Relationship of Pyridoxine and its Derivatives to the Mechanism of Action of Isoniazid.* (20621)

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In most instances of metabolic antagonism the essential metabolite and the inhibitory agent have been similar in chemical structure. The structural appearance of isoniazid‡ lead to the speculation that its mode of action might represent interference with the utilization of nicotinamide or some other essential metabolite of similar structure. This report deals with the screening of certain vitamins, some of which are structurally similar to isoniazid, for possible isoniazid reversal.

Materials and methods. The method chosen to study the mode of action of isoniazid was similar to that of McIlwain(1), and Lampen and Jones(2). Various organisms with known nutritional requirements were inhibited with the drug and reversal of the inhibition was initially attempted with nicotinic acid, nicotinamide, pyridoxine, thiamine, riboflavin, pantothenic acid, and p-aminobenzoic acid. Studies were later limited to pyridoxine and its derivatives when the initial results showed that only these compounds gave a competitive reversal of inhibition. The final steps were to study the effects on various species of *Mycobacterium*. No attempt was made to study any organism in any medium when a concentration of greater than 10^{-2} M of the isoniazid was required for inhibition. The organisms employed in this study were obtained from the American Type Culture Collection, Washington, D.C., unless otherwise stated. Synthetic media were used whenever possible to control the amount of essential metabolites present. Some comparisons were made between synthetic and nonsynthetic media and Table I lists the organisms, media, references, and the method of measuring and detecting growth. *Lactobacillus plantarum (arabinosus)* 17-5 was chosen because of its wide external nutritional requirements which included those of major interest, nicotinic acid and pyridoxine. It also discriminates between pyridoxine and its derivatives, pyridoxal and pyridoxamine, the latter 2 being more active than pyridoxine as growth promoters. In all experiments using *Lactobacillus* changes in growth were observed grossly over 72 hours and the medium titrated for acid production at that time. *Streptococcus pyogenes* was used only because of its external pyridoxine requirement. *Saccharomyces carlsbergensis* was chosen because of its use in assay of vit. B_6 and its uniform response to B_6 and its derivatives. Final turbidity readings for determination of growth were made after 18 hours incubation at 30°C. The effect of isoniazid inhibition was studied in relation to the growth of 2 *Escherichia coli* strains which do not require added B_6, one mutant strain of which requires nicotinic acid or its amide. The final turbidity readings were made after 18 hours incubation at 37°C. The *E. coli* strains are similar to the *Mycobacterium* which also have no external requirement for pyridoxine or its derivatives. Both are known to synthesize vit. B_6. *Mycobacterium butyricum* grows rapidly in a synthetic media and has been used for the assay of isoniazid(6). Cultures were observed for growth at the end of 24 and 48 hours and the end of one week. The BCG and H37Rv strains were incubated for at least 3 weeks before final growth and turbidity observations were made.

Results. Using the standard technic of microbiological assay with *Lactobacillus plantarum* and its synthetic medium containing all the maximum growth requirements, it was possible to inhibit the organism with isoniazid. Inhibition began with isoniazid concentrations of 4.8×10^{-3} and was complete at 10^{-2} M. Table II gives the concentrations of the isoniazid in the medium with the acid titration values produced after 72 hours growth.

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† Sandia Base with station at Los Alamos.

‡ Kindly supplied as Nydrazid by Squibb and Co., and as Rimifon by Hoffmann-LaRoche, Inc.

TABLE I. List of Microorganisms, Media, and Method of Measuring Growth.

Microorganism	Growth medium	Method of measuring growth response
Lactobacillus plantarum (arabinosus) 17-5 ATCC No. 8014	Modified Snell and Wright synthetic(3)	Titration of acid produced*
Streptococcus pyogenes ATCC No. 10389	Meat infusion broth	Gross growth observations
Escherichia coli†	Nutrient and tryptone broths	Turbidity‡
Escherichia coli strain 303-138 nicotinic acid mutant ATCC No. 9723†	Synthetic medium of MacLeod plus nicotinic acid(4)	"
Saccharomyces carlsbergensis ATCC No. 9080	Synthetic medium as used in vit. B_6 assay(3)	"
Mycobacterium tuberculosis var. bovis BCG§	Modified Kirchner's synthetic (5) and Dubos medium	Gross growth observations and turbidity
Idem., var. hominis H37Rv ATCC No. 9360	Dubos medium	*Idem.*
Mycobacterium butyricum ATCC No. 362	Synthetic as used in assay of isoniazid(6)	Gross growth observations pellicle type

* All titrations were done with 0.1 N NaOH.
† Isolated in this laboratory.
‡ Turbidity readings on Coleman Model 14 Universal Spectrophotometer.
§ BCG strain obtained from Chicago Research Laboratories.

