The Action of Isoniazid on the Transaminases of *Mycobacterium tuberculosis* (B.C.G.)

By JEAN YOUATT
Bacteriology Department, University of Melbourne, Victoria, Australia

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One of the proposed mechanisms for the action of *iso*nicotinic acid hydrazide (isoniazid) has been the inhibition of enzyme systems which require pyridoxal phosphate (Cymerman-Craig, Rubbo, Willis & Edgar, 1955). However, it appeared unlikely that the inhibition of an enzyme system present in many bacteria could account for the highly specific activity of isoniazid in low concentrations against species of *Mycobacterium*.

Sakai (1954) found that the aspartic–glutamic acid transaminase of *Mycobacterium tuberculosis* B.C.G. strain (B.C.G.) was inhibited 90–100% by 0·01 M-isoniazid with an isoniazid-sensitive strain, whereas the transaminase of the resistant B.C.G. was not inhibited. Transamination of asparagine, glycine, phenylalanine, leucine, valine, alanine and aspartic acid by avian tubercle bacilli was described by Ito & Sugano (1954), who also observed the inhibition of the reaction by 0·01 M-isoniazid. Yonemura & Iisuka (1955) described the transamination of twenty amino acids in the presence of α-oxoglutarate, by a virulent strain of bovine tubercle bacilli. They found no transamination between any amino acid, other than glutamic acid, and pyruvic or oxaloacetic acid.

Yamamoto (1955) found that cysteine desulphydrase of sensitive and resistant strains of B.C.G. was inhibited by isoniazid. The inhibition was partially reversed by pyridoxal and adenosine triphosphate.

The results with enzymes of *Mycobacterium* closely parallel those obtained with organisms naturally insensitive to isoniazid, such as *Escherichia coli*, with which it was shown that enzymes requiring pyridoxal phosphate were inhibited by isoniazid at concentrations of the order of 0·01 M (Yoneda, Kato & Okajima, 1952).

The experiments described below were made with the aim of determining whether the inhibition of transaminase in *Mycobacterium* could account for the highly specific action of isoniazid in low concentrations.

METHODS

Cultures. Surface cultures of B.C.G. were grown on Sauton medium (Soltys, 1952) to which 3 g. of casein hydrolysate (British Drug Houses Ltd.) was added/l. Dispersed cultures were grown in Sauton medium containing 0·05% of polyoxyethylene sorbitan mono-oleate (Tween 80). Suspensions of the organisms in pentane were used to inoculate the surface of the medium. Subcultures were made at intervals of one week and cultures were incubated at 37° for 7–14 days.

A culture of B.C.G. resistant to isoniazid was obtained by surface inoculation of Sauton medium containing 1 μg. of isoniazid/ml. In this first subculture growth was slow but thereafter the organisms grew readily in successive transfers to media with 10, 20 and 40 μg. of isoniazid/ml.

Preparation of suspensions and extracts. Surface cultures were suspended in 0·9% NaCl by gentle grinding in a Potter–Elvehjem homogenizer. This treatment resulted in some cell damage, as evidenced by leakage of cell contents into the suspending medium. Cell-free extracts were prepared by shaking suspension for 2 min. in a Nossal disintegrator (Nossal, 1953). Dialysis of the extracts overnight against distilled water at 5° was essential to remove the amino acids, of which glutamic acid, aspartic acid and alanine were consistently detected by paper chromatography. Tyrosine was also found in most cell extracts. These internal free amino acids were also found when the cultures were grown with ammonium chloride as their only source of nitrogen.

Detection and estimation of transaminase activity. Unless otherwise stated, transaminase activity was measured under the following conditions. The final concentrations of the reagents, which were adjusted to pH 8, were: keto acid, 0·05 M; amino acid, 0·025 M; Sørensen's phosphate buffer, 0·1 M; cell suspensions, 20–50 mg. (dry wt.)/ml. and cell extracts, 10–20 mg. (dry wt.)/ml. A volume (1 ml.) of the reaction mixture was incubated at 35° for the periods indicated in the tables, after which 1·5 ml. of 0·67 M-acetate buffer, pH 4, was added. After centrifuging, 2 ml. of supernatant was transferred to a Warburg flask with 0·5 ml. of a suspension of *Proteus mirabilis* in the side arm. Carbon dioxide production was measured at 35°. The use of *Proteus* suspensions for this purpose is described below.

In qualitative examinations the supernatants were spotted on Whatman no. 1 paper and chromatograms were run in butanol–acetic acid–water (4:1:1) and developed with ninhydrin in butanol (0·25%, w/v).

