting the bone marrow was prewashed in 70% alcohol and dried.]

After filtering, the cell suspension was centrifuged at room temperature at 2500 rpm for 10 min, and the supernate was replaced with a medium containing a mixture of the patients's serum in THBSS in a ratio of serum/THBSS, 1:3. The amount of serum-enriched medium added was calculated to yield the number of 0.3-ml aliquots of the cell suspension needed for the particular experiment; the volume of medium added usually approximated the volume of washed bone marrow cells.

Cultures were incubated in 10-ml siliconized vacutainers in a total volume of 1 ml. Each tube contained 0.3 ml of final marrow suspension, the compounds tested were dissolved in THBSS and added in 0.1-ml volume and the total volume was adjusted with THBSS to 0.9 ml. The cultures were incubated in triplicate for 4 hr at 37° in a Dubnoff metabolic incubator-shaker at 80 cycles/min. The tubes were covered loosely with a sheet of Parafilm. One hour before incubation was terminated, 0.1 ml of radionuclide was added. The final nucleated cell counts varied between 2×10^6 and 12×10^6/ml depending upon the initial cellularity of the bone marrow. Cultures were terminated by initiating the DNA, RNA, or protein extraction procedures.

Determination of radioactivity in nucleic acid and protein. Extraction with perchloric acid. The Cooper and Rubin (29) modification of the technique of Feinendegen and Bond (30) was used.

At the end of the 4-hr incubation, 2 ml of THBSS were added to each tube and the tubes were placed in a refrigerated centrifuge at 4° and centrifuged for 10 min at 600*g*. The supernate was discarded; and the cells in each tube were washed with another 2 ml of THBSS, which was again discarded after centrifugation. Fixation of the washed cells with cold ethyl alcohol–acetic acid (3:1) solution for 10 min was followed by storage of the cells in 70% ethyl alcohol at 4° overnight. The following morning the cells were extracted with 2% perchloric acid (PCA) for 50 min at 4° (extract I). The supernate was discarded. The precipitate was extracted with 10% PCA for 5 hr at room temperature (extract II). The supernate contained most of the RNA and U-^{3}H labeled.

The remaining precipitate was extracted with 10% PCA at 65° for 2 hr (extract III) (NB, Cooper and Rubin extracted for 4 hr).

TABLE I. Methods of Nucleic Acid and Protein Extraction.[a]

DNA, RNA, and protein extraction procedure	Relative recovery of label (%)		
	TdR-^{3}H	U-^{3}H	Leu-^{3}H
Shock lysis 1. RBC hemolysis 2. 10% TCA precipitation 3. Count	100	100	100
PCA Extraction 1. Prepare precipitate with acid alcohol 2. Extract with 2% PCA at 4° 3. Extract with 10% PCA at 24°; this contains the RNA 4. Extract with 10% PCA at 65°; this contains the DNA 5. Remaining precipitate treated with alkali and acid to extract protein	60	48	26

[a] After the bone marrows were cultured for 4 hr, the culture was terminated by initiation of either the shock lysis-TCA or PCA extraction procedures for recovery of labeled DNA, RNA, and protein.

This supernate contained most of the DNA and TdR-^{3}H label. Both extracts II and III were prepared for scintillation counting by adding 0.2 ml into 15 ml of scintillation fluid.

To the remaining precipitate, 5 ml of 2 *N* NaOH was added; and the mixture was heated at 100° in a water bath for 10 min and cooled. The supernate was saved; and, to the precipitate, 5 ml of 2 *N* HCl was added, and the tube was shaken. After centrifugation, the 2 supernates were combined. This contained most of the Leu-^{3}H-labeled protein. This was likewise prepared for scintillation counting.

Precipitation with trichloroacetic acid (TCA). After incubation, the samples were subjected to hypotonic-shock lysis to destroy erythrocytes then restoring tonicity. The samples were spun at 4° for 10 min at 600*g*; and the supernates were discarded. This procedure was repeated once to remove remaining red blood cells (31). The acid-insoluble materials were prepared by adding 2 ml of 10% TCA to the above sediment. This was then spun and decanted. To the resulting sediment was added 0.5 ml of Hyamine and 15 ml of scintillation fluid. The sample was then ready for liquid scintillation counting in a refrigerated Beckman LS250. Since quenching was constant from sample to sample in any one experiment, and each experiment contained its own controls, quench correction was not necessary.