TABLE II. Concentrations of Isoniazid Used to Obtain Inhibition of *Lactobacillus plantarum*.

Conc. of isoniazid, M	ml of 0.1N NaOH/10 ml*
0	1.8†
0	12–13‡
7.3×10^{-4}	12.5
3.65×10^{-3}	5.0–6.5
5.80×10^{-3}	3.0–3.8
7.30×10^{-3}	2.5–3.3
1.0×10^{-2}	1.2–2.8

* Avg range of titration results at 72 hr, all determinations.
† Control in which basal medium was deficient in nicotinic acid.
‡ Control, complete medium with all essential growth requirements present.

Table III is a summary of the results obtained when reversal of the isoniazid inhibition in *Lactobacillus* was attempted with some of the more common metabolites. The only substance tested that had any effect was pyridoxine. Inhibition by a concentration of 10^{-2} M of isoniazid was completely reversed by 4.8 x 10^{-4} M of pyridoxine when added to the complete synthetic medium.

Table IV demonstrates the reversal of the isoniazid inhibition in *Lactobacillus* with varying concentrations of pyridoxine and its derivatives. A competitive type of reversal is demonstrated. The data show that the derivatives of pyridoxine, pyridoxamine, and pyridoxal, were much more effective in reversing the isoniazid inhibition. Pyridoxamine dihydrochloride was from 2000-4000 times as effective as pyridoxine while the pyridoxal hydrochloride was 1000-2000 times as effective in reversing the inhibition of 10^{-2} M concentration of isoniazid.

With *Saccharomyces carlsbergensis* it was possible to demonstrate again the competitive reversal of the isoniazid by pyridoxine and

TABLE III. Common Metabolites Tested for Reversal of Isoniazid Inhibition of *Lactobacillus plantarum*.

Conc. of isoniazid, M	Metabolite	Added conc., M	Growth*
0	Complete basal medium†		4+
1×10^{-2}	" "	"	—
"	Nicotinic acid	$.04–4.0 \times 10^{-3}$	—
"	Nicotinamide	$.04–4.0 \times$ "	—
"	Pyridoxine·HCl	$.24–4.8 \times 10^{-4}$	3+–4+
"	Thiamine·HCl	$.15–3.0 \times$ "	—
"	Ca pantothenate	$.11–2.3 \times$ "	—
"	p-aminobenzoic acid	$.37–3.7 \times$ "	—
"	Riboflavin	$.13–1.3 \times$ "	—

* Determined by titration of acid produced.
† All essential metabolites present in basal medium in conc. of no more than 2 μg/10 ml.

TABLE IV. Competitive Reversal of Isoniazid Inhibition of *Lactobacillus plantarum* by Pyridoxine and Its Derivatives.

Conc. of isoniazid, M	Metabolite conc., M	ml of 0.1 N NaOH/10 ml of media
	Pyridoxine • HCl	
0	9.7×10^{-7}	12.7
1×10^{-2}	9.7×10^{-7}	2.3
"	4.8×10^{-6}	2.6
"	9.7×10^{-6}	2.8
"	2.4×10^{-5}	3.1
"	4.8×10^{-5}	3.4
"	2.4×10^{-4}	10.7
"	4.8×10^{-4}	11.4
"	1.9×10^{-3}	12.6
	Pyridoxamine • 2HCl	
0	*	13.1
1×10^{-2}	*	1.1
"	4.1×10^{-9}	4.6
"	2.1×10^{-8}	8.3
"	4.1×10^{-8}	10.0
"	1.2×10^{-7}	11.6
"	2.1×10^{-7}	12.8
"	4.1×10^{-7}	13.8
"	8.2×10^{-7}	13.8
	Pyridoxal • HCl	
0	*	13.3
1×10^{-2}	*	2.8
"	4.9×10^{-8}	4.3
"	1.5×10^{-7}	5.1
"	2.5×10^{-7}	7.2
"	4.9×10^{-7}	7.9
"	9.8×10^{-7}	8.5
"	2.5×10^{-6}	11.8
"	4.9×10^{-6}	12.8

* All basal media contained 2 μg (9.7×10^{-7} M) pyridoxine/10 ml.

its derivatives. In this organism, however, no great differences were noted between pyridoxine, pyridoxamine, and pyridoxal, except that the 2 latter derivatives were slightly less effective. Table V demonstrates some of the results when the concentration of metabolite was constant and the amount of inhibitor varied. Similar competitive reversal of inhibition was obtained when the inhibitor concentration was constant and the metabolite varied.

