The Destruction of *iso*Nicotinic Acid Hydrazide in the Presence of Haemin

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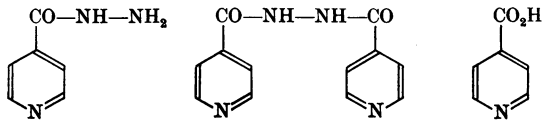
It has been shown (Fisher, 1954) that haemin antagonizes the antibacterial effect of *iso*nicotinic acid hydrazide (isoniazid; *iso*nicotinoylhydrazine) on *Mycobacterium tuberculosis* H37Rv. In investigating this phenomenon, we have found that haemin catalyses the oxidative destruction of isoniazid (I) and that the products are 1:2-di*iso*nicotinoylhydrazine (II) and *iso*nicotinic acid (III).



(I) (II) (III)

MATERIALS AND METHODS

The isoniazid, after recrystallization from ethanol and drying over calcium chloride and then over phosphorus pentoxide, had m.p. 170°. Potentiometric titration with alkali (0·1 N-KOH), making use of the pK 10·8 (Albert, 1953) and ultraviolet spectrophotometry (at 262 mμ.), indicated a purity of 99·84%. This material was used to evaluate the published methods of iodometric titration. That conducted in the presence of NaOH (Alicino, 1952) gave a titre of 92%, and that using $NaHCO_3$ (Canbäck, 1952) gave 95·5%, not improved by allowing a longer reaction time. The principal reaction is

$$R.CO.NH.NH_2 + 2I_2 + H_2O \rightarrow R.CO_2H + N_2 + 4HI. \quad (i)$$

It was found that di*iso*nicotinoylhydrazine (II) can be titrated similarly:

$$R.CO.NH.NH.CO.R + 2I_2 + 2H_2O \rightarrow 2R.CO_2H + N_2 + 4HI. \quad (ii)$$

Because isoniazid, shaken with haemin in air, was found to give a mixture of (I), (II) and (III) (see below), titration with I_2 was used (with the factor 100/95·5) to obtain rapidly some general information on the progress of this decomposition. Thus, a sample (5 ml.) was withdrawn from the reaction mixture, kieselguhr (0·05 g. Hyflo-Supercel) added, then N-HCl (2–3 drops to give pH 2). The haemin was filtered off and washed. The filtrates were adjusted to pH 6–7 with N-NaOH, $NaHCO_3$ (0·2 g.) was added, then 0·01 N-I_2 (25 ml.). After 15 min., 0·5 N-H_2SO_4 (10 ml.) was added, and the excess of I_2 titrated with 0·01 N sodium thiosulphate, standardized against both KIO_3 and $K_2Cr_2O_7$.

The *iso*nicotinic acid was dried over P_2O_5 at 20° and 20 mm. (Found: C, 58·1; H, 4·2; N, 11·2. Calc. for $C_6H_5O_2N$: C, 58·5; H, 4·1; N, 11·4%.)

Di*iso*nicotinoylhydrazine (II) has previously been prepared only by the action of *iso*nicotinoyl chloride on hydrazine hydrate (Graf, 1933). A more convenient preparation was found, as follows. Isoniazid (1 g.), dissolved in ethanol (100 ml.), was stirred at room temp. with freshly precipitated yellow HgO (1·58 g.; 1 equiv.) for 18 hr. The solution was filtered from metallic mercury, which was then washed with ethanol. The combined filtrates were briefly shaken with carbon, filtered and taken to dryness. The solid was recrystallized from water (40 ml.), giving 0·35 g. of white crystals of (II), m.p. 260° (cf. 254–255°, Graf, 1933). (Found: C, 59·7; H, 3·7; O, 13·1. Calc. for $C_{12}H_{10}O_2N_4$: C, 59·5; H, 4·1; O, 13·2%.)

The haemin was standardized spectrophotometrically (at 555 mμ.) and found to be 92±5% pure. It was weighed out for each experiment and dissolved in the minimal volume of N-NaOH, then diluted with half of the buffer solution to be used and adjusted to pH 7·5 (glass electrode).

