Inhibition of the Alanine- α -Oxoglutaric Acid Transaminase of Pig-Heart Muscle by Cyclic Hydrazides

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*iso*Nicotinic acid hydrazide (isoniazid) has anti-tuberculous activity both *in vivo* and *in vitro* (Fox, 1951). In man its principal toxic effect, peripheral neuritis (Biehl & Vilter, 1954; Oestreicher, Dressler & Middlebrook, 1954), can be prevented by the concurrent administration of vitamin B₆ (pyridoxine) (Biehl & Vilter, 1954; Biehl & Nimitz, 1954). It is of interest to discover whether the specific anti-mycobacterial activity of isoniazid is related to its anti-vitamin B₆ action.

Inhibition by isoniazid of the growth of a number of bacterial cultures has been demonstrated (Yoneda & Asano, 1953; Lichstein, 1955) as well as inhibition of pyridoxal phosphate-dependent decarboxylases (Davison, 1956; Hoare, 1956), and of transaminases from *Mycobacterium tuberculosis* var. *avium* (Ito & Sugano, 1954) and the Bacille Calmette Guérin strain (B.C.G.) of tubercle bacilli (Sakai, 1954); reversal of this inhibition was reported by most of these authors. These observations, together with the similarity in the chemical structure of isoniazid and pyridoxal, suggest that isoniazid may compete with vitamin B₆ in enzyme systems such as decarboxylases and transaminases which are dependent on this co-factor. This hypothesis is supported by the high antituberculous activity of 2-methylisoniazid, which closely resembles pyridoxal phosphate in structure, in contrast to the markedly lower efficiency of the 2-ethyl analogue, and the inactivity of the higher 2-alkyl compounds as well as of 3-methyl- and 2:5-dimethyl-isoniazid (Isler *et al.* 1955).

Evidence for the reversal by vitamin B₆ of the antituberculous activity of isoniazid *in vitro* is conflicting. Pope (1956) found that pyridoxal and pyridoxamine caused reversal of the antituberculous activity, but Oestreicher *et al.* (1954) obtained reversal only with pyridoxal. Boone & Woodward (1953) and Ungar, Tomich, Parkin & Muggleton (1954) failed to detect reversal by either compound. Failure to obtain reversal of the antituberculous activity *in vivo* by concurrent admin-

istration of vitamin B₆ in mice and guinea pigs has also been reported (Ungar *et al.* 1954; Grunberg & Blencowe, 1955; Wasz-Hockert, McCune & Tompsett, 1956; Brun, Cayré & Viallier, 1954).

As an alternative to the theory that isoniazid acts as a competitor to vitamin B₆ Vilter, Biehl, Mueller & Friedman (1954) suggested that inhibition of vitamin B₆-dependent enzymes is due to the formation of a pyridoxal isonicotinoylhydrazone, by interaction between the aldehyde group of pyridoxal and the hydrazide radical of isoniazid, but they did not isolate this compound. Pyridoxal isonicotinoylhydrazone was subsequently prepared by Sah (1954), who reported that it inhibited the growth of tubercle bacilli both *in vivo* and *in vitro*.

Evidence bearing upon one or other hypothesis should be given by a study of the effect of a group of cyclic hydrazides and related compounds on a pyridoxal-dependent enzyme such as transaminase. The isoniazid-vitamin B₆ antagonism may be due either to a 4-substituted pyridine structure, in which case only isoniazid would be inhibitory, or to Schiff's-base formation, when all hydrazides would be effective. Investigation of the effect of five hydrazides on the alanine- α -oxoglutaric acid transaminase of pig heart showed that this enzyme system was affected equally by all the five substances used, a result in favour of Schiff's-base formation. As only one of these hydrazides has antituberculous properties there appears to be no correlation between the antituberculous and the anti-vitamin B₆ activities of isoniazid.

MATERIALS AND METHODS

The following hydrazides and related compounds were of analytical purity: isonicotinic acid hydrazide, m.p. 169.5–170.5°, nicotinic acid hydrazide, m.p. 160.5–161.5°, picolinic acid hydrazide, m.p. 100°, benzoic acid hydrazide, m.p. 112–113°, isonicotinamide, m.p. 155–156°, nicotinamide, m.p. 128–129°, benzamide, m.p. 126–128°, isonicotinic acid hydrazide methiodide, m.p. 212–213° (decomp.). L-Alanine, 4-deoxy pyridoxine hydrochloride and phosphate, pyridoxal ethyl acetal hydrochloride and pyridoxal 5-phosphate were supplied by Roche Products Ltd.