The first 2 organisms discussed above are representative of the type that requires an external source of pyridoxine. Table VI shows some results with the laboratory strain of *Escherichia coli* which is an example of the type without external pyridoxine requirement. The strain isolated in the laboratory was inhibited in nutrient broth by the amounts of isoniazid shown in the table and reversal by pyridoxine and its derivatives was only partial. Pyridoxal and pyridoxamine appeared only slightly more active in reversing the inhibition than did pyridoxine.

Results with the mutant strain of the *E. coli* requiring nicotinic acid were slightly different. Pyridoxine at no time reversed completely the inhibition. Pyridoxamine and pyridoxal produced a complete reversal of inhibition and were at least 2 to 3 times more active.

It is interesting to observe that *Streptococcus pyogenes* could not be inhibited by isoniazid in any concentration up to 10^{-2} M. This was attempted in the commonly used meat infusion broth. Although this organism

TABLE V. Competitive Reversal of Isoniazid Inhibition by Pyridoxine • HCl in *Saccharomyces carlsbergensis*.

Conc. of pyridoxine, M	Conc. of isoniazid, M	Opt. density at 650 mu
0	0	.1
4.86×10^{-5}	0	1.88
"	7.3×10^{-4}	1.80
"	2.19×10^{-3}	1.75
"	$2.9 \times$ "	1.45
"	$3.65 \times$ "	1.24
"	$5.8 \times$ "	.79
"	$7.3 \times$ "	.65
"	1.0×10^{-2}	.32

TABLE VI. Inhibition of *E. coli* (Laboratory Strain) with Isoniazid and Partial Reversal of Inhibition with Pyridoxine and Pyridoxamine.

Conc. of isoniazid, M	Conc. of metabolite, M	Optical density†
	Pyridoxine • HCl	
0	0	.57
5.8×10^{-3}	0	.12
"	4.8×10^{-5}	.19
"	2.4×10^{-4}	.19
"	$4.8 \times$ "	.22
"	1.44×10^{-3}	.28
"	$2.9 \times$ "	.36
	Pyridoxamine • 2HCl*	
0	0	.55
5.8×10^{-3}	0	.11
"	4.1×10^{-5}	.16
"	2.1×10^{-4}	.21
"	$4.1 \times$ "	.24
"	1.26×10^{-3}	.36
"	$1.7 \times$ "	.45

* Pyridoxal gave similar results up to concentrations of 2.45×10^{-4} M.

† All measurements made at 620 mu.

has an external requirement of pyridoxine, it was felt that in all probability the non-synthetic medium contained sufficient pyridoxine or its derivatives to prevent any inhibition. Inhibition of the common strain of *E. coli* was also attempted in tryptone broth without success. Tryptone broth may have sufficient vit. B_6 or one of its derivatives to compete with the isoniazid. Although nutrient broth is not a synthetic medium its vit. B_6 content may be sufficiently low to have little effect on the isoniazid inhibition. The initial amount of pyridoxine and its derivatives in many common laboratory media may account for the wide discrepancies in reports of inhibition of other microorganisms by isoniazid.

In an attempt to study the reversal of isoniazid inhibition in the various strains of *Mycobacteria* no effect of any nature was noted. Using the BCG strain in modified Kirchner's and Dubos medium and the H37Rv strain in Dubos medium, inhibition by concentration of isoniazid from 0.073 to 7.3×10^{-5} M was not reversed by concentrations up to 1×10^{-3} M of pyridoxine and its derivatives. In the *M. butyricum* using the synthetic isoniazid assay medium no reversal was noted when the concentrations of isoniazid ranged from 3.6 to 36×10^{-5} M and the concentration of pyridoxine and its derivatives ranged up to 5×10^{-4} M.

