Means of Increasing the Tolerated Dose of Isoniazid in Mice. III. Certain Vitamins. (23337)

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In efforts to reduce toxic reactions caused by use of isoniazid (INH) in therapy of tuberculosis, it has become increasingly evident that this chemical alters normal metabolism and may be "detoxified" or transformed by supplemental use of substances essential to normal enzymic processes. Thus it has already been demonstrated that various amino acids, in particular glycine supplemented with sodium glucuronate(1) and cysteine(2), or certain alpha keto acids(3), or cycloserine(4), or glycerine(5) or propylene glycol(3) as solvents, are severally effective in increasing the tolerated dose of INH administered orally in DBA strain of mice. Hence, it seems logical that similar biotransformation may also occur if INH is supplemented with vitamins. The present report gives results of an investigation of the effect of certain vitamins on acute and chronic toxicity of INH in DBA mice.

Methods. In this strain of mice, the amount of INH which is 100% lethal by oral administration is 6 mg/20 g mouse (300 mg/kg). Consequently, the effectiveness of the various vitamins was tested initially with a somewhat larger dose, 8 mg, of isoniazid. The procedure consisted of mixing in a test tube the required amount of INH and of vitamin in water to such volume that the desired concentrations were contained in 0.5 ml for oral administration. When necessary, the hydrogen ion concentration was adjusted to pH 5. Ergosterol required mineral oil rather than water for suspension.

Results. Effect of certain vitamins on acute oral toxicity of isoniazid in mice. Initial tests with 8 mg INH/20 g mouse and an equimolar concentration of each of 15 vitamins are summarized in Table I in the order of effectiveness. With 3 others and the isomeric isonicotinic acid, a 5:1 molar ratio was used. On the assumption that, as food accessory substances, only minute amounts of vitamins are necessary, smaller amounts of these 4 were tested but were much reduced in their activity as detoxicants. It is possible that those with little or no effect might be active in other concentrations, except that with increased amounts some of the vitamins, such as menadione or choline, are toxic *per se*. Also in this Table are subsequent tests with other concentrations of the 4 vitamins, pyridoxal-HCl, pyridoxine-HCl, p-aminobenzoic acid, and nicotinic acid, which were better than 50% effective in increasing survival rate of the mice. Pyridoxal-HCl is outstanding in that doses ranging from 8 to 20 mg/mouse protected all but one of the animals against this lethal 8 mg dose of INH. This vitamin was therefore selected for a more thorough study.

Effect of repeated administrations. Various concentrations of INH were administered by gavage at 24 and 72 hour intervals with pyridoxal-HCl in molar equivalent proportions; and also one test was made of 8 mg INH with 8 mg pyridoxal, *i.e.*, a 1:0.66 molar ratio. As seen in Table II, this latter mixture prolonged survival time better than the others administered daily. Indeed it was comparable to the most effective mixture given at 72-hour intervals, namely 20 mg INH with 30 mg pyridoxal. This result may have been due to the fact that the INH and pyridoxal formed a hydrazone in the test tube before administration which was only gradually hydrolyzed *in vivo*. Thus in the mixture of 8 mg INH and 8 mg pyridoxal, which contained such a hydrazone, the amount of INH gradually liberated would theoretically be 5.3 mg. During this gradual hydrolysis, some INH was being excreted. At the same time the free INH (2.7 mg) of the original mixture was also gradually eliminated. Accordingly, at no time would there be a toxic dose. This is demonstrated by the fact that up to 8 daily doses were administered without lethal effect.

TABLE I. Effect of Certain Vitamins on Acute Oral Toxicity of Isoniazid in DBA Mice.

8 mg INH

Vitamin	Vit. amt, mg*	No. surviving/No. tested	% survival
Pyridoxal-HCl	8	10/10	100
"	12*	16/16	100
"	15	10/10	100
"	20	9/10	90
"	30	6/10	60
Pyridoxine-HCl	60†	16/20	80
"	30	2/10	20
P-Aminobenzoic acid	12	6/10	60
" "	8*	15/26	58
Nicotinic acid	36†	24/46	52
" "	25	3/10	30
" "	7*	2/20	10
Nicotinamide	36†	7/15	47
Menadione	10	7/16	44
[Isonicotinic acid]	36†	16/46	35
Alpha-tocopherol	25	3/10	30
Choline-HCl	8	1/ 6	17
Folic acid	25	1/ 6	17
Ergosterol	23	2/17	12
Pyridoxamine-HCl	14	0/ 6	0
Ascorbic acid	10	0/ 6	0
Biotin	14	0/10	0
Riboflavin	20	0/10	0
Ca pantothenate	28	0/ 6	0
Vitamin A	17	0/10	0
Thiamine-HCl	20	0/20	0
Inositol	13	0/20	0

* Indicates equimolar ratio.