Estimation of glutamic acid with Proteus mirabilis. *P. mirabilis* was grown on meat-infusion agar enriched with freshly prepared yeast extract and 0·1% sodium glutamate. The organism was not dangerous to handle, could be grown on a simple medium with yields up to 4 g. dry wt./l. and had a glutamic acid decarboxylase activity of the order of 0·4 μmole of glutamic acid/hr./mg. dry wt., at 37° and pH 4·5. The following methods of preparation gave suspensions of satisfactory specificity: 1, lyophilized suspensions resuspended in buffer at pH 4; 2, suspensions at pH 4 which had been shaken with glass beads in the Nossal

disintegrator for 1 min.; 3, freshly harvested suspensions with 0·03 M-isoniazid. (Freshly harvested suspensions showed production of CO_2 in the absence of added glutamic acid. This was suppressed by the presence of isoniazid.) Cell-free extracts could be prepared from 2 by centrifuging. None of these preparations showed gas production with L-glutamine, DL-aspartic acid, L-ornithine, DL-lysine, DL-arginine, DL-tyrosine, DL-histidine, or α-oxoglutaric, pyruvic or oxaloacetic acid, or with these keto acids in combination with DL-aspartic acid, DL-valine, DL-leucine, DL-isoleucine, DL-methionine, DL-alanine, DL-phenylalanine. One molecule of CO_2 was produced for each molecule of glutamic acid and γ-aminobutyric acid was identified by paper chromatography. The decarboxylase was not inhibited by isoniazid at 0·1 M in either cell suspensions or extracts.

Requirement for pyridoxal phosphate. The addition of pyridoxal phosphate to suspensions and extracts of B.C.G. was found to be unnecessary and it was omitted from all experiments except the surveys of the range of transaminase activities and experiments where the reversal of isoniazid inhibition was measured. The pyridoxal phosphate of the aspartic–glutamic acid transaminase of B.C.G. appears to be firmly bound to the apoenzyme. Stimulation by pyridoxal phosphate was observed only when the enzyme had been precipitated with saturated ammonium sulphate and dialysed against acetate buffer pH 4 for several days. The protein was precipitated at this pH but redissolved in buffer at pH 8. The addition of pyridoxal phosphate to this preparation caused a 50% increase in activity. Halpern & Grossowicz (1956) have recently described the stability of the transaminase of *Mycobacterium phlei*.

Use of isoniazid as an inhibitor. Unless otherwise stated, suspensions and extracts were pre-incubated with isoniazid for 30 min. before the addition of the substrates. Prolonged exposure of the cells to isoniazid before harvesting was also tested.

RESULTS

Range of transaminase activities

Each of the following amino acids was incubated with α-oxoglutaric acid, pyruvic acid and oxaloacetic acid in the presence of cell suspensions and extracts with pyridoxal phosphate: DL-alanine, L-asparagine, DL-aspartic acid, L-arginine, L-cysteine, DL-cystine, L-glutamic acid, L-glutamine, glycine, DL-histidine, DL-hydroxyproline, DL-isoleucine, DL-leucine, DL-lysine, DL-methionine, DL-phenylalanine, DL-proline, DL-serine, DL-threonine, DL-tyrosine, DL-tryptophan, DL-valine. By the use of paper chromatography, evidence of transaminase activity was found in the following systems: α-oxoglutarate and the amino acids, aspartic acid, leucine, isoleucine, valine, asparagine, alanine, methionine, and phenylalanine; pyruvic acid and glutamic acid; oxaloacetic acid and glutamic acid. Asparagine is rapidly metabolized by the organisms to aspartic acid and may not be directly concerned in the transaminase reaction. No variation in the range of transaminase activity was found with the age of the culture or with its resistance to isoniazid.

Inhibition of transaminases by isoniazid

Differences in the degree of sensitivity of transaminases to isoniazid are shown in Table 1. Even with incubation for 3 hr. (*B*) transamination of alanine was barely detectable. The reaction was shown to be linear with time, over 1½ hr. (*A*). Where there is overlap between the two series it will be seen that the only change in the pattern of behaviour was a small increase in the sensitivity to isoniazid of the aspartic–glutamic acid transaminase. No differences were detected between sensitive and resistant B.C.G. greater than were found with different preparations of sensitive organisms.

Isoniazid increased the leakage of glutamic acid from whole-cell suspensions. It was therefore not possible to determine the inhibition of the transaminase reaction with whole cells.

