Inhibition of Isoniazid Acetylation *in vitro* and *in vivo*.* (22505)

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Previous studies have shown that isoniazid (INH) and sulfanilamide are acetylated by the same enzymic system, and that competitive inhibitors of sulfanilamide acetylation are inhibitors, also, of INH acetylation in pigeon liver extracts(1,2). The present report concerns the inhibition of INH acetylation *in vitro*, and its application *in vivo* as exemplified by the effect of p-aminosalicylic acid (PAS), and of sulfanilamide on the INH levels in the blood plasma of rabbits. A colorimetric method is described for the determination of free INH in the presence of aromatic amines.

Materials and methods. Pigeon liver extract was prepared as previously described(2). One ml of the extract was added to each reaction tube after all other additions had been made. In addition to pigeon liver extract all tubes contained the following substances, in final concentration, to a total volume of 3 ml: potassium phosphate buffer, pH 7.4, 0.02M; potassium acetate 0.02M; potassium citrate 0.02M; sodium adenosine triphosphate 0.004M; INH, 329 μg (8×10^{-4}M); acetylation inhibitors as indicated in Table I. The tubes were incubated for 30 min. at 37°C in the presence of air. 0.2 ml aliquots of the appropriately diluted incubation mixture were taken for the determination of free INH. The extent of acetylation was taken to be the difference between the free INH content of the incubated tubes and that of the zero-time control. In the absence of an acetylation inhibitor, approximately 50% of the added INH was acetylated during the 30 minute incubation period. Female albino rabbits were used for the *in vivo* experiments. They were maintained on a constant diet of Purina Rabbit Chow (100 g per day) containing all necessary vitamins and dietary factors, and water *ad libitum*. All drugs were given by mouth as a solution or suspension in tap water. A modification of the chlorodinitrobenzene method of Scott(3) was used for the measurement of INH in tissue extracts and plasma. Acetyl-INH and isonicotinic acid do not react with this reagent, and none of the acetylation inhibitors used in this investigation interfered with the determination of INH. Thus free INH can be selectively measured in the presence of its acetyl derivative and of aromatic amines and amides.

Procedure for tissue extracts. 0.2 ml of extract containing 10 to 20 μg of INH is pipetted into a 20 x 170 mm Pyrex test tube containing 200 mg of dry powdered borax. Ten ml of a 2.5% (W/V) solution of chlorodinitrobenzene in dehydrated ethanol is added. After mixing, the tube is placed in a boiling water bath for 20 minutes to allow evaporation of the alcohol. The tube is then cooled for 1 minute in ice-water, made up to 10 ml volume with methanol, agitated, and centrifuged at 3000 r.p.m. for 2 minutes. The optical density of the supernatant is measured at 530 mμ against a blank prepared in the same way but omitting INH. The amount of INH present in each sample is ascertained from a calibration curve prepared from appropriate standards run with each set of determinations. The optical density measured at 530 mμ is a linear function of INH concentration within the range 0 to 20 μg and reproducibility is $\pm$ 3% at the 15 μg level. *Procedure for blood plasma*—0.5 ml of clear plasma is pipetted into a centrifuge tube containing 6 ml of dehydrated ethanol. After mixing and standing for 5 minutes the tube is centrifuged. Five ml of the clear supernatant is transferred to a 20 x 170 mm Pyrex test tube containing 200 mg of powdered borax. Five ml of a 5% (W/V) solution of chlorodinitrobenzene in ethanol is added, and the treatment continued as described above for extracts. Appropriate standards containing known amounts of INH added to plasma are run with each set of determinations.

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TABLE I. Inhibition of Isoniazid Acetylation in Pigeon Liver Extracts.

Inhibitor	Inhibitor conc., M/l × 10^{-4}	Inhibition, %
2-Hydroxybenzamide (salicylamide)	10	36
2-Chlorobenzamide	10	0
4-Hydroxybenzamide	10	10
2-Hydroxy-5-bromobenzamide (5-bromosalicylamide)	1.3	52
2,5-Dihydroxybenzamide (gentisic acid amide)	2.5	54
3,4,5-Trihydroxybenzamide (gallic acid amide)	8	29
2-Hydroxy-3-methylbenzamide (o-cresotamide)	10	36
2-Hydroxy-4-aminobenzamide (p-aminosalicylamide)	10	86
2-Hydroxy-4-aminobenzoic acid (PAS)	10	49
4-Aminobenzoic acid (PABA)	10	41
4-Aminophenylacetic acid	20	36
0-Hydroxybenzal isonicotinylhydrazone (Nupasal-213)*	5	70
1-Hydrazinophthalazine (Apresoline)†	10	53
Sulfanilamide	9	30
N^1, N^1-Diethylsulfanilamide	2	74
N^1-Acetylsulfanilamide (sulfacetamide)	8	13
Sulfamethylthiodiazole	8	54
Sulfamethazine	8	16
6-Aminonicotinamide	10	50
4-Amino-5-imidazolecarboxamide	20	31

* Provided through the courtesy of Smith and Nephew Research Ltd., Ware, Herts., England.

† Provided through the courtesy of Dr. W. Murphy, Ciba, Montreal.

Experimental conditions are described under "Materials and methods."

Acids were brought to pH 7.4 with NaOH.