Results. Initial experiments were designed to compare the efficiency of PCA extraction in recovering labeled DNA, RNA, and protein vs the recovery of label after hypotonic shock lysis followed by TCA precipitation. Table I shows that the simpler and less time-consuming hypotonic-shock lysis technique yielded higher recovery of all labels. Most of the subsequent experiments were therefore terminated by the shock lysis-TCA procedure.

The effect of both D- and L-pencillamine in varying doses on the percentage incorporation of TdR-^{3}H into bone marrow DNA was studied. Figure 1 shows that L-penicillamine inhibits by 10–20% more than the D form in all concentrations used. The shaded area of the curve represents the serum concentrations expected to be reached after single oral doses ranging between 500 and 1000 mg of drug (31). The higher clinically used doses of penicillamine inhibits by 15% (D-penicillamine) and 35% (L-penicillamine) in this *in vitro* data.

Figure 2 plots percentage of TdR-^{3}H incorporation vs hours of incubation with 1 mg/ml of D-penicillamine. TdR-^{3}H was added 30 min before the end of incubation.

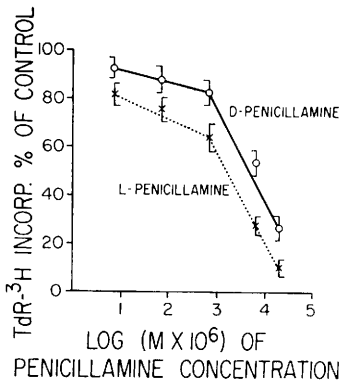


FIG. 1. Dose–response curve: Incubation of a penicillamine sensitive human bone marrow in varying concentrations of D-penicillamine (O—) and L-penicillamine (x - -); (vertical lines) ± 1 SD.

The percentage of TdR-^{3}H incorporation is time dependent, with significant inhibition occurring after 1 hr incubation with penicillamine.

To help assess possible mechanisms of penicillamine inhibition of TdR-^{3}H incorporation into DNA, the incorporation of formate-^{14}C and dU-^{3}H into DNA was studied. Penicillamine proved to reduce not only TdR-^{3}H, but also formate-^{14}C and dU-^{3}H incorporation into DNA (Table II), suggesting that penicillamine inhibition of TdR-^{3}H incorporation into DNA is, at least in part, not due to intracellular TdR-^{3}H transport blockage, acceleration of *de novo* synthesis of thymidine, or inhibition of thymidine kinase.

Table III shows the average inhibition of TdR-^{3}H incorporation in 9 experiments in 9 different patients. Bone marrow cells from different patients showed different sensitivity to penicillamine. Penicillamine inhibited TdR-^{3}H incorporation into DNA, and serine-^{14}C and Leu-^{3}H incorporation into protein; there was lesser inhibition of U-^{3}H incorporation into RNA. In all instances, L-penicillamine was a more potent inhibitor than D-penicillamine.

Preincubation for 1 hr with concentrations of L-valine equimolar to the concentration of penicillamine, or with 0.01 mg of pyridoxine HCl or pyridoxal phosphate did not significantly reduce the penicillamine effect (Table IV). Concentrations of pyridoxine HCl or of pyridoxal phosphate equimolar to the higher concentrations of penicillamine were themselves toxic to bone marrow, each reducing DNA synthesis by approximately 40% (data not in Table IV). It should be noted that these concentrations exceed, by over 100,000 times, the normal concentration in human blood.

The penicillamine effect was not reduced by preincubation of the drug with metals chelated by penicillamine (Table V). In these experiments, the chloride salts of Cu^{2+} (33) and Fe^{3+} (34) and $ZnSO_4$ (35), were

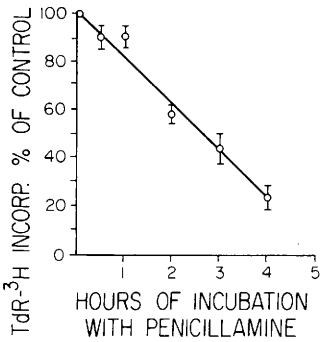


FIG. 2. Time response to D-pencillamine (1 mg/ml): Human bone marrow cultured for various times in the presence of 1 mg/ml of D-penicillamine; TdR-^{3}H added 30 min before termination of culture; (vertical lines) 1 SD.