Table 1. *Sensitivity to isoniazid of different transaminase reactions in extracts of isoniazid-sensitive B.C.G.*

Reaction mixtures: α-oxoglutarate 0·05 M, amino acid 0·025 M, phosphate buffer 0·1 M, all at pH 8. Extracts of sensitive B.C.G. 20 (*A*) and 10 (*B*) mg. dry wt./ml. Pre-incubated with isoniazid for 30 min. at 35°. Reaction time: (*A*) 1½ hr.; (*B*) 3 hr.

	Amino acid	Rate of transamination (μmoles of glutamic acid/hr./ml.)	% inhibition by isoniazid 0·1 M	0·01 M	0·001 M
(*A*)	Aspartic acid	3·3	74	35	8
	Leucine	5·8	100	100	52
	Isoleucine	4·82	100	100	54
	Valine	4·42	100	100	55
(*B*)	Aspartic acid	1·74	94	49	27
	Leucine	2·1	100	100	40
	Isoleucine	3·74	100	89	43
	Valine	3·45	98	94	53
	Alanine	0·13	100	47	8
	Phenylalanine	0·77	69	22	0
	Methionine	0·8	89	66	45

Sakai (1954) reported that the aspartic–glutamic acid transaminase of B.C.G. was 90–100% inhibited by 0·01M-isoniazid, whereas in these experiments less inhibition was observed. An explanation of the discrepancy was found in the concentration of keto acid employed. Sakai used α-oxoglutarate at a concentration of 0·005M. Table 2 shows that the rate of transamination is very sensitive to changes in substrate concentration and the degree of inhibition depends on the relative concentrations of isoniazid and keto acid.

Above 50 μmoles of α-oxoglutarate/ml. the enzyme is approaching saturation and the degree of inhibition by a given concentration of isoniazid is independent of the concentration of keto acid. Table 3 shows the inhibition by a range of isoniazid concentrations with two concentrations of α-oxoglutarate. From the linear plot of log [isoniazid] against the percentage inhibition the concentration of isoniazid producing 50% inhibition was in each case 30 μmoles/ml.

Effect of hydrazones of isoniazid on transaminase

Davison (1956) described the formation of a hydrazone by isoniazid and pyridoxal phosphate and was able in this way to explain the inhibition of enzymes requiring pyridoxal phosphate. Thus it would be expected that the addition of pyridoxal phosphate would bring a reversal of isoniazid inhibition and that stable hydrazones of isoniazid should not inhibit the transaminase reaction. In the present study it was found that an equimolar concentration of pyridoxal phosphate completely antagonized the isoniazid inhibition of transaminase, and this antagonism was also observed to a lesser degree (30%) with pyridoxine and adenosine triphosphate. Acetylacetone *iso*nicotinoylhydrazone and the isoniazid hydrazone of pyridoxal produced no inhibition of the isoleucine–glutamic transaminase at 0·01M, at which concentration isoniazid inhibited 90–100%. The isoleucine–glutamic transaminase was selected for this experiment because of its greater sensitivity to isoniazid than the aspartic–glutamic transaminase.

The failure of the hydrazones to inhibit the transaminase reaction was of particular interest because both of these compounds had a tuberculostatic activity similar to isoniazid (Table 4). The hydrazones were stable in the presence of heavy suspensions of B.C.G. in Sauton medium. With picryl chloride as a reagent for isoniazid (Cuthbertson & Ireland, 1952) free isoniazid could be detected with certainty at 0·001M. The hydrazones were present at 0·025M. The test was therefore capable of detecting the release of free isoniazid down to 4% of the theoretical isoniazid. The tuberculostatic activity of the hydrazones could not be explained on the grounds of the release of

Table 2. *Effect of keto acid concentration on the rate of transamination and the degree of inhibition by isoniazid*

Reaction mixtures: α-oxoglutarate concentrations as indicated in the table, DL-aspartic acid 0·025M, phosphate buffer 0·1M, all at pH 8. In series (*A*) isoniazid was present at 8·3 μmoles/ml. and in series (*B*) at 25 μmoles/ml. An extract of sensitive B.C.G. was used (10 mg. dry wt./ml.). Pre-incubated with isoniazid for 30 min. at 35°. Reaction time 1½ hr. at 35°.