Results. The inhibitory activity of each of the 20 compounds listed in Table I is dependent upon the presence of an amino, amido, or hydrazino group in the molecule. The contribution to inhibitory potency made by other substituent groups has been discussed previously(2) and will be mentioned only briefly here. The strongly activating effect of the 5-bromo and 5-hydroxy groups of 5-bromosalicylamide and gentisamide, respectively, appears to be mediated through the 2-hydroxy group, since benzamide and 5-bromobenzamide (not shown in Table I) have no activity at 10^{-3}M. This is also indicated by the fact that gallamide which possesses the 5-hydroxy group, but lacks the 2-hydroxy, exerts only about one-sixth the inhibition of gentisamide. p-Aminosalicylamide appears to have the combined effect of PAS and salicylamide.

N^1, N^1-diethylsulfanilamide was the most effective sulfonamide tested. It is surprising that sulfamethylthiodiazole, which is poorly acetylated *in vitro* and *in vivo* proved to be somewhat more effective than sulfanilamide, which has a high rate of acetylation. The inhibition by sulfamethylthiodiazole may be due to blockage of active centers of the acetylating enzyme.

The primary amino group of 6-aminonicotinamide can be acetylated, thus accounting for its inhibitory effect. When this compound was fed to rabbits, 6-acetylaminonicotinamide was detected chromatographically as a urinary excretion product (unpublished results).

As previously mentioned(1), PAS, administered concurrently with INH to rabbits, produced a marked increase in the free INH plasma level. This is illustrated by the experimental results shown in Table II. The free INH concentration of plasma was measured 1, 2, and 4 hours after a single dose of INH, as indicated in the table. After a 48 hour interval, INH and PAS sodium were administered concurrently and plasma INH again measured. (Twenty-four hours after the administration of a single dose of INH there was no detectable INH in the plasma and hence no carry-over from one experiment to the next.) In the presence of PAS, the INH plasma levels after 4 hours were more than doubled in each individual case. This effect of PAS on INH plasma levels has been recently corroborated by Mandel *et al.*(4), who found that concurrent administration of PAS with INH resulted in detectable eleva-

TABLE II. Effect of PAS (Sodium) on Plasma Levels of Free INH in 3 Rabbits.

Wt, kg	Drug dose (mg/kg) INH	Drug dose (mg/kg) PAS (sodium)	Plasma levels of free INH (mg/100 ml) at 1 hr	2 hr	4 hr
6	50	—	4.8	2.4	1.4
	50	500	6.3	5.2	2.9
6	50	—	3.1	2.1	1.1
	50	500	4.9	3.4	2.4
3	50	—	2.24	—	.64
	50	500	2.86	—	1.86

TABLE III. Effect of Sulfanilamide (SAM) on Plasma Levels of INH in Rabbits after a Single Oral Dose.

Rabbit No.	Drug dose (mg./kg.) INH	SAM	Plasma level of free INH (mg./100 ml.) At 1 hr	At 3 hr
1	50	—	3.3	.65
	50	150	3.8	1.72
2	50	—	3.9	.57
	50	150	3.95	1.70

tion of the active INH level in the blood serum of tuberculous patients. The effect of sulfanilamide on the INH level of plasma is shown in Table III. The greater effectiveness of sulfanilamide as compared with PAS can partly be attributed to the higher excretion rate of the latter.

Discussion. When INH is administered orally to humans, from 50 to 90% of it is eliminated in the urine as 1-isonicotinyl-2-acetylhydrazine(5,6). Acetyl-INH has negligible antitubercular effect(7) and is rapidly excreted. The increased plasma levels of free INH attained by the concurrent administration of INH and PAS or sulfanilamide to rabbits are undoubtedly due to the effect of the latter compounds on INH acetylation. Mandel *et al.* have suggested that the increased therapeutic effect of INH plus PAS over INH alone may be due in part to the enhanced blood levels of free INH resulting from the inhibition of INH acetylation by PAS. To some extent, a somewhat similar effect may be the explanation for the more rapid and profound therapeutic effect observed by Selikoff, *et al.*(8) when the total daily dose of INH was divided into 3 or 4 doses. Thus, there are indications that the maintenance of therapeutic blood concentrations of INH may be of importance in tuberculosis therapy, views to the contrary not withstanding(9-11). As is known to be the case with sulfonamides, it is probable that the mean blood level of free INH, and not the total amount of INH absorbed is the important factor in the effectiveness of INH therapy.

The possibility of increasing the therapeutic effectiveness of a given dose of INH by the concurrent administration of a non-toxic acetylation inhibitor should be explored. For this purpose PAS is not the ideal inhibitor of acetylation, since it is acetylated only to the extent of 40 to 50% in humans and is excreted largely as a glycine conjugate(12). A sulfonamide retaining adequate solubility in the acetyl form would appear to show greater promise, as would the amide of PAS and other compounds listed in Table I. For that matter, PAS could still be retained in the composition for its property of delaying the development of bacterial resistance to INH.

Summary. The acetylation of INH by pigeon liver extracts was found to be inhibited by various amino, amido, and hydrazino compounds. PAS, administered to rabbits in conjunction with INH, produced a marked increase in the plasma level of free INH. A similar effect was obtained with sulfanilamide. A colorimetric method for the determination of free INH in the presence of aromatic amines is described.

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