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Pyridoxal was prepared from pyridoxal ethyl acetal hydrochloride by hydrolysis with 0.2N-NaOH. α -Oxoglutaric acid was obtained from L. Light and Co. Ltd. Buffer solutions were prepared from appropriate mixtures of 0.067M- $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and KH_2PO_4 .

Enzyme preparation. The preparation from chilled pig hearts obtained from the slaughterhouse was carried out at 4°. Minced heart muscle (300 g.) was homogenized for 2 min. in 900 ml. of 0.03M-phosphate buffer, pH 7.2, and the homogenate was centrifuged at 400 g for 5–10 min. The supernatant and gelatinous clot of mitochondria were decanted from the residue of cell debris and adjusted to pH 5.4 with 10% (v/v) acetic acid and recentrifuged for 20 min. at 1000 g. The sediment containing the transaminase was dissolved in 150 ml. of 0.067M-phosphate buffer, pH 7.4, and frozen solid in batches of about 25 ml. Immediately before use a batch of the suspension was thawed and homogenized for 2 min., any unused material being discarded.

Determination of transaminase activity. The enzyme suspension was incubated with L-alanine, α -oxoglutaric acid and inhibitors; the amount of glutamic acid produced was estimated by decarboxylation with *Clostridium welchii* by the method of Gale (1945). In order to produce a consistent degree of inhibition it was necessary (see below) to pre-incubate the extract with the inhibitor. Enzyme suspension (6 ml.) was pre-incubated for 30 min. at 37° with 6 ml. of 0.067M-phosphate buffer containing the required concentration of inhibitor. At the end of this time 3 ml. each of 0.1M-solutions of L-alanine and α -oxoglutaric acid in phosphate buffer were added. After 10 min. two 3 ml. samples were transferred to centrifuge tubes, which were plunged into boiling water for 5 min. to precipitate protein which was then removed by centrifuging. A portion (1 ml.) of the supernatant was added to 2 ml. of 0.1M-acetate buffer, pH 4.4, in the main compartment of a Warburg flask and the pH was brought back to 4.4 by the addition of 2 or 3 drops of 2N-acetic acid. The bacterial suspension (0.5 ml.) (see below) was placed in the side arm of the flask and, after equilibration at 37°, tipped into the main compartment. Incubation was continued until no further CO_2 was evolved.

Preliminary experiments showed (Table 1) that isoniazid did not affect the estimation of glutamic acid by this method, although the decarboxylase of *C. welchii* is known to be catalysed by pyridoxal. Incubation of the bacterial suspension with 0.133M-isoniazid before addition of glutamic acid also had no effect on subsequent decarboxylation (Table 1).

The bacterial suspension was prepared from stock cultures of *C. welchii*, strain S.R. 12, N.C.T.C. 6784, maintained on Robertson's cooked meat medium as supplied by the Wellcome Research Laboratories, Langley Court, Beckenham, Kent. The *C. welchii* was grown for 16 hr. at 37° in 2 l. of Lemco broth containing 2% of glucose and harvested by centrifuging. The cells were washed once in water and resuspended in 50 ml. of 0.1M-acetate buffer, pH 4.4.

The activity of the transaminase is expressed in terms of glutamic acid as μ moles formed in 18 ml. of incubation mixture in 10 min. Experiment showed that the rate of production of glutamic acid was linear over this period. Hydrazide inhibition is expressed as the percentage depression of transaminase activity compared with that of a control. The results shown in the tables are the mean of two or more experiments.

RESULTS

Transaminase activity was depressed by addition of isoniazid, but consistent results were not obtained unless the enzyme was pre-incubated with the inhibitor before addition of the substrates. This pre-incubation diminished the activity of the enzyme even in the absence of inhibitors; the optimum time for consistent inhibition without too great a loss of enzyme activity was 30 min. (Table 2).

Table 1. *Effect of addition of isoniazid on glutamic acid decarboxylation by Clostridium welchii*

For experimental details see text. The theoretical yield from 5 μ moles of glutamic acid is 112 μ l. of CO_2 .

Concn. of isoniazid in flask (M)	CO_2 evolved (μ l.)
0	124
1.33×10^{-1}	126
1.33×10^{-2}	124
1.33×10^{-3}	125

Bacterial suspension pre-incubated with isoniazid for 1 hr.

0	110
1.33×10^{-1}	109

Table 2. *Effect on transaminase activity of pre-incubation of the enzyme extract with and without 3.3×10^{-2} M-isoniazid*

For experimental details see text.