† Indicates 5 millimols vitamin/millimol INH.

In miscellaneous low titer group, all are equimolar unless otherwise marked.

Only then was there evidence of possible accumulation in the tissues. The combination of 20 mg INH and 30 mg pyridoxal given at 72-hour intervals was of special interest, inasmuch as in these amounts each chemical alone had been found lethal within an hour. Yet this hydrazone was administered twice weekly for 8 doses before the 50% mortality endpoint was reached. From these tests it may be concluded that concomitant pyridoxal permits mice to tolerate repeated administration of as much as 3 times the lethal dose of INH for several weeks. In addition, a test of repeated administrations of each of 2 other vitamins, nicotinic acid and p-aminobenzoic acid, with 8 mg INH at 72 hour intervals is included in Table II. Of the 2, nicotinic acid was slightly superior, but in view of the fact that a 5:1 molar ratio was necessary, it was not as effective/mole as pyridoxal.

Blood levels. Assay of INH blood plasma levels in DBA mice at various time intervals after administration of INH-pyridoxal mixtures was carried out by the "pyridyl" method (6). The mixture of 8 mg INH and 12 mg pyridoxal (molar equivalents), *i.e.*, the hydrazone, gave a constant level of approximately 1 mg% for 7 hours. This lower level may indicate the extent to which the hydrazone, formed in the test tube before administration, has been hydrolyzed *in vivo* during the first hour after administration. Also purified hydrazone (20 mg) in one test gave a constant level of approximately 1 mg% for 7 hours. However, the one-hour level after an injection of a mixture of 8 mg INH with 8 mg pyridoxal was 4.2 mg%. This higher level may be accounted for by the amount of free INH in solution (2.7 mg) in excess of that used in forming the hydrazone. For it was found that the plasma level assayed 1 hour after a 2 mg oral dose of INH was 3.4 mg%. It remains to be seen whether a low but persis-

TABLE II. Effect of Repeated Oral Administrations of Certain Vitamin-Isoniazid Mixtures in DBA Mice.

Isoniazid, mg	Vitamin	mg	Time intervals, hr*	No. of mice	No. mice surviving the following No. of doses													
					1	2	3	4	5	6	7	8	9	10	11	12	13	14
8	Pyridoxal-HCl	8	24	10	10	10	10	10	10	10	10	10	6	4	4	4	3	2
15	"	22.5	24	9	9	9	5	2										
20	"	30	24	30	25	24	23	9										
8	"	12	72	16	16	16	15	13	12	12	10	9	3					
10	"	15	72	9	8	8	8	7	4	3	3							
20	"	30	72	20	20	20	20	20	17	14	14	11	9	6				
8	Nicotinic acid	36	72	16	14	12	10	10	10	10	10	9	7	7	4			
8	p-Aminobenzoic acid	8	72	10	8	8	7	7	7	5	5	0						

* 24 hr indicates daily, Monday through Friday inclusive; 72 hr, twice weekly, Monday and Thursday.

tent INH blood level is more efficacious in treatment of human tuberculosis than an early higher peak followed by rapid decrease in INH concentration in the blood stream.

Effect of certain vitamins on INH inhibition of Mycobacterium tuberculosis. One method has been applied to determine the effect of some of these vitamins on the bacteriostatic action of INH on one avirulent strain of *Mycobacterium* B103. The test was carried out by a procedure previously reported (1). The vitamins, tested in molar equivalent amounts, included: pyridoxal-HCl, pyridoxine-HCl, p-aminobenzoic acid, nicotinic acid and its isomer isonicotinic acid, nicotinamide, and menadione, and for comparison the purified hydrazone of INH and pyridoxal. Of themselves none of the vitamins except possibly menadione demonstrated any inhibitory action on growth for the period of observation. When mixed with INH, there was questionable interference by nicotinamide, but with the others no antagonism to the bacteriostatic action of INH. In contrast, the purified hydrazone by itself was as effective as INH in inhibiting growth, and in mixture with INH did not interfere with its activity as a tuberculostatic agent.