Discussion and conclusions. Of the more common vitamins tested for reversal of isoniazid inhibition in *Lactobacillus plantarum,* nicotinic acid, nicotinamide, and pyridoxine and its derivatives were structurally similar to isoniazid. The concentrations of isoniazid required to inhibit *Lactobacillus* and the other organisms studied were much greater than are required to inhibit *Mycobacteria.* Although relatively high concentrations of isoniazid were required to inhibit *Lactobacillus plantarum* and *Saccharomyces carlsbergensis,* when pyridoxine and its derivatives were supplied externally, a typical competitive type reversal of the inhibition was obtained. With *Lactobacillus,* pyridoxal and pyridoxamine were much more effective than pyridoxine in reversing this inhibition. This result may be expected as the lactic acid bacteria normally demonstrate a greater response to pyridoxamine and pyridoxal. With the strains of *E. coli,* larger amounts of vit. B_6 and derivatives were required to produce reversal of inhibition and reversal was not always complete and not as typically competitive. The fact that *E. coli* can synthesize pyridoxine may result in a more complex, atypical mode of inhibition and reversal. Using *Mycobacteria,* which synthesize pyridoxine, no reversal of isoniazid inhibition was obtained by addition of any amounts of pyridoxine or its derivatives to the media. If vit. B_6 is involved in the inhibition of growth of *Mycobacterium* by isoniazid it does not appear to be a substrate phenomenon as in the case of *Lactobacillus* and *Saccharomyces.* The above results, however, do not preclude the possibility of a metabolic antagonism between isoniazid and vit. B_6. It is possible that in the *Mycobacteria* isoniazid may produce inhibition by interfering with the initial synthesis of pyridoxine or by interfering with the utilization of pyridoxine or its derivatives for the synthesis of essential substances such as enzyme systems in which they serve as prosthetic groups, as in decarboxylase or transaminase reactions. Zeller and Barsky(7,8) demonstrated inhibition of monamine oxidase on bacterial and mammalian enzymes with isoniazid and isonicotinyl-2-isopropyl hydrazine. No attempt to reverse this inhibition was mentioned. Yoneda *et al.*(9,10) were able to reverse isoniazid inhibition of indole formation and amino acid decarboxylation activity in *E. coli* with vit. B_6 and its derivatives. The present results also indicate that isoniazid is an antimetabolite of vit. B_6 in certain microorganisms. Antagonism between isoniazid and vit. B_6 was not manifested in the *Mycobacterium* however, and does not serve to explain the mode of action of isoniazid on the tubercle bacillus.

Preliminary results in this laboratory(11) indicate that the uptake of C^{14} labeled isoniazid by these organisms is very small and as yet no conclusion can be drawn in relationship to the mode of action of isoniazid.

Summary. 1. It was possible to inhibit growth of organisms other than *Mycobacterium* with isoniazid by controlling the amount of pyridoxine and its derivatives in

the media. *Lactobacillus plantarum* and *Saccharomyces carlsbergensis* were inhibited in a synthetic medium by isoniazid concentrations of less than 10^{-2} M. This inhibition was competitively reversed by pyridoxine, pyridoxamine, and pyridoxal. In reversing isoniazid inhibition of *Lactobacillus*, pyridoxamine, and pyridoxal were from 1000 to 4000 times more effective, than pyridoxine. 2. Two strains of *E. coli* were inhibited with isoniazid with partial reversal with pyridoxine and derivatives. Larger amounts of the metabolite were required and the reversal was not as typically competitive. 3. No reversal of isoniazid inhibition could be obtained with vit. B_6 or its derivatives in any of the *Mycobacteria* tested. If isoniazid acts as an antagonist of pyridoxine or its derivatives in the *Mycobacteria*, it is not a simple substrate competition or uptake phenomenon.

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Failure of Grafted Ovaries to Assume Adrenalcortical Function in Rats.* (20622)

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It has been shown that under specific conditions the ovary of the mouse can and does assume life-sustaining corticoadrenal functions(1,2). Working independently and in different laboratories, the authors have made autoplastic intrasplenic ovarian grafts in young adult and in immature rats in an effort to determine the effectiveness of such a graft on post adrenalectomy survival time in the rat.

The recent article by Lichton, Goldblatt, and Stolpe(3) prompts us to make our data available.

Methods. The animals used were of sturdy albino stock of no specific strain origin, and in each case were fed standard laboratory diets. The experiments on the young adult rats were performed by Hill, and those experiments on the immature rats were done by Bernstorf. The mature rats were bilaterally spayed, and at the same time the left ovary was grafted into the spleen. At various intervals following recovery, the right adrenal was removed. The left and second adrenal was removed approximately 2 weeks following first adrenal removal (Table I). Following the completion of adrenalectomy, the weights of all animals were recorded every few days. If the animals were discovered soon after death they were examined for remnants of adrenal tissue and for the general condition of the graft. The immature rats were spayed at weaning, and 17 of the 19 animals used

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