	α-Oxoglutarate (μmoles/ml.)	Glutamic acid produced (μmoles/ml./hr.) Without isoniazid	With isoniazid	% inhibition
(*A*)	100	10·2	6·7	34
	50	6·7	4·7	30
	25	4·8	3·6	25
	12·5	2·5	1·8	28
	6·25	0·55	0·0	100
(*B*)	100	6·0	4·4	27
	50	5·8	3·7	38
	25	4·8	2·1	56
	12·5	2·9	0·17	94
	6·25	0·67	0·0	100

Table 3. *Inhibition of transaminase by varying concentrations of isoniazid, with constant α-oxoglutarate concentration*

Reaction mixture: α-oxoglutarate, 50 and 70 μmoles/ml. respectively in (*a*) and (*b*); DL-aspartic acid, 0·025M; phosphate buffer, 0·1M; extract of sensitive B.C.G. 10 mg. dry wt./ml. All reagents were at pH 8. Pre-incubated with isoniazid for 30 min. at 35°. Reaction time, 3 hr. at 35°.

Isoniazid (μmoles/ml.)	% inhibition (*a*)	(*b*)
90	—	75
80	—	73
70	71	66
60	70	—
50	65	66
30	60	—
20	—	37
10	22	22
5	10	9

Table 4. *Tuberculostatic concentrations of isoniazid and its hydrazones*

Compound	Tuberculostatic end-point*
Isoniazid	5×10^{-7} M
Acetylacetone *iso*nicotinoylhydrazone	2×10^{-6} M
Pyridoxal *iso*nicotinoylhydrazone	10^{-6} M

* The lowest concentration of isoniazid which prevented growth of *Mycobacterium tuberculosis* H 37 Rv in Youman's medium after 14 days' incubation at 37°.

free isoniazid unless the hydrazones were 50–100% broken down.

An attempt was made to see whether a hydrazone was stable inside the cell. An amount (1 g. dry wt.) of B.C.G. was suspended in 0·05% Tween 80 and incubated with 0·01 M-pyridoxal *iso*nicotinoylhydrazone for 24 hr. at 37°. After thorough washing and resuspension in water the cells were ruptured at 115° for 2 min. The resulting aqueous extract was separated by centrifuging and extracted with ethyl acetate in which the hydrazone would be more soluble than free isoniazid. The remaining aqueous extract and the ethyl acetate extract were concentrated to approx. 0·2 ml. Each was tested for free isoniazid before and after acid hydrolysis. Free isoniazid was detected only in the ethyl acetate extract after hydrolysis. The experiment therefore produced no evidence of the release of free isoniazid in the cell. It is important to the understanding of the mode of action of isoniazid that the behaviour of these hydrazones be studied further. They are inhibitory to the growth of *Mycobacterium*, and strains which are resistant to isoniazid are also resistant to the hydrazones. As the hydrazones cannot combine with pyridoxal phosphate they appear to discount the suggestion that the chemical inactivation of enzymes requiring pyridoxal phosphate explains the mode of action of isoniazid.

Transaminase activity in cells after long exposure to isoniazid

The evidence so far obtained indicated that transaminase inhibition could not explain the specific action of isoniazid. In order to test the effect of isoniazid on transaminases after incubation under conditions known to be bactericidal, cultures in Sauton medium were incubated with 100 μg. of isoniazid/ml. for 2- to 7-day periods. The isoniazid was added after the normal incubation period of 7 days at 37°. The extracts obtained from these cells still showed the aspartic–glutamic and isoleucine–glutamic acid transaminase reactions with rates comparable with extracts of untreated cells. No additions of pyridoxal phosphate were made to these extracts.

DISCUSSION

Reference has been made to Davison's (1956) experiments with isoniazid and pyridoxal phosphate. He observed that the rate of combination of isoniazid and pyridoxal phosphate was similar to the rate of inhibition of enzymes requiring pyridoxal phosphate, and suggested that the rate of inhibition by isoniazid might be used to demonstrate the requirement for pyridoxal phosphate. Davison did not record whether the rate of inhibition was affected by the substrate concentration. By this criterion the glutamic acid decarboxylase of *Proteus mirabilis* would not require pyridoxal phosphate. It would be of interest to know what the effect of isoniazid would be on an enzyme in which the pyridoxal phosphate was known to be firmly bound to the apoenzyme, for example the histidine decarboxylase described by Guirard & Snell (1954). The varying inhibition with the transaminases of B.C.G. and the insensitivity of the *Proteus* decarboxylase suggest that the inhibition by isoniazid may be determined by the ease with which the coenzyme may be removed from the apoenzyme.

Davison showed that the reaction between isoniazid and pyridoxal phosphate was rapid but the bactericidal effect of isoniazid is slow. Further, if Davison is correct in suggesting that all enzymes requiring pyridoxal phosphate are inhibited at the same rate, it would be necessary to postulate that the inhibition of all such enzymes resulted in the slow development of conditions intolerable to the organisms.