Time of pre-incubation (min.)	Glutamic acid production when extract pre-incubated without isoniazid (μ moles/10 min./18 ml.)	Percentage depression of glutamic acid production by isoniazid
0	5.61	Variable (10–40)
15	5.23	40
30	5.06	55
45	4.70	50
60	4.83	55

Table 3. *Inhibition of transaminase activity by hydrazides*

For experimental details see text.

Percentage inhibition of glutamic acid production by

Concn. of inhibitor (M)	isoNicotinic acid hydrazide	Nicotinic acid hydrazide	Benzohydrazide	Picolinic acid hydrazide	isoNicotinic acid hydrazide methiodide
(a) Incubation with 6 ml. of enzyme extract					
3.3×10^{-3}	20	12	20	20	10
3.3×10^{-2}	60	58	77	40	45
3.3×10^{-1}	90	90	92	90	72
(b) Incubation with 2 ml. of enzyme extract					
3.3×10^{-3}	18	33	22	13	—
3.3×10^{-2}	65	62	73	50	—

Table 4. *Inhibition of transaminase activity by pyridoxal derivatives and antimetabolites*

For experimental details see text. With the exception of 4-deoxypyridoxine and 4-deoxypyridoxine phosphate these experiments were carried out at pH 8.

Percentage inhibition of glutamic acid production by

Concn. of inhibitor (M)	4-Deoxy-pyridoxine	4-Deoxy-pyridoxine phosphate	Pyridoxal	Pyridoxal + 3.3×10^{-3} M-isoniazid	Pyridoxal isonicotinoylhydrazone	isoNicotinic acid hydrazide
3.3×10^{-3}	5	10	0	52	0	10
3.3×10^{-2}	25	15	35	58	35	55
3.3×10^{-1}	85	90	75	90	(63)*	90

* Saturated solution of the hydrazine, concentration less than 0.33M.

The inhibitory action of various concentrations of the hydrazides of isonicotinic acid (I), nicotinic acid (II), picolinic acid (III) and benzoic acid (IV) and of isonicotinic acid hydrazide methiodide (V) is shown in Table 3. All were equally effective and gave approximately 50% inhibition at a concentration of 3.3×10^{-2} M.

The rather high concentration of inhibitor required to obtain approximately 50% inhibition of the transaminase suggested that the effective concentration of hydrazide in the incubation mixture might be reduced by interaction with free pyridoxal present in the crude extract. In that event a reduction in the volume of extract used should result in greater inhibition of the enzyme with the same amount of inhibitor. However, comparison of the inhibitory action of the hydrazides, by using only 2 ml. of the enzyme preparation made up to 6 ml. with phosphate buffer, showed no significant change in the percentage inhibition of initial rate of reaction (Table 3).

Pre-incubation of three of the corresponding amides—isonicotinamide, nicotinamide and benzamide—with the extract caused no inhibition of the transaminase with a final concentration of 3.3×10^{-2} M of these substances, thus emphasizing that the $-\text{CO}\cdot\text{NH}\cdot\text{NH}_2$ radical was essential for inhibition.

If inhibition of the transaminase by isoniazid involves competition or reaction with the coenzyme pyridoxal phosphate (VI), excess of pyridoxal phosphate might be expected to reverse the inhibition. Unfortunately it was not practicable to demonstrate the reversal of inhibition by isoniazid in the crude enzyme extract used because, at the high concentrations of pyridoxal phosphate required to exceed that of the isoniazid, an unidentified compound was produced which on incubation with *C. welchii* caused an uptake of gas, masking the production of carbon dioxide from glutamic acid. This effect was not investigated further. Addition of pyridoxal and the pyridoxal phosphate antimetabolites 4-deoxypyridoxine and 4-deoxypyridoxine phosphate all produced inhibition (Table 4). Addition of isoniazid, together with pyridoxal, produced greater inhibition than pyridoxal alone, while the preformed Schiff's base, pyridoxal isonicotinoylhydrazone (VII), produced no more inhibition than pyridoxal alone (Table 4). Because of the insolubility of the Schiff's bases in neutral solution, these experiments had to be carried out at pH 8. At this pH isoniazid inhibition was the same as at pH 7.4, the activity of the enzyme was only slightly reduced and all compounds were soluble except for pyridoxal isonicotinoylhydrazone at a concentration of 0.33M.

