tered to the patients in sufficient concentration for a sufficient length of time to prevent the convulsant action of the current. The concentration of carbon dioxide varied from 15 to 30% and the duration of administration from 25 to 170 seconds. A description of the technic and of other details has been given by Pollock *et al.*[5]

Administration of carbon dioxide produced, in all our cases, the following convulsive phenomena: Initial periocular twitching followed by either an extensor hypertonus in all extremities or, more often, slight flexor spasm in the arms and extension in the legs, consecutive transitory plastic tonus, increasing occipital rigidity and opisthotonus, finally, a high degree of extensor rigidity appeared in all limbs, the fingers in *main d'accoucheur* position, toes in flexion. The dilated pupils reacted to light on all but 2 occasions. The tendon and skin reflexes could not be elicited on account of the muscular rigidity. Pathological reflexes were not seen during or after the convulsions though in some cases they preceded them. No tongue biting, incontinence, or postconvulsive stupor appeared. The respiratory and circulatory changes observed were those described by Meduna and Gyarfas.[2] The fits did not appear to be self-limiting. E.E.G., though obscured by muscular movements, was always clearly not of the grand mal type. The electrical activity of the cortex returned to normal shortly after discontinuing inhalation of carbon dioxide.

The clinical symptoms, the E.E.G., and the absence of postseizure stupor, correspond to decerebrate seizures. Our observations indicate, therefore, that the apparently different types of convulsive phenomena described by Meduna and Gyarfas[2] represent only different phases of one activity.

Summary. 1. Combination of E.S.T. with CO_2 inhibits the convulsion.

2. Inhalation of 30% CO_2 and 70% O_2 produced in all cases observed convulsive phenomena of decerebrate character.

16904

Mode of Action of Desoxypyridoxine.

W. W. UMBREIT AND J. G. WADDELL. (Introduced by H. Molitor.)

From the Merck Institute for Therapeutic Research, Rahway, N. J.

Antagonisms caused by metabolite analogues are generally accepted as being due to a competition between the analogue and the natural substrate for some enzyme system of the cell.[1] The inhibitor is thought to occupy a space on the surface of an enzyme which would normally be used by the metabolite. The relatively high ratios of analogue to metabolite usually required are considered as a reflection of relative affinity of the enzyme protein for the metabolite and its analogue. While these concepts have served as useful tools and have perhaps been entirely valid in many cases, it has also been apparent that they must be amplified to cover more complex situations; for example, where the substance used is a structural analogue of a vitamin which functions in the form of a coenzyme with a low dissociation constant. Such a case is offered by the action of desoxypyridoxine in competing with the vitamin B_6 group. This compound, 2,4-dimethyl-3 hydroxy-5 hydroxymethylpyridine, was reported by Ott[2] to act as a powerful antagonist of pyridoxine in the chick. Two moles of desoxypyridoxine counteracted 1 mole of pyridoxine when the latter was limiting. Emerson[3] showed that, in the rat, approximately 50 parts of desoxypyridoxine brought on the

1 Woolley, D. W., *Advances in Enzymology*, 1946, **6**, 129.

2 Ott, W. H., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 125.

3 Emerson, G. A., *Fed. Proc.*, 1947, **6**, 406.

signs of B_6 deficiency when a purified diet supplemented with pyridoxine was employed and that this ratio changed to 175 parts when a natural diet was fed. Additional studies by Mushett *et al.*,[4] Porter *et al.*[5] have demonstrated that desoxypyridoxine produces the pathological and biochemical symptoms associated with a vitamin B_6 deficiency so that there is no question that desoxypyridoxine antagonizes vitamin B_6. These studies revealed, however, that the antagonism was evident only when the vitamin B_6 supply was sub-optimal. With adequate vitamin B_6, desoxypyridoxine had little effect even at relatively high ratios. These conclusions are difficult to explain on a metabolite-analogue competition basis so that it was early realized that a more complex situation was present. Direct enzymatic studies were therefore undertaken to attempt to determine the mechanism of inhibition by desoxypyridoxine. The enzyme employed was tyrosine decarboxylase for which pyridoxal phosphate, a member of the vitamin B_6 group, is the coenzyme.[6] It was assumed that these studies would bear some relationship to the mode of action of desoxypyridoxine in the animal body but the concepts developed experimentally here may require some modification when applied to the complex situation in the animal.

