Antagonist Studies: Comparison of Desoxypyridoxine and Pyridoxine-Deficiency Therapy on Various Vitamin B_6 Enzymes.* (20709)

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The use of antagonist therapy to duplicate or accentuate a vitamin deficiency is a common practice. Many anti-metabolites produce gross and histological changes similar to, if not identical with, those observed on a deficient regimen. Although many investigators have studied the effect of vitamin deficiencies on enzymatic systems normally containing the lacking metabolite, little work has been done pertaining to the action of vitamin antagonists on these same systems. The stimulus for this study arose from the observation that differential toxic effects on cancer and host tissue can be best obtained if tumor-bearing animals are permitted *ad libitum* access to a complete diet while on antagonist therapy. Although it is appreciated that quantitative biochemical differences between normal and cancer cells may for the most part explain this phenomenon, the successful carcinostatic results reported with pyridoxine and riboflavin antagonists(1-4), without recourse to metabolite-deficient diets, raise further question to the belief that identical biochemical lesions are produced by antagonist therapy and metabolite-deficiency therapy.

The present communication reports a comparison of the effect of vit. B_6 antagonist, desoxypyridoxine, and a vit. B_6 deficiency on 3 vit. B_6-containing enzymes in the mouse.

Methods. C57 male black mice from 6-8 weeks of age were employed throughout. A total of 80 mice were used in these studies. Twenty mice were treated with the vitamin antagonist and 20 were held as controls. Twenty mice were placed on the vit. B_6-deficient diet and the remaining 20 received the identical diet plus the vitamin. The desoxypyridoxine-treated mice received an *ad libitum* diet of Rockland chow and water as did their saline controls. The vit. B_6 antagonist was administered intraperitoneally at a level of 150 mg/kg body weight per day for a 14-day period. Under these conditions, no loss of weight or other outward signs of a vit. B_6 deficiency were observed. Each control animal received 0.25 ml of saline daily. All animals were sacrificed one hour after the final injection. The mice used in the deficiency studies were housed in plastic cages with raised screen floors and received a high protein diet consisting of: vitamin-free casein 50, salt mixture 4(5), Cerelose 40, corn oil 5 g; cod liver oil (4500 U.S.P. units of vit. A and 450 U.S.P. units of vit. D per g) 250, choline chloride 150, calcium pantothenate 4.0, niacin 4.0, riboflavin 0.6, thiamine hydrochloride 0.6, inositol 20.0, biotin 0.03, folic acid 0.02, and 2-methyl-1,4-napthoquinone 0.04 mg. Control animals received an identical ration except that 0.6 mg of pyridoxine hydrochloride was included. All animals on the deficient regimen lost weight, an average decrease of 40% of the initial weight being observed for the 4-week experimental period. No significant enzymatic differences were noted between control animals receiving the pyridoxine-supplemented diet and mice fed a stock chow diet. Throughout the experimental period the food consumption of the control animals in the vit. B_6 deficiency studies was restricted to a level comparable to that ingested by the deficient animals. Water was supplied *ad libitum*.

All animals were killed by cervical rupture, the livers being immediately removed and chilled in ice cold saline. The tissues were then blotted dry and weighed. Portions of each liver were taken and analyzed for glu-

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TABLE I. Effect of High Protein-Induced Vit. B_6 Deficiency on Liver Enzymatic Activities.*

Systems	Control	Deficient
Transaminase†	229 ±13	143 ±10
Dopa decarboxylase‡	1.37± .07	1.16± .03
Cysteine desulfhydrase§	8.2 ± .7	4.2 ± .5

* Values are the avg of 8 individual observations, run in duplicate and include the stand. error. $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$

† Reaction mixture consisted of 0.1 ml of a 0.5% homogenate, 0.5 ml 0.67 M L-aspartic acid, 0.1 ml 0.1 M alpha-keto-glutaric acid and 0.1 M phosphate buffer (pH 7.4) to 2.0 ml final vol; incubated 10 min. at 37°C. See text for the definition of units.

‡ Reaction mixture consisted of 0.5 ml of a 10% homogenate, 0.3 ml 0.1 M DL-dopa, 0.05 ml octyl alcohol and octyl alcohol-saturated phosphate buffer (0.02 M, pH 6.9) to give a final vol of 2 ml; incubated 15 min. at 37°C. See text for definition of units.