DISCUSSION

The results of these experiments demonstrate that the five hydrazides tested inhibit the alanine- α -oxoglutaric acid transaminase of pig-heart muscle to about the same extent. Only isoniazid has therapeutic value as an antituberculous agent; conversion of the ring nitrogen into the quaternary form (e.g. V), or its replacement by carbon (e.g. IV), destroys antituberculous activity, while a change in the position of the hydrazide radical either abolishes activity completely (e.g. II) or reduces it to one-hundredth (e.g. III) (Albert, 1956; McMillan, Leonard, Meltzer & King, 1953). There is thus no apparent correlation between antituberculous and antitransaminase activity in these compounds, but these experiments do not finally exclude this possibility since the bacterial cell may exhibit selective permeability towards different hydrazides. The bacteriostatic power of these compounds might then be a reflexion of their degree of penetration into the cell.

It is of interest to consider the possible mechanisms by which isoniazid and the other hydrazides may effect inhibition of the transaminase reaction. The concentration of hydrazide required to produce 50% inhibition is twice that of α -oxoglutaric acid, and it might seem on first consideration that the hydrazide was forming a complex with the keto acid and causing inhibition by removal of a substrate. However, the necessity for pre-incubating the enzyme with the hydrazide to obtain inhibition excludes this possibility. A more plausible assumption is that the inhibition is effected in some way through the coenzyme, pyridoxal phosphate. There are then two main ways in which inhibition may be brought about: competition of the hydrazide with pyridoxal phosphate for the enzyme protein, or modification of the coenzyme in such a way that it is no longer capable of catalysing transamination. From these results it is unlikely that the hydrazides compete directly with pyridoxal phosphate for the enzyme. If this were so, it would be expected that isoniazid, the only hydrazide tested with a 4-substituted pyridine structure, would alone be inhibitory or at least be more effective than the others. In fact all five were equally potent as inhibitors and more effective in low concentration than the competitive inhibitors, deoxypyridoxine, deoxypyridoxine phosphate and pyridoxal. It seems probable, then, that hydrazides act by modifying the coenzyme in such a way that it is no longer capable of combining with the substrate, e.g. by formation of a Schiff's base as suggested by Davison (1956) to account for inhibition by hydrazide of the cysteine sulphinic acid decarboxylase of the liver. This theory is supported by the necessity of the hydrazide radical for

inhibition of transamination (cf. the inactivity of the corresponding amides) and the reports of inhibition of other pyridoxal phosphate-dependent enzyme systems by hydrazide compounds (Gale, 1946).

The question then arises whether inhibition is caused by combination of the hydrazide with enzyme-bound pyridoxal phosphate, or by reaction with any free pyridoxal phosphate in solution, to form a Schiff's base which might compete for the enzyme site. The latter is unlikely since pyridoxal *isonicotinoyl*hydrazone is a less effective inhibitor than isoniazid in the same concentration and at the same pH (Table 4). The degree of inhibition of pyridoxal-dependent enzyme systems by hydrazides appears to be related to the strength of the pyridoxal phosphate-enzyme association. The inhibition of bacterial decarboxylases by semicarbazide and hydrazine was reported by Gale (1946) to be much greater in those systems where the co-factor was easily separated from the enzyme than in those in which it was tightly bound. The concentration of hydrazide (3.3×10^{-2} M) required to obtain 50% inhibition in this work, as compared with that (3×10^{-4} M) required for 50% inhibition of the decarboxylase studied by Davison (1956), is in agreement with the idea that the coenzyme-enzyme association in transaminases is particularly strong (Meister, 1955).

Thus hydrazides may act as inhibitors of transaminases and other pyridoxal phosphate-dependent enzyme systems by combination with the aldehyde group of the pyridoxal phosphate, provoking dissociation of the coenzyme from the enzyme. The variation in effective concentration of hydrazides necessary to produce inhibition of different enzyme systems may be explained by the known variations in strength of the pyridoxal phosphate-enzyme association with different enzyme proteins.

SUMMARY

1. The effect of *isonicotinic* acid hydrazide, *nicotinic* acid hydrazide, *picolinic* acid hydrazide, *benzohydrazide* and *isonicotinic* acid hydrazide methiodide on the alanine- α -oxoglutaric acid transaminase of a pig-heart-muscle extract was studied. All were found to produce approximately 50% inhibition of glutamic acid production at a concentration of 3.3×10^{-2} M.

2. The corresponding amides were without effect on the transaminase.

3. The mechanism of hydrazide inhibition is discussed on the basis of these findings.

4. Of the five hydrazides tested only *isonicotinic* acid hydrazide is of therapeutic value as an antituberculous drug. It is concluded that there is no correlation between the antituberculous and anti-

vitamin B₆ activities of these hydrazides, unless the bacterial cell is selectively permeable to isoniazid alone.