The substrate competition concept is based upon the early studies of Quastel and Wooldridge[7] upon malonate inhibition of succinate transformations and this concept has largely colored the thinking of subsequent observers. However, when one is dealing with a vitamin analogue, the points of competition may be multiplied and a simple concept may require modification. The case of vitamin B_6 may be diagrammed as in Fig. 1. The known coenzyme form of the vitamin B_6 group is pyridoxal phosphate, yet in the diet pyridoxine, pyridoxal, and pyridoxamine are, within limits of stability and adsorption, essentially equally effective and have been shown to be converted into pyridoxal phosphate.[8] Desoxypyridoxine might act at points A, B, or C or it might be converted into desoxypyridoxine phosphate which could compete by uniting with the apo-tyrosine decarboxylase, or by displacing pyridoxal phosphate from the holoenzyme. Since the

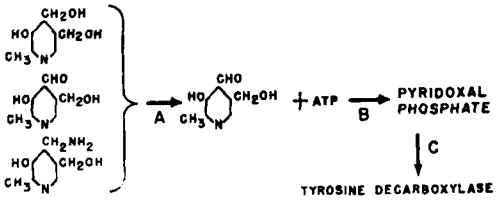

FIG. 1.
Reactions of members of the vitamin B_6 group.

analogue could act at one or at more than one of these points, it hardly seemed likely that a simple concept of substrate competition would suffice to describe the action of desoxypyridoxine. It therefore appeared worthwhile to attempt to distinguish experimentally between the possibilities mentioned particularly since enzymatic studies involving the possible interactions outlined above have been somewhat lacking.

Methods and materials. *Streptococcus faecalis*, strain R, was used as a source of tyrosine decarboxylase. For the preparation of the active (holo) enzyme the organism was grown at 25°C, 16-18 hours, in a medium composed of 1% each of tryptone, yeast extract, and glucose with 0.5% K_2HPO_4. The cells were harvested by centrifugation, washed with distilled water, and the vacuum dried preparation used as a source of the enzyme. The apoenzyme was prepared similarly using a medium deficient in the vitamin B_6 group.[9] The decarboxylation of tyrosine was measured at 37°C in 0.06 M acetate buffer at pH 5.5 as previously described.[6] Pyridoxal and desoxypyridoxine were used as their hydro-

[4] Mushett, C. W., Stebbins, R. B., and Barton, M. N., *Trans. N. Y. Acad. of Sciences*, 1947, **9**, 291.

[5] Porter, C. C., Clark, I., and Silber, R. H., *J. Biol. Chem.*, 1947, **167**, 573.

[6] Umbreit, W. W., Bellamy, W. D., and Gunsalus, I. C., *Arch. Biochem.*, 1945, **7**, 185.

[7] Quastel, J. H., and Wooldridge, W. R., *Biochem. J.*, 1927, **21**, 1224.

[8] Bellamy, W. D., Umbreit, W. W., and Gunsalus, I. C., *J. Biol. Chem.*, 1945, **160**, 461.

[9] Bellamy, W. D., and Gunsalus, I. C., *J. Bact.*, 1945, **50**, 95.

chlorides. Pyridoxal phosphate was standardized against a relatively pure sample[10] and was used as the calcium salt. Desoxypyridoxine phosphate will be described later. Adenosine triphosphate (ATP) was used as the sodium salt. Conventional Warburg technics were employed throughout.

Results. As outlined above, several possible sites of action of desoxypyridoxine are evident. These may be divided into two categories: (1) the inhibitions possible if the active agent is desoxypyridoxine, (2) the inhibitions possible if the active agent is desoxypyridoxine phosphate. While the second category proved to be correct, as will be shown later, it was still necessary to prove that there does not also exist an effect of desoxypyridoxine itself. This proof is provided in the first section. The tyrosine decarboxylase system employed did not include reactions A (Fig. 1) hence any effect of desoxypyridoxine (or its phosphate) on the conversion of pyridoxine or pyridoxamine to pyridoxal phophate could not be studied in this system.