§ Reaction mixture consisted of 2 ml of supernatant fluid equivalent to 20% homogenate in 0.1 M phosphate buffer (pH 7.4) and 1 ml of 0.03 M L-cystine; incubated for 2 hr at 37°C. Values are expressed as micromoles of ammonia nitrogen liberated per 2 hr per g tissue wet wt.

tamic-aspartic transaminase, dihydroxyphenylalanine (dopa) decarboxylase and cysteine desulfhydrase using the methods of Tonhazy *et al.*(6), Dietrich(7), and Greenstein *et al.* (8), respectively. L-cystine was used as the substrate in the cysteine desulfhydrase studies. Transaminase and dopa decarboxylase activities are reported as microliters of CO_2 liberated per mg tissue dry weight per 10 minutes (Q_T^{10}) and 15 minutes (Q_T^{15}) incubation period, respectively. Cysteine desulfhydrase activity is reported as micromoles of ammonia liberated per 2 hours per g tissue wet weight. All determinations were conducted at 37°C and at least 8 individual determinations were carried out per group.

Results. It has been demonstrated by other investigators, employing growth(9) and tissue analyses(10) as criteria, that a high protein diet accelerates the depletion of the vit. B_6 reserves in animals receiving a vit. B_6-deficient diet. The effect of such a regimen on the activity of 3 vit. B_6-containing enzyme systems (transaminase, dopa decarboxylase and cysteine desulfhydrase) is summarized in Table I. The activity of all 3 systems is significantly lowered, cysteine desulfhydrase being the most sensitive to vit. B_6 deprivation, followed by transaminase and dopa decarboxylase. These results are similar to those obtained by other investigators in other species(11-14).

The effect of desoxypyridoxine on transaminase, dopa decarboxylase and cysteine desulfhydrase activity is presented in Table II. Desoxypyridoxine administered at a level of 150 mg/kg body weight to mice receiving an *ad libitum* diet containing ample vit. B_6 was observed to severely antagonize transaminase activity. Dopa decarboxylase was antagonized to a lesser but still significant extent. Cysteine desulfhydrase activity was, however, uninhibited.

The participation of pyridoxal phosphate as a prosthetic group in the 3 enzymatic systems studied has been demonstrated beyond reasonable doubt. The metabolically active form of desoxypyridoxine, desoxypyridoxine-5-phosphate, has been shown by Umbreit and

TABLE II. Effect of Desoxypyridoxine Therapy on Liver Enzymatic Activities.*

System	Saline control	Desoxypyridoxine-treated
Transaminase†	210 ±27 (8)	108 ±45 (8)
Dopa decarboxylase‡	1.12± .07 (14)	.92± .03 (14)
Cysteine desulfhydrase§	7.3 ± .7 (15)	9.5 ± .8 (15)

* Figures in parentheses represent number of individual observations. Values are avg of duplicate determinations and include stand. error. $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$

† Reaction mixture consisted of 0.1 ml of a 0.5% homogenate, 0.5 ml 0.67 M L-aspartic acid, 0.1 ml 0.1 M alpha-keto-glutaric acid and 0.1 M phosphate buffer (pH 7.4) to 2.0 ml final vol; incubated 10 min. at 37°C. See text for the definition of units.

‡ Reaction mixture consisted of 0.5 ml of a 10% homogenate, 0.3 ml 0.1 M DL-dopa, 0.05 ml octyl alcohol and octyl alcohol-saturated phosphate buffer (0.02 M, pH 6.9) to give a final vol of 2 ml; incubated 15 min. at 37°C. See text for definition of units.

§ Reaction mixture consisted of 2 ml of supernatant fluid equivalent to 20% homogenate in 0.1 M phosphate buffer (pH 7.4) and 1 ml of 0.3 M L-cysteine; incubated for 2 hr at 37°C. Values are expressed as micromoles of ammonia nitrogen liberated per 2 hr per g tissue wet wt.

Waddell(15) to compete with pyridoxal phosphate for the apoenzyme. The degree to which desoxypyridoxine will antagonize a pyridoxal phosphate-containing system is for the most part affected by the amount of pyridoxal phosphate available and the dissociation constant of the coenzyme-apoenzyme complex. Under the conditions of the experiment the amount of pyridoxal phosphate present can be assumed to have remained relatively constant. Thus, the only remaining variable of consequence is the dissociation constant of the various vit. B_6-containing systems. Since this variable is also the main factor affecting the rate at which enzymatic activity is depleted through vitamin deprivation, one might expect similar effects on enzymatic systems containing pyridoxine, regardless of whether these systems are antagonized by desoxypyridoxine or a pyridoxine deficiency.

Experimentally, however, this is not the case. Transaminase and dopa decarboxylase behave according to expectation. Cysteine desulfhydrase, although very sensitive to vit. B_6 deprivation, is not antagonized by desoxypyridoxine therapy.