EXPERIMENTAL

Isoniazid (0·01 M), plus haemin or other additions where used, in 0·05 M phosphate buffer (pH 7·5) was shaken in a long-necked open flask at about 180 oscillations per minute (17–20°).

On one occasion the products of the reaction were isolated, making use of the high solubility of (I) and (III) in cold water as compared with that of (II), and of the solubility of (I) in boiling ethanol under conditions where (III) was present as the sodium salt and hence insoluble. Thus, 0·01 M isoniazid and haemin (0·001 M) in buffer (200 ml., as above) were shaken in air for 24 hr., kieselguhr (2 g.) was added and the haemin precipitated at pH 2 by adding 5 N-HCl. The filtrate was evaporated to 10 ml., adjusted to pH 7 and chilled. The di*iso*nicotinoylhydrazine was filtered off and recrystallized from water as white needles, m.p. 260° (0·02 g.). The filtrate was taken to dryness and extracted twice with boiling ethanol (2+1 ml.). The evaporated extract gave white crystals of isoniazid (0·11 g.), which, recrystallized from ethanol, gave 0·05 g., m.p. 166–170°. The ethanol-insoluble residue of sodium *iso*nicotinate was dissolved in water (2 ml.) and adjusted to pH 3. The solution was evaporated, and the solid extracted three times with ethanol (3 × 15 ml.). The extract was concentrated to 3 ml., chilled, and the crystals were dried at 110°, giving *iso*nicotinic acid (0·02 g.), m.p. 300–305° (decomp.; sealed tube). Mixed melting points and chromatography were used to confirm the identity of these three substances.

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Paper chromatography. The most suitable developing solvent was aqueous 0·5 M-NH_4Cl. Ammonia (2 N) gave better resolution, but increased the rate of oxidation in air. Insufficient resolution was given by the following: water, dimethylformamide, butanol–acetic acid, $(NH_4)Cl$ in 0·01 N-HCl. The papers were dried in warm air (30 min.), and read under an ultraviolet lamp emitting principally at 254 mμ. (A Thermal Syndicate mercury burner, Model T/M 5/369 E was used, with a Chance Brothers Silica Filter OX 7/19874.) All spots were dark against the fluorescent background of the paper. Under Wood's light (365 mμ.), di*iso*nicotinoylhydrazine alone fluoresced (orange). Only the isoniazid spots gave a blue colour when sprayed with a solution of KIO_3 and starch in dilute H_2SO_4. The following R_F values were found (in 0·5 M-NH_4Cl) by the ascending method:

*iso*Nicotinic acid	0·81
Isoniazid	0·73
Di*iso*nicotinoylhydrazine	0·68

The values were not changed when all three substances were present, nor when phosphate or aminotrishydroxymethylmethane (tris) buffers were used to dissolve them. Haemin prevented resolution and had to be removed by precipitation before the solution was applied to the paper. (When 2 N-NH_3 was used as a developer, haemin actually changed the relative position of the spots. These irregularities are attributed to its colloidal nature.)

Quantitative paper chromatography. A sample (5 ml.) of the reaction mixture was adjusted to pH 2 with 5 N-HCl (one small drop), and the haemin removed by centrifuging. A sample (0·06 ml.) was applied to each of three sheets of Whatman no. 1 filter paper (45 × 50 cm.) as a band about 9 cm. long, and standard solutions (0·06 ml.) of substances (I), (II) and (III) (see Table 1) were similarly applied along the same starting line, one to a paper. The paper was developed in 0·5 M-NH_4Cl until the front was 1 cm. from the top of the paper (5 hr.). The bands (now six in number) were quickly marked in their new positions under 254 mμ. light, and each standard band and the corresponding band of the reaction mixture were cut out. Three further bands of paper, of the same area, were removed at R_F values corresponding to each of the three substances, the eluates from these blank bands being used in the solvent cells during spectrophotometry. The nine strips of paper were eluted with water overnight (see Brimley & Barrett, 1953) and the eluates were made up to 5 ml. The ultraviolet absorption was then measured in a photoelectric spectrophotometer (see Table 1 for analytical wavelengths). The following recoveries were repeatedly obtained (±2%):