Action of desoxypyridoxine. Since the results show that desoxypyridoxine as such has no effect upon the systems shown in Fig. 1, only a brief description of the experiments will be given. It was first necessary to test the possibility that desoxypyridoxine interfered with tyrosine decarboxylase presumably by displacing the pyridoxal phosphate. Beiler and Martin[11] have already reported that desoxypyridoxine was ineffective in this system up to concentrations as high as 300 γ per ml. In our hands concentrations as high as three times this level had no effect. Further, such concentrations had no effect upon the aspartic-glutamic transaminase from heart muscle[12,13] or upon the tryptophanase system of *E. coli*[14] both of which have somewhat higher dissociation constants than tyrosine decarboxylase and hence might be expected to show some degree of competition. Once pyridoxal phosphate is associated with the tyrosine decarboxylase protein, desoxypyridoxine does not displace it. Yet if the latter were allowed to reach the apoenzyme first it might prevent the combination of pyridoxal phosphate and apoenzyme. For this purpose a preparation was employed which at saturation had a Q_{CO_2} of 676. It responded in a linear manner to pyridoxal phosphate up to 8 mγ (calculated as the free acid) at which point the Q_{CO_2} was 460. It was reasoned that the ideal conditions for demonstrating any effect of desoxypyridoxine upon the combination of apoenzyme and coenzyme would be under conditions where the coenzyme was decidedly limiting since any competition would be immediately evident in a decreased rate of tyrosine decarboxylation. A point was therefore chosen approximately halfway up the region of linear response (3.8 mγ free acid) with the result (Table I) that desoxypyridoxine had no effect upon the combination of coenzyme and apoenzyme in spite of the fact that it was given adequate access to the enzyme before the pyridoxal phosphate was supplied. Similar results were obtained with a purified horse-heart transaminase preparation carried through the second precipitation in the procedure of O'Kane and Gunsalus[13] at which point it was 85% resolved.

Since from these data, desoxypyridoxine does not act on tyrosine decarboxylase (C, Fig. 1) the possibility remained that it acted by competing with pyridoxal for conversion to pyridoxal phosphate (point B, Fig. 1). It was reasoned that if ATP were made the limiting factor in the conversion of pyridoxal to pyridoxal phosphate, effects of replacing pyridoxal by desoxypyridoxine on the surface of the converting enzyme would be immediately evident. In this type of experiment a preparation was employed which had a very active converting system. Not very much is known about this enzyme and its occurrence in a given preparation is not exactly pre-

[10] Gunsalus, I. C., Umbreit, W. W., Bellamy, W. D., and Foust, C. E., *J. Biol. Chem.*, 1945, **161**, 743.

[11] Beiler, J. M., and Martin, G. J., *J. Biol. Chem.*, 1947, **169**, 345.

[12] Green, D. E., Leloir, L. F., and Nocito, V., *J. Biol. Chem.*, 1945, **161**, 559.

[13] O'Kane, D. E., and Gunsalus, I. C., *J. Biol. Chem.*, 1947, **170**, 425.

[14] Wood, W. A., Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, 1947, **170**, 313.

TABLE I.
Lack of Effect of Desoxypyridoxine on Combination of Pyridoxal Phosphate and Apo-tyrosine Decarboxylase.

Pyridoxal phosphate, mγ	Ratio $\frac{\text{Desoxypyridoxine}}{\text{Pyridoxal phosphate}}$	Rate of tyrosine decarboxylation, Q_{CO_2}
0	0	61.6
1.92	0	158.8
3.84	0	242
3.84	3000	240
3.84	6000	246
7.68	0	448
11.52	0	540
15.36	0	576
19.20	0	676

Desoxypyridoxine, when supplied, incubated with apoenzyme 30 minutes before pyridoxal phosphate supplied.

TABLE II.
Effect of Desoxypyridoxine on the Synthesis of Pyridoxal Phosphate from Pyridoxal and ATP.

γ ATP*	Ratio $\frac{\text{Desoxypyridoxine}}{\text{Pyridoxal}}$	Q_{CO_2} on tyrosine
10	0	160
10	500	173
10	10,000	162
50	0	360
50	500	368
50	10,000	390
100	0	448
100	500	448
100	10,000	444

* $Na_4(ATP) \cdot 3H_2O$.

dictable. We have had preparations capable of converting 50% of the pyridoxal supplied to pyridoxal phosphate while others capable of converting only 1 to 2% or even less have been obtained under apparently the same conditions of cultivation and treatment of the organisms. However, in this case, a preparation actively converting 50% of the pyridoxal supplied was employed. ATP was made the limiting factor and two pyridoxal levels, one low (0.05 γ) and another relatively high (1 γ) were employed. The desoxypyridoxine was incubated with the enzyme for 30 minutes, to allow access to the enzyme, the pyridoxal and ATP were added simultaneously, incubated for 10 minutes to allow reaction B to proceed and the rate of tyrosine decarboxylation measured. The data for the lower level of pyridoxal are given in Table II; since the results with the higher level were similar they have been omitted. Within the limits of error of measurement there is no effect of desoxypyridoxine upon the formation of pyridoxal phosphate from ATP and pyridoxal. This is somewhat surprising since the competition theory as it is usually applied would predict that this reaction would be the site of action of desoxypyridoxine.