This latter phenomenon is not understood. The direct participation of desoxypyridoxal phosphate as an activator in the cysteine desulfhydrase system is doubtful in view of the work of Meister *et al.*(14), since these investigators observed a marked depression of cysteine desulfhydrase activity in vit. B_6-deficient rats receiving desoxypyridoxine. It is possible that pyridoxal phosphate combines with the protein moiety of cysteine desulfhydrase in such a manner as to make the configuration at the 4 position of the pyridine nucleus critical in regard to apoenzyme-prosthetic group affinity. If this were the case, replacement of the aldehyde by a methyl group at this position could produce a compound totally ineffective in displacing pyridoxal phosphate in the cysteine desulfhydrase system. Desoxypyridoxine phosphate, on the other hand, can displace pyridoxal phosphate bound to the apoenzyme of either transaminase or dopa decarboxylase; apparently, in these cases the aldehyde grouping at position 4 is essential for enzymatic action. This is in keeping with the postulation that the first step in transamination and decarboxylation reactions is a Schiff base formation involving the pyridoxal moiety of the enzyme and the amino acid concerned(16,17). The observation of Heyl *et al.*(18) that L-cysteine reacts with a pyridoxal to form a thiazolidine compound, rather than the Schiff bases produced by most other amino acids, would tend to exclude the involvement of a Schiff base reaction in the enzymatic degradation of cysteine. The stability of cysteine desulfhydrase preparations in the presence of substrate, together with the low degree of dissociation of the thiazolidine compound formed by the reaction of pyridoxal with L-cysteine(18), permits the surmise that very little of this material is formed in the presence of cysteine desulfhydrase, and may indicate that the pyridoxal phosphate is bound to the apoenzyme of cysteine desulfhydrase in such a manner as to make the aldehyde group unavailable.

These results present the possibility that vitamin antagonists may be synthesized to function solely as specific anti-enzymes, and not as antagonists for all the biochemical functions of the vitamin. This study accentuates the well-recognized potentialities of anti-metabolites as biochemical tools capable of more clearly defining pathways of intermediary metabolism. More important, perhaps, the data present the possibility that vitamin antagonists may be useful as highly precise chemotherapeutic agents, particularly in the presence of a normal dietary intake.

Summary. 1. Mice receiving a vit. B_6 deficient-high protein diet were observed to have significantly lower transaminase, dopa decarboxylase and cysteine desulfhydrase activities as compared to similar animals receiving an identical diet supplemented with pyridoxine. Cysteine desulfhydrase was the most sensitive to vit. B_6 deprivation, followed by transaminase and dopa decarboxylase. 2. The administration of desoxypyridoxine to mice receiving an *ad libitum* stock diet containing adequate vit. B_6 significantly decreased transaminase and dopa decarboxylase activity. However, cysteine desulfhydrase activity was uninhibited. 3. A possible explanation of the observed results is discussed,

the potentialities of vitamin antagonists as precise chemotherapeutic agents being indicated.

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Poliomyelitis Virus in Tissue Culture VI. Use of Kidney Epithelium Grown on Glass.* (20710)

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Tissue culture methods have become essential to the conduct of research with poliomyelitis virus and indeed with certain other viruses as well(1). For this purpose rolled or stationary test-tube cultures of tissue have been chiefly used in which the tissue fragment is imbedded in a plasma clot. This paper reports a simplification of the technic for preparing cultures, in that the tissue fragment can be made to stick on glass in the absence of plasma and clotting solution (chick embryo extract or thrombin). The new cells then grow out directly on the glass surface. Before the culture is used, the tissue fragment, easily removed to leave behind a sheet of newly grown cells, can be transferred to seed another culture tube. A variety of tissues from man, monkeys, and mouse have been cultured on glass without plasma. However, this paper will deal exclusively with the culturing and use of monkey kidney tissue.†

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Materials and methods. The technic of preparing test tube cultures of monkey tissue in plasma as used in this laboratory has been described in detail(4,5). Similar conditions have been followed in the present experiments in preparing the plasma-clot test-tube cultures, (hereafter called "plasma tubes"). Test tube cultures prepared in the absence of plasma are called "dry tubes", and their preparation is given in detail below.

Complete medium for all cultures, in the presence or absence of plasma clot, consisted of 9 parts of mixture No. 199(6) and 1 part bovine serum.‡ The No. 199 was made up in

† Epithelial and fibroblastic cells derived from human cancer and capable of growth on glass are also sensitive to poliomyelitis virus(2,3). For certain purposes where one does not want a cell line of cancerous origin, or a cell which requires human serum for its growth, or a cell line which may suddenly be lost because of mold or other contamination, the use of primary explants from readily available normal tissue offers advantages.