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REFERENCES

- Albert, A. (1956). *Nature, Lond.*, **177**, 525.
 Biehl, J. P. & Nimitz, H. T. (1954). *Amer. Rev. Tuberc.* **70**, 430.
 Biehl, J. P. & Vilter, R. W. (1954). *Proc. Soc. exp. Biol., N.Y.*, **85**, 389.
 Boone, I. U. & Woodward, K. T. (1953). *Proc. Soc. exp. Biol., N.Y.*, **84**, 292.
 Brun, J., Cayré, R. M. & Viallier, J. (1954). *C.R. Acad. Sci., Paris*, **148**, 1817.
 Davison, A. N. (1956). *Biochim. biophys. Acta*, **19**, 131.
 Fox, H. H. (1951). *Abstr. 12th Int. Congr. Chem., N.Y.*, p. 299.
 Gale, E. F. (1945). *Biochem. J.* **39**, 46.
 Gale, E. F. (1946). *Advanc. Enzymol.* **6**, 1.
 Grunberg, E. & Blencowe, W. (1955). *Amer. Rev. Tuberc.* **71**, 898.
 Hoare, D. S. (1956). *Biochim. biophys. Acta*, **19**, 141.
 Isler, O., Gutmann, H., Straub, O., Fust, B., Böhm, E. & Studer, A. (1955). *Helv. chim. acta*, **38**, 1033.
 Ito, F. & Sugano, T. (1954). *Kekkaku*, **29**, 368.
 Lichstein, H. C. (1955). *Proc. Soc. exp. Biol., N.Y.*, **88**, 519.
 McMillan, F. H., Leonard, F., Meltzer, R. I. & King, J. A. (1953). *J. Amer. pharm. Ass.* **42**, 457.
 Meister, A. (1955). *A Symposium on Amino Acid Metabolism*. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
 Oestreicher, R., Dressler, S. H. & Middlebrook, G. (1954). *Amer. Rev. Tuberc.* **70**, 504.
 Pope, H. (1956). *Amer. Rev. Tuberc.* **73**, 735.
 Sah, P. P. T. (1954). *J. Amer. chem. Soc.* **76**, 300.
 Sakai, J. (1954). *Kekkaku*, **27**, 161.
 Ungar, J., Tomich, E. G., Parkin, K. R. & Muggleton, P. W. (1954). *Lancet*, **267**, 220.
 Vilter, R. W., Biehl, J. P., Mueller, J. F. & Friedman, B. I. (1954). *Fed. Proc.* **13**, 776.
 Wasz-Hockert, O., McCune, R. M. & Tompsett, R. (1956). *Amer. Rev. Tuberc.* **74**, 471.
 Yoneda, M. & Asano, N. (1953). *Science*, **117**, 277.

The Blocking Activity of Sympathomimetic Amines on Adrenaline Hyperglycaemia

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Previous workers have found that while a di- or mono-hydroxybenzene ring must be present for a sympathomimetic amine to have hyperglycaemic activity, the analogous deoxy compound can still affect the blood pressure. In view of this, the effect on the hyperglycaemic and blood-pressure-raising activities of replacing the catechol or benzene ring of sympathomimetic amines by a pyridine ring was studied.

Ellis (1951) suggested that in amines with hyperglycaemic activity the side chain is responsible for the attachment to the enzyme complex concerned in adrenaline hyperglycaemia, whereas the catechol nucleus is the active grouping in the production of hyperglycaemia. Thus, on this hypothesis, it may be possible for a compound having a side chain similar to that of adrenaline to attach itself, and, while exerting no hyperglycaemic effect owing to the lack of the appropriate aromatic structure, block the hyperglycaemic effect of adrenaline administered subsequently. In agreement with this hypothesis Ellis found that ephedrine did, in fact, largely

inhibit the adrenaline effect in rats, and this was confirmed for rabbits by Komrad & Loew (1951).

Some information on how far this ephedrine-blocking effect is dependent on an *N*-methylated hydroxyisopropylamine side chain attached to a benzene nucleus has now been obtained by using norephedrine and its pyridine analogue and the pyridine analogue of α -aminopropiophenone. Synthetic methods for preparing these two pyridine analogues, namely 3-(2-amino-1-hydroxypropyl)-pyridine (IV) and 3-(2-amino-1-oxopropyl)pyridine (II) respectively, have been developed.

The 3-(2-amino-1-oxopropyl)pyridine was prepared by the action of nicotinic anhydride on α -benzamidopropionic acid, according to Dakin & West (1928), followed by a hydrolysis of the benzamido group. In order to obtain the corresponding hydroxypropyl derivative, which was prepared by catalytic reduction of the ketone, it was necessary first to reduce the benzamido derivative and then hydrolyse, since hydrogenation of the free amine could not be achieved without simultaneous