	%
*iso*Nicotinic acid	94
Isoniazid	72
Di*iso*nicotinoylhydrazine	92

It was found that isoniazid was not appreciably decomposed by the ultraviolet light used to locate it, nor was any lost by volatilization when drying the paper. Moreover, the same loss was obtained when water was used as developer. It was found that the principal loss was due to oxidation in air to *iso*nicotinic acid, usually a slow process in the absence of haemin (see Results), but accelerated here by the more efficient contact with the oxygen of the air. The quantitative changes were determined as follows. Mixtures of isoniazid (0·005 M) and di*iso*nicotinoylhydrazine (0·0025 M), and of isoniazid and *iso*nicotinic acid (0·005 M of each) were applied to a paper and developed in 0·5 M-NH_4Cl as before. The recoveries of (I) and (II) were as above, but that of *iso*nicotinic acid had increased from 94 to 105%. Henceforth, the factor 94/105 was applied to the apparent yields of *iso*nicotinic acid obtained from the reaction mixture (the isoniazid is automatically converted by comparison with the standard run at the same time). This enrichment of the bands of *iso*nicotinic acid at the expense of isoniazid must cease when the bands have become fully resolved during development, but the R_F values are so close that their separation takes several hours.

RESULTS

When isoniazid (0·01 M) was shaken in air at 17–20° with haemin (0·001 M) in phosphate buffer (pH 7·5), a rapid loss of isoniazid was found (Fig. 1), as shown by the diminishing consumption of iodine when samples were titrated. After 24 hr. shaking, samples of isoniazid, di*iso*nicotinoylhydrazine (II) and *iso*nicotinic acid were isolated in a pure condition (see 'Materials and Methods'). No other constituents could be found, though any binding of these pyridines to the haemin before acidification was not investigated. The use of tris buffer (pH 7·5) in place of phosphate led to similar results.

When the haemin was omitted, a small but definite loss of isoniazid occurred (Fig. 1), and the same products were formed, as was easily demonstrated when this reaction was accelerated by bubbling hot moist air through a solution of isoniazid at 85° and pH 7·5 for 20 hr. In an atmosphere of nitrogen, no reaction occurred (observed for 100 hr.). The use of 0·0001 M haemin led to a rate of destruction intermediate between that caused by 0·001 M haemin and by air alone (Fig. 1). Similarly, it was shown that (II) is oxidized to (III) rapidly in the presence of haemin and more slowly in its absence. The haemin-free oxidation of isoniazid is greatly accelerated in alkaline solutions.

The presence of 0·0001 M hydrogen peroxide (in the 0·001 M haemin experiment) removed what seems to be a small initial lag, whereas the addition of catalase slightly decreased the rate of oxidation (Fig. 2). The same products as before were obtained in both cases.

To find whether di*iso*nicotinoylhydrazine is an intermediate in the conversion of (I) into (III), or arises by a chemical reaction between (I) and (III), a solution of (I) and (III) (0·01 M of each) was made in phosphate buffer (pH 7·5) previously boiled for 5 min. and flushed with oxygen-free nitrogen. Samples were kept under nitrogen at 20°, and at 85°, respectively, for 42 hr. Paper chromatography failed to reveal the presence of di*iso*nicotinoylhydrazine. Hence it would seem that under

our experimental conditions this substance is not generated by interaction of the final product with the starting material, but is an actual intermediate. Haemin was not included in this experiment, because (II) is freely formed in its absence at 85° (see experiments conducted in air, above).

Finally a sample of the normal reaction mixture (isoniazid, 0·01 M; and haemin 0·001 M; 20° and pH 7·5) was submitted to quantitative paper chromatography after 24 hr. shaking. The results are given in Table 1. It is evident that after this time, half of the isoniazid is unchanged and the remainder has been converted into roughly equal amounts of di*iso*nicotinoylhydrazine and *iso*nicotinic acid.

DISCUSSION

Fisher (1954) discovered that haemin interferes with the action of isoniazid on the tubercle bacillus H37Rv, the activity being reduced 20 000-fold when the haemin:isoniazid molecular ratio is 1:2. He formed the opinion that no destruction of isoniazid by haemin occurred outside the bacillus.