Discussion. Interest in the use of vitamins with INH has been stimulated by observations of many clinicians that toxic manifestations in 40% of tuberculous patients under treatment with INH include peripheral neuritis and other symptoms similar to those observed in pyridoxine deficiency. In applying Zeller's observation(7) that toxicity of INH is largely due to inactivation of amine oxidases, Reilly and collaborators(8) demonstrated that the INH toxicity could be partially reversed in mice by use of small amounts of pyridoxine. Similarly Biehl and Vilter(9) concluded that simultaneous pyridoxine prevented development of neuritis, and from metabolic studies suggested that INH inactivated amino acid decarboxylase by virtue of forming a hydrazone with the essential coenzyme, pyridoxal phosphate. Without discussion of mechanism, Tirunarayanan and Vischer(10) reported that among 6 B vitamins studied as possible detoxifying agents for INH in guinea pigs, only pyridoxine was effective. It is pertinent that pyridoxine and pyridoxal normally have similar activity *in vivo*. Accordingly, it may be that depletion of pyridoxal from tissues, which occurs with INH alone, would be prevented by the use of the pyridoxal isonicotinyl hydrazone.

It is of interest that absorption of INH may be more prolonged due to gradual hydrolysis of the hydrazone, and hence effectiveness without toxic manifestations may be extended, as suggested from the constant lower blood level.

It should be noted that our report includes study of doses of INH ranging from 8 to 20 mg/mouse, all of which are well above the 6 mg lethal dose. The implication may be that pyridoxal used with sublethal doses of INH would reduce or even eliminate some of the toxic manifestations observed in human tuberculous patients under treatment with this chemotherapeutic agent.

Summary. Certain vitamins have been found effective in increasing the tolerated oral dose of isoniazid (INH) in DBA mice. Four of the group, pyridoxal-HCl, pyridoxine-HCl, p-aminobenzoic acid, and nicotinic acid, permitted 50% or more, mouse survival in acute toxicity tests with 8 mg INH (400 mg/kg). Only pyridoxal-HCl, in 1- or 2-molar equivalent concentration, protected 100% of mice against this dose. This vitamin in appropriate concentration formed a hydrazone with INH and the compound containing as much as 20 mg INH/mouse was tolerated for 8 semi-weekly administrations. Blood plasma levels of INH following administration of the hydrazone were somewhat lower than those after a sublethal dose of INH alone, but were constant and more prolonged, presumably due to gradual *in vivo* hydrolysis of the hydrazone. Six vitamins, as tested, did not interfere with *in vitro* bacteriostatic action of INH on one avirulent strain of *Mycobacterium*, but the pyridoxal isonicotinyl hydrazone *per se* was as effective as INH alone as a tuberculostatic agent.

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Endogenous Respiration of *Serratia marcescens* and Oxidation of Added Carbohydrates.* (23338)

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During an investigation of the oxidative patterns of *Serratia marcescens*, cells grown in one of several media consistently yielded an anomalous pattern of substrate oxidation when the data were corrected for endogenous metabolism in the classical manner. Subsequent study of the data indicated that this unusual behavior may be due to suppression of the endogenous metabolism of the organisms. The role of endogenous metabolism during oxidative dissimilation of various substrates has been variously reported. Production of $C^{14}O_2$ from C^{14} "labeled" *Streptomyces coelicolor* was found to be the same during oxidation of glucose and pyruvate as it was in endogenous metabolism(1). Wilner and Clifton(2) reported that the endogenous respiration of a strain of *Bacillus subtilis* was not suppressed during exogenous respiration, and that correction for the former was necessary to obtain satisfactory equations for carbon assimilation. Conversely, endogenous respiration of *Pseudomonas saccharophila* was reported to be inhibited during oxidation of assimilable substrates(3,4), and the oxidation data for various carbohydrates by *Prototheca zopfi* were more amenable to analysis if the endogenous respiration of the organisms was disregarded(5). The present report suggests that the conflicting conclusions of the cited studies may be a result of different growth conditions, in addition to the differences related to species specificity.

Methods. S. marcescens† was grown in media containing 5% skim milk solids, 3% Armour's peptone,‡ and 2% of one of the following carbohydrates. *Neutral glucose*: glucose added to medium prior to sterilization at 121°C for 15 minutes. The pH was 7.0-7.2 without adjustment. *Neutral gluconate*: gluconic acid was dissolved in a few ml water and adjusted to pH 6.0 prior to addition to the medium. Following sterilization the pH dropped to approximately 4.0. Sterile NaOH was added to raise the pH to approximately 6.0. *Acid gluconate*: gluconic acid was prepared as above and added to the medium. However, after sterilization the pH was allowed to remain at pH 4.0. After 16 hours growth on a reciprocal shaker at 30°C, the cells were harvested by centrifugation, washed once with water, and stored at 5°C until used. The Warburg experiments were carried out by conventional manometric technics. Gluconic acid was obtained as glucono-delta lactone from the Eastern Chemical Corporation.

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‡ Mention of commercial products does not imply endorsement by the U. S. Dept. of Agriculture over similar products not mentioned.