TABLE II. Inhibition of Formate-^{14}C, TdR-^{3}H, and dU-^{3}H Incorporation into Human Bone Marrow DNA.

Type of DNA extract	L-Penicillamine	Inhibition (%) of label		
	(mg/ml)	Formate-^{14}C	TdR-^{3}H	dU-^{3}H
Shock Lysis TCA	0.1	30	49	—
	1.0	42	79	45
PCA Extract (DNA fraction)	0.1	2	50	—
	1.0	18	60	—

TABLE III. Average Inhibition of Incorporation of Radionuclides in the *in vitro* Bone Marrow Culture of 9 Different Patients ± 1 SD.

Amino acid (mg/ml)	Inhibition (%)			
	TdR-^{3}H	U-^{3}H	Leu-^{3}H	Serine-^{14}C
D-Penicillamine				
0.1	7 ± 7	5 ± 7	5 ± 10	8 ± 9
1.0	29 ± 11	14 ± 11	23 ± 9	14 ± 6
L-Penicillamine				
0.1	20 ± 8	6 ± 9	16 ± 11	12 ± 2
1.0	46 ± 18	16 ± 8	40 ± 14	44 ± 15

incubated for 30 min at room temperature with autologous serum to attach metal to transport protein prior to adding penicillamine. Preincubation with up to 10 times the normal serum concentrations of iron, copper, and zinc failed to lessen penicillamine inhibition of TdR-^{3}H incorporation into DNA.

Various sulfhydryl-containing amino acids, and β-mercaptoethanol, were investigated as possible DNA synthesis inhibitors. These compounds were used in equimolar concentrations to those amounts of penicillamine which inhibited DNA synthesis. The compounds which contained a free sulfhydryl group inhibited DNA synthesis (Table VI). Penicillamine disulfide inhibited only in the larger concentrations, suggesting perhaps that reduction of the disulfide bond occurs in the culture milieu, releasing penicillamine. The analogous amino acids (valine, serine, glycine, methionine) without free sulfhydryl groups, and the reducing agent ascorbic acid, in 0.1 to 1 mg/ml concentrations, did not demonstrate inhibitory activity.

Discussion. The experiments here reported demonstrate that D-penicillamine has potent metabolic effects at the cellular level (9, 36) when used in concentrations greater than those believed attained in therapy of Wilson's disease. In short-term human bone marrow culture, it appears to inhibit DNA and protein synthesis, with only slight depression of RNA synthesis. The L-enantiomorph proved a more powerful inhibitor of cellular metabolism; this is not surprising, since it is more toxic clinically.

The concentrations of D-penicillamine, which significantly inhibited DNA synthesis, are above those achieved by single oral doses ranging between 500 and 1000 mg. The daily dose employed in Wilson's disease is variable, but 4 g/day is not unusual; use of up to 8 g/day has been reported (37). The concentrations of penicillamine which may occur in plasma have only recently been studied (32). A mean of 3.4% with a high of 5.4% (*i.e.*, 0.03 mg/ml of plasma) of an orally administered dose up to 600 mg may be present per

TABLE IV. Failure to Reduce Penicillamine Inhibition by 1-hr Preincubation of Bone Marrow Cells with L-Valine, Pyridoxine HCl, or Pyridoxal PO_4.

Amino acid (mg/ml)	Inhibition (%) of TdR-^{3}H	Inhibition (%) of TdR-^{3}H after 1-hr preincubation with:		
		L-Valine .78 mg/ml	Pyridoxine HCl 0.01 mg/ml	Pyridoxal PO_4 .01 mg/ml
D-Penicillamine 1.0	38	37	34	31
L-Penicillamine 1.0	70	68	66	72

TABLE V. Failure to Reverse Penicillamine Inhibition of TdR-^{3}H Inhibition into DNA by Preincubating Human Bone Marrow Cells for 1 hr with Iron, Copper, or Zinc.

L-Penicillamine (mg/ml)	Preincubation for 1 hr with (μg/ml):	Inhibition (%) of TdR-^{3}H incorporation
1.0	—	48
1.0	Fe^{+3} 1	52
1.0	5	54
1.0	10	41
1.0	Cu^{+2} 1	31
1.0	5	58
1.0	10	50
1.0	Zn^{+2} 1	47
1.0	5	61
1.0	10	51

liter of plasma 1–2 hr after oral ingestion. The drug is fairly evenly distributed throughout body water. Up to 15% of the absorbed drug is present 48 hr after administration, suggesting that serum levels may progressively rise as a cumulative result of multiple oral doses.