Action of desoxypyridoxine phosphate. The previous section has shown that desoxypyridoxine does not influence either the conversion of pyridoxal to its phosphate or the activity of the phosphate once formed. There remained, therefore, the possibility that desoxypyridoxine was not the active agent in the competition but that it was itself converted into desoxypyridoxine phosphate which was the competitive agent. Evidence bearing on this point was first sought in the conversion system (B, Fig. 1). The previous section has shown that when ATP was the limiting factor and adequate pyridoxal was available, desoxy-

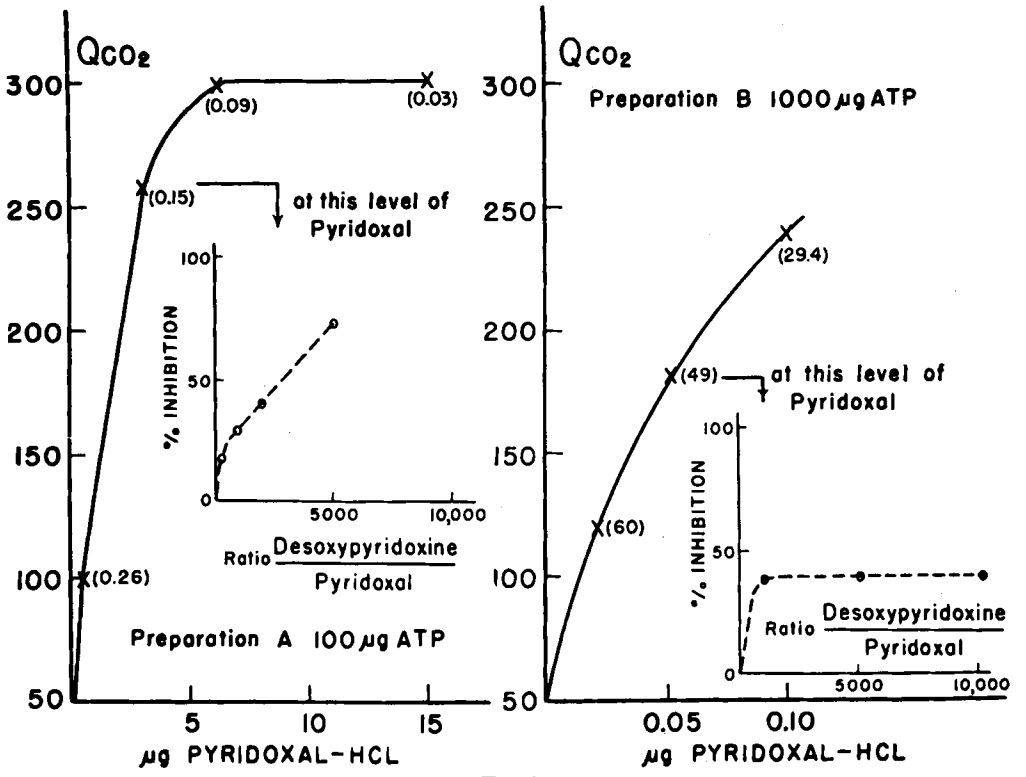

FIG. 2.

Enzymatic conversion of desoxypyridoxine to its phosphate and its interference with tyrosine decarboxylation.

In each case, 1 mg of the enzyme preparation was incubated with ATP and desoxypyridoxine for 15 min., pyridoxal added, in amounts indicated, incubated for 10 min., then tyrosine added and the rate of tyrosine decarboxylation determined. The solid lines show the rate of tyrosine decarboxylation at various levels of pyridoxal supplied in the absence of desoxypyridoxine and serve to show that pyridoxal is the limiting factor and that ATP is in excess. The values in parentheses give the percent of conversion of pyridoxal to pyridoxal phosphate. On the inset graphs the dashed curves show the extent of inhibition by various increments of desoxypyridoxine at the level of pyridoxal indicated.

pyridoxine could not compete to any detectable extent with pyridoxal. However, if pyridoxal were limiting so that some portion of the enzyme were free from pyridoxal, while ATP was at saturation or above, desoxypyridoxine might then become attached to the enzyme and be phosphorylated, the latter form interfering somehow with reaction C which would be inhibited. Data showing that such an inhibition does occur are given in Fig. 2.