The evidence against transaminase as the site of action of isoniazid against *Mycobacterium* was that the transaminase activity remained after incubation with isoniazid under conditions known to be bactericidal and that transaminase activity was not inhibited by stable hydrazones of isoniazid which were known to have similar tuberculostatic activity to isoniazid. As far as could be determined, these compounds were stable in the presence of B.C.G. and the activity could not be attributed to the release of free isoniazid in the medium.

In the cell it is possible to visualize the equilibrium equation below, where X is the sensitive enzyme and INH is isoniazid.

$$\text{X} + \text{pyridoxal INH hydrazone} \rightleftharpoons \text{pyridoxal} + \text{INH} + \text{X} \rightleftharpoons \text{pyridoxal} + \text{INH–X}.$$

In such a system it is not possible to equate X with the transaminase system since this was not inhibited either by the pyridoxal *iso*nicotinoylhydrazone or by equimolar mixtures of isoniazid and pyridoxal.

SUMMARY

1. No qualitative differences in the range of transaminase activities of sensitive and resistant strains of *Mycobacterium tuberculosis* (B.C.G.) were found. The enzymes in extracts of sensitive and resistant organisms were equally sensitive to isoniazid.

2. Below a concentration of 0·05 M the amount of α-oxoglutarate employed in estimating the aspartic–glutamic transaminase activity affected the rate of the reaction and the degree of inhibition by a given concentration of isoniazid.

3. The presence of equimolar concentrations of pyridoxal phosphate prevented the inhibition by isoniazid. Stable hydrazones of isoniazid did not inhibit transaminase activity. These compounds, which were stable in the presence of heavy suspensions of B.C.G. organisms, had tuberculostatic activity comparable with isoniazid.

4. Transaminase activity was found in extracts of cells which had been exposed to isoniazid under bactericidal conditions.

5. It appears improbable that the bactericidal activity of isoniazid can be explained by its action on a transaminase or other enzyme requiring pyridoxal phosphate.

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Determination of Iron in Biological Material by Spectrophotometry of Ferric Perchlorate

By I. D. P. WOOTTON
*Postgraduate Medical School, London, W.*12

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The determination of iron in inorganic samples by conversion into ferric sulphate or ferric perchlorate has been described by Bastian, Weberling & Pallila (1953, 1956). These salts absorb strongly at a region in the ultraviolet where interference from other elements is negligible. It therefore seemed likely that iron in biological material could be estimated by digestion with perchloric acid followed by spectrophotometry of the diluted digest. An accurate and convenient method results; as described, samples should contain 0·2–1 mg. of iron, but much smaller quantities of iron can be estimated by modification of the volumes. For convenience, the present procedure yields 100 ml. of solution for the optical measurement. It is, however, quite practicable to finish with a final volume of 10 ml. With this modification, iron can be estimated in the range 0·02–0·1 mg.

METHOD

Reagents. AnalaR perchloric acid (60%; sp.gr. 1·54) is taken to be 10N. By tenfold dilution of the concentrated acid, N-perchloric acid is prepared. AnalaR concentrated nitric acid is used. All reagents must be iron-free. Several batches of AnalaR acids have been used; they have all given acceptably low blank values.

Samples. An appropriate amount of material containing up to 1 mg. of iron is weighed or measured into a 250 ml. conical flask. For blood, 2 ml. is taken; for tissue such as liver or spleen, 2 g. of finely chopped material is weighed out; for urine, quantities varying from 5 to 50 ml. or more are necessary.

In the special case of serum-iron estimation, this method does not present any advantage over existing procedures. This is because the concentration of iron in the serum is exceedingly low and the method chosen must therefore be very sensitive. In addition, preliminary treatment of the serum with a precipitant of acid protein is necessary to remove traces of haemoglobin; the resulting filtrate is suitable for immediate development of colour with one of the conventional reagents.

Digestion. Nitric acid (5 ml.) is added to the contents of the flask and the mixture is boiled gently on a hot-plate for at least 1 hr., or longer if necessary, until a brown or yellow mobile liquid results. Further additions of nitric acid may be necessary to prevent drying. To the cooled solution 5 ml. of 10N-perchloric acid is added and the flask is returned to the hot-plate. The temperature is raised and the contents are allowed to evaporate to dryness. The heating should be adjusted so that 1½–2 hr. elapse between the addition of the perchloric acid and complete dryness. The residue is treated with 10 ml. of 10N-perchloric acid, which is heated until boiling to achieve complete solution of the residue. The cooled solution is washed quantitatively into a 100 ml. volumetric flask and