The observation that preincubation with B_6 or metals chelated by penicillamine did not correct inhibition of DNA synthesis is in keeping with the observations made by Littman *et al.* (12), who showed that B_6 could not fully correct the inhibition by penicillamine of growth of the B_6-dependent mouse sarcoma 180 tumor; and those of Jaffe (6), who clearly demonstrated continued depression by penicillamine of serum rheumatoid factor in spite of concurrent B_6 supplementation. Additionally, Moore *et al.* (38) have shown that the action of 1-formylisoquinoline thiosemicarbazone, a drug which inhibits DNA synthesis by chelating the iron moiety of ribonucleoside diphosphate reductase, is not reversed by the presence of iron. In fact, the inhibition was increased by iron.

These observations suggest a B_6-independent mode of penicillamine effect. Furthermore, the role of metal chelation is still uncertain. However, it must be borne in mind that we used molar concentrations of pyridoxal PO_4 which were only 1/163 that of penicillamine, and molar concentrations of iron only 1/37 that of penicillamine. This was necessitated by the toxicity and difficulty with protein precipitation brought about by higher concentrations of these agents.

The exact mode of penicillamine inhibition of nucleic acid and protein synthesis is not yet determined. The presence of a free SH group does appear necessary. This suggests, as one possibility, reduction of disulfide bonds of enzymes and other proteins (including nucleoproteins) by penicillamine and other SH-containing amino acids.

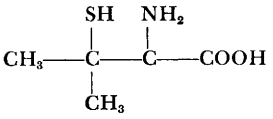
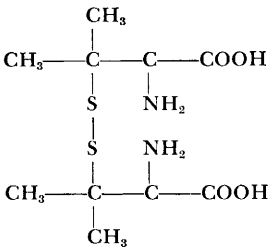
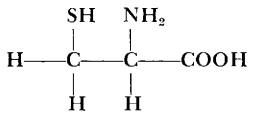
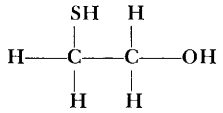
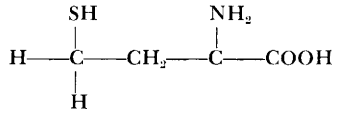
Penicillamine inhibition of DNA synthesis was present whether or not the culture milieu included serum. Thus, penicillamine effect on a growth factor in serum would appear not to be the mode of inhibition.

L-Penicillamine HCl in aqueous solution is acidic; 1 mg/ml concentrations of the drug shifted the pH of our incubation medium by 0.2 units. That such a pH shift is not involved in L-penicillamine inhibition was determined by experiments in which the pH of the culture media was readjusted to prepenicillamine values. L-Penicillamine effect was unchanged.

The possibility that penicillamine (being β-mercaptovaline) might be acting as a valine antagonist was studied by preincubation of bone marrow cells with L-valine. Equimolar concentrations of valine had no significant effect on penicillamine inhibition of DNA synthesis.

Figure 3 schematically presents the utilization of thymidine to form DNA thymine.

TABLE VI. Sulfhydryl Group Inhibition of TdR-^{3}H Incorporation into DNA.[a]

Compound	Conc (mg/ml)	Inhibition (%) of TdR-^{3}H incorporation
L-Penicillamine 	0.1	10
	1.0	42
D-Penicillamine disulfide 	0.1	0
	1.0	15
L-Cysteine 	0.08	14
	0.8	29
ß-Mercapto-ethanol 	0.05	20
	0.5	52
L-Homocysteine 	0.9	52

[a] The various sulfhydryl-containing amino acids were used in concentrations equimolar to that of penicillamine.