Two types of preparations were used, one (preparation A) in which the conversion of pyridoxal to pyridoxal phosphate was relatively weak (0.2-0.3%) and another (preparation B) in which the conversion was relatively good (50-60%). The figures in parentheses on the graphs give the per cent of the pyridoxal supplied which appeared as pyridoxal phosphate (after a 10 minute incubation period). The pyridoxal phosphate was determined by comparing the activity found with that given by purified pyridoxal phosphate with the same preparation. As previously mentioned the conversion of pyridoxal to the phosphate by ATP is not a quantitative reaction and such preparations respond to pyridoxal phosphate in much lower quantities than to pyridoxal and ATP. For example, in the original enzyme preparation described[6] pyridoxal phosphate was 18-21 times as active as pyridoxal and ATP[10] indicating a conversion of approximately 5%. Different preparations of the dried cells of this organism

require different quantities of pyridoxal and ATP to reach either the maximum activity or the activity corresponding to a given level of pyridoxal phosphate. The cause of this difference is not known but it might possibly be related to different degrees of permeability of the cell to pyridoxal (thus preparation A might require 2 γ pyridoxal-hydrochloride to reach an internal concentration equivalent to that which preparation B was capable of reaching with only 0.06 γ). There are some indications that this is indeed the explanation but proof is lacking. However, by the use of two preparations of widely different "converting abilities" it was hoped to avoid complications arising from this effect.

The level of pyridoxal was chosen which gave good activity but which was below the saturation level. Desoxypyridoxine did, under these circumstances, show inhibitions as shown on the inserted graphs in each case. However, relatively high levels of desoxypyridoxine were required. Where the conversion to pyridoxal phosphate is relatively weak, desoxypyridoxine exerts its greatest effect. These data provide presumptive evidence that when ATP is in excess and pyridoxal limiting, there is some degree of inhibition by desoxypyridoxine. Since various other possibilities have been eliminated in the first section, the remaining possibility is that desoxypyridoxine is converted into desoxypyridoxine phosphate and that the phosphorylated derivative is the actual inhibitor.

Unfortunately desoxypyridoxine phosphate is not available nor is its preparation a matter of simple chemistry. The synthesis used by Beiler and Martin[11] is not a good one. Yields of pyridoxal phosphate using pyridoxal as the starting material are rarely more than 0.5% and there is no reason to expect any better yields with desoxypyridoxine. Somewhat improved syntheses have been obtained from pyridoxal by modifications of the original procedure[6] but rarely is more than a 5% conversion obtained. In the case of pyridoxal phosphate an assay method[6] is available which permits one to follow purification of this substance, but with desoxypyridoxine there is as yet no method of specifically estimating the phosphorylated form in a mixture of phosphorylated compounds. As a source of desoxypyridoxine phosphate we therefore employed a preparation* made by treating desoxypyridoxine with phosphoryl chloride, neutralizing the resulting mixture and separating the desoxypyridoxine phosphate as the calcium salt by alcohol precipitations. Such a material is not pure and may contain other substances. However, its organic phosphate content (4.52%) was assumed to be due to desoxypyridoxine phosphate and the amount added was calculated on this basis.

From the data cited previously, desoxypyridoxine phosphate interferes with reaction C (Fig. 1) in some manner. There are two parts to this reaction, one, a combination of the apoenzyme and pyridoxal phosphate, and two, the decarboxylation of tyrosine. The desoxypyridoxine phosphate does not measurably prevent the activity of tyrosine decarboxylase once the coenzyme is attached.

A vacuum dried preparation of *S. faecalis R* grown on a yeast extract medium had a Q_{CO_2} on tyrosine of 393. When 100,000 times as much desoxypyridoxine phosphate was supplied as the pyridoxal phosphate contained in the preparation a Q_{CO_2} of 362 was observed (8% inhibition); with 1 million times as much desoxypyridoxine phosphate the Q_{CO_2} was 372 (5% inhibition). These inhibitions are not significant. Beiler and Martin[11] have reported some degree of inhibition (approximately 30%) of tyrosine decarboxylase with a preparation of desoxypyridoxine phosphate. This type of inhibition has not occurred in our experiments.

Desoxypyridoxine phosphate does, however, interfere markedly with the combination of pyridoxal phosphate and apoenzyme. Data on this point are shown in Fig. 3 in which are plotted the results of a variety of conditions employed in determining the inhibitions at various ratios of desoxypyridoxine phosphate to pyridoxal phosphate. Curve A is the average of several types of experiments designed so that the desoxypyridoxine phosphate would have an equal or better chance of reaching the apoenzyme surface than the

* We are indebted to the Research Laboratories of Merck & Co., Inc., for this preparation.

pyridoxal phosphate. The individual treatments are plotted as discrete points on the curve. In one case desoxypyridoxine phosphate and pyridoxal phosphate were mixed and added to the preparation simultaneously on the assumption that this could provide an equal chance for both to reach the enzyme. They were then incubated for various periods up to 30 minutes before tyrosine decarboxylation was measured. In another treatment

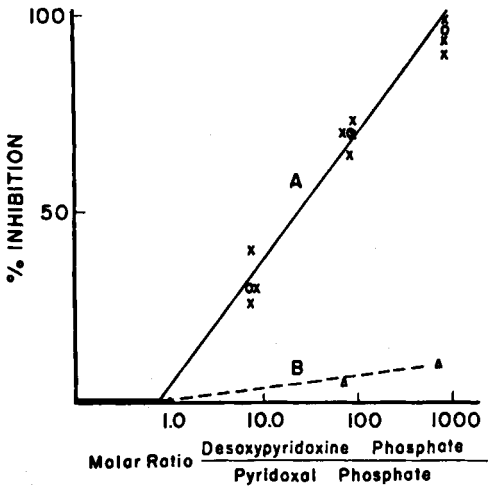

FIG. 3.

Competition between desoxypyridoxine phosphate and pyridoxal phosphate for tyrosine decarboxylase apoenzyme.

In curve A, desoxypyridoxine phosphate incubated with apoenzyme for 30 min. before pyridoxal phosphate added, or desoxypyridoxine phosphate and pyridoxal phosphate added simultaneously. After 10 min. of contact with pyridoxal phosphate, tyrosine was added and the rate of tyrosine decarboxylation determined. In curve B, the pyridoxal phosphate was added before the desoxypyridoxine phosphate. Pyridoxal phosphate used at level of 20 mγ (barium salt) per 3 ml, which is close to the top of the linear portion of the response curve of this preparation.

desoxypyridoxine phosphate was supplied first, incubated with the apoenzyme for 30 minutes, pyridoxal phosphate supplied, equilibrated for 10 minutes and tyrosine decarboxylation measured. Within the errors of measurement all of these combinations where desoxypyridoxine phosphate had an equal or better chance to reach the apoenzyme than pyridoxal phosphate, essentially the same degree of inhibition is observed. If, however, as shown in curve B, pyridoxal phosphate is supplied to the apoenzyme first and the combination between the two permitted, desoxypyridoxine phosphate supplied later has much less effect; in fact, the total inhibitions reached at the highest ratio was only 12%.

These data constitute evidence that the inhibition of tyrosine decarboxylase is due to the competition between desoxypyridoxine phosphate and pyridoxal phosphate for the apoenzyme. Pyridoxal phosphate has a greater affinity for this enzyme than desoxypyridoxine phosphate and if it is permitted to combine with the apoenzyme before desoxypyridoxine phosphate is present, the latter has but little effect.

A moment's consideration of the data presented will show that this locus of action of desoxypyridoxine is capable of explaining the results observed with desoxypyridoxine in growth studies. It is only when vitamin B_6 is deficient that there is any great opportunity for either the phosphorylation of desoxypyridoxine or its combination with apoenzymes and thus it is only under these circumstances that a marked inhibitory effect is observed. It is also evident that when one is dealing with a vitamin analogue the simple concept of substrate competition must be modified to include the other possibilities of competition. Further, there is no assurance that other analogues of vitamin B_6 might not act at different loci, hence any general viewpoint of metabolite analogue competition must be amplified. With such amplification it would seem reasonable that many of the present unexplainable phenomena of metabolite analogue competition might become understandable.

Summary. Upon the basis of the data presented, it is concluded that desoxypyridoxine exerts its inhibiting effect by being first converted into desoxypyridoxine phosphate which then competes with pyridoxal phosphate for the apoenzyme. This conclusion offers an explanation for the observation that in the animal, desoxypyridoxine exerts its antagonistic effect primarily under conditions of restricted vitamin B_6 intake.