The *de novo* pathway, if accelerated, would decrease TdR-^{3}H incorporation into DNA. Transport blockage of TdR-^{3}H into the cell nucleus, or inhibition of thymidine kinase, would also decrease TdR-^{3}H incorporation into DNA. The experiments utilizing formate-^{14}C (Table II) support the hypothesis that a general slowing of DNA synthesis is responsible for true decreased TdR-^{3}H incorporation into DNA, and would tend to exclude the above three possibilities. Other experiments revealed reduction of dU-^{3}H incorporation into DNA comparable to that with TdR-^{3}H. Such results lend support to the concept that penicillamine slows DNA synthesis. That penicillamine did not cause

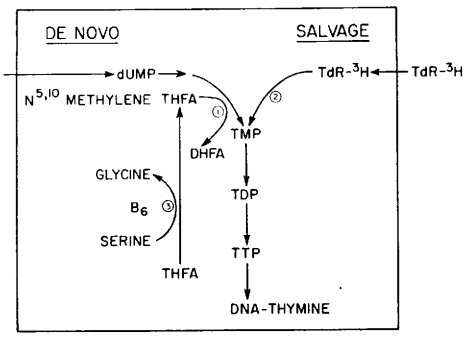


FIG. 3. *De novo* and salvage pathways involved in the synthesis and incorporation of thymidine into DNA: (1) thymidylate synthetase; (2) thymidine kinase; (3) serine methyltransferase (serine hydroxymethylase) (serine hydroxymethyltransferase).

morphologic damage to bone marrow cells was shown by comparing cells incubated with L-penicillamine (1 mg/ml) for 4 hr at 37° with controls. Wright–Giemsa stains of such cells showed no significant morphologic differences between penicillamine-treated and control cells.

There have been claims that penicillamine may be useful as therapy for various autoimmune diseases. The use of penicillamine in rheumatoid arthritis is associated, within weeks to months, with a drop in rheumatoid factor titer, normalization of erythrocyte sedimentation rate and marked improvement in anemia (6). Because of the sustained depression of rheumatoid factor titer for months after withdrawal of penicillamine therapy, Jaffe (6) has proposed an effect of penicillamine at the cellular level; our data supports such an effect.

Chronic aggressive hepatitis, a liver disease manifesting autoimmune phenomena (39), and responsive to prednisone and Imuran (40), has recently been treated with penicillamine (41, 42). Effective treatment of autoimmune hemolytic anemia in man with penicillamine was reported by Ritzman and Levin (10). Edwards and Gengozian (9) also described therapy of Coombs-positive autoimmune hemolytic anemia with penicillamine, in a patient refractory to prednisone and 6-mercaptopurine. For 60 days after termination of therapy, antibody was not measurable.

Perhaps the first hint that penicillamine possessed antimetabolic properties was the report by Wilson and Du Vigneaud (16) in 1949 of penicillamine inhibition of growth of young rats. Other workers studied penicillamine inhibition of the growth of the pyridoxine-dependent mouse sarcoma 180 tumor (12, 13). Littman *et al.* (12, 13) concluded that both enantiomorphs are active against sarcoma 180 cells, the L form more so than the D. They also presented evidence that activity as a pyridoxine inhibitor was probably not responsible for tumoricidal effect. It was also interesting that penicillamine was in fact just as active an inhibitor of tumor growth as was 5-fluorouracil. Hourani *et al.* (14) have also demonstrated oncostatic activity of D-pencillamine *in vivo* against the S-91 mouse melanoma.

As a result of the present findings, we have embarked on a study of the chemotherapeutic value of penicillamine vs human malignancies *in vitro* and *in vivo*. Preliminary studies in our laboratory suggest that D-penicillamine in concentrations of 0.1 mg/ml may inhibit the incorporation of TdR-^{3}H by 50% in a culture of human colon adenocarcinoma cells, while manifesting no toxicity vs the normal bone marrow *in vitro* of the same patient; this suggests a favorable therapeutic ratio may also occur *in vivo* using this drug against such a tumor.

Summary. The sulfhydryl-containing amino acid penicillamine inhibited uptake of thymidine-^{3}H, deoxyuridine-^{3}H, and formate-^{14}C into DNA of human bone marrow cells *in vitro*. Pencillamine inhibition of leucine-^{3}H incorporation into protein was also inhibited; less inhibition of uridine-^{3}H uptake into RNA was demonstrated. Significant inhibition was present with a concentration of penicillamine not much more than that presumed present in plasma of patients ingesting the drug. Penicillamine inhibition was not significantly affected by prior incubation of bone marrow cells with L-valine, iron, copper, zinc, pyridoxine, or pyridoxal phosphate.

The L-enantiomorph was more inhibitory than the D form.

These findings may help explain the inhibitory effects of penicillamine on animal tumors, and suggest possible value of this drug in chemotherapy of human neoplasms.

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