However, it is evident from Fig. 1 and Table 1 that haemin acts on isoniazid in a simple phosphate buffer at pH 7·5. This reaction does not depend on the presence of phosphate, for it proceeds at about the same rate in tris buffer. It is an oxidative reaction, occurring readily in air, but not in nitrogen. Half of the isoniazid is destroyed in 24 hr. at 20° (Table 1). The reaction is catalytic, diminishing as the haemin is diminished, but still taking place, although slowly, in the absence of haemin (Fig. 1). In all cases the products formed are di*iso*nicotinoylhydrazine (II) and *iso*nicotinic acid (III), the former changing to the latter on further aeration (rapidly if haemin is present,

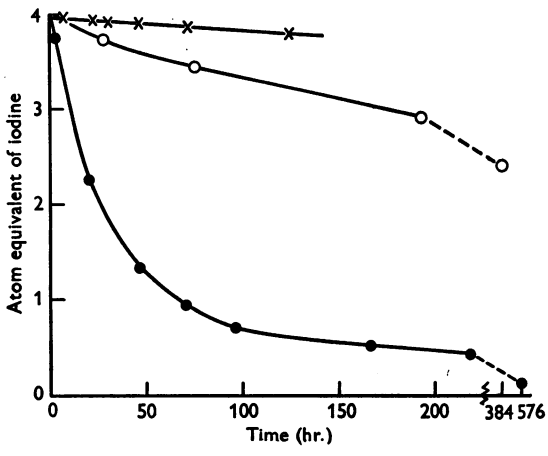


Fig. 1. Oxidative decomposition of 0·01 M isoniazid, alone ×; in the presence of 0·0001 M haemin ○; and in the presence of 0·001 M haemin ● (all in 0·05 M phosphate buffer at pH 7·5). The ordinate is the combined amount of isoniazid and di*iso*nicotinoylhydrazine remaining, expressed as atom equivalents of iodine as determined by titration. See Eqns. i and ii.

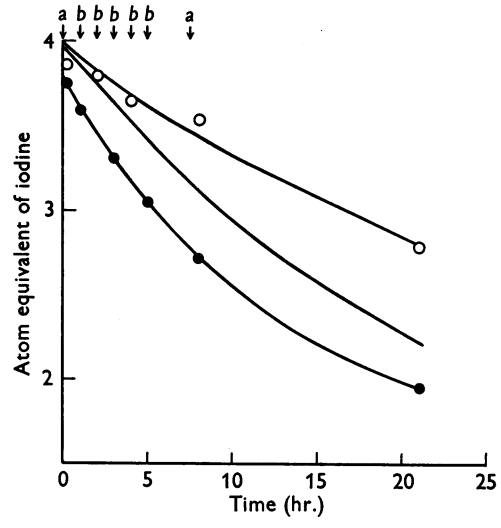


Fig. 2. Oxidative decomposition of 0·01 M isoniazid and 0·001 M haemin, alone, —— (taken from Fig. 1); with 0·0001 M-H_2O_2 added, ●; and with approx. 0·7% catalase added at the times indicated by the arrows, where $a = 0·5$ ml. and $b = 0·1$ ml., ○. (All were in 0·05 M phosphate buffer at pH 7·5.)

Table 1. *Chromatographic analysis (paper) of the solution obtained by shaking isoniazid* (0·01 M) *and haemin* (0·001 M) *in air at* 20° *and* pH 7·5 *for* 24 *hr.*

Substance	Analytical wavelength ($\lambda_{max.}$) in mμ.	$\log_{10} \epsilon$	Standard (eluate) Concentration (M)	Standard (eluate) Optical density	Recovery of standard (%)	Reaction solution (eluate) optical density	Yield* (%)
Isoniazid	262	3·647	0·005	0·185	72†	0·181	48·9
Di*iso*nicotinoylhydrazine	266	3·880	0·0025	0·198	90	0·114	28·8
*iso*Nicotinic acid	262	3·565	0·005	0·203	94	0·124	27·3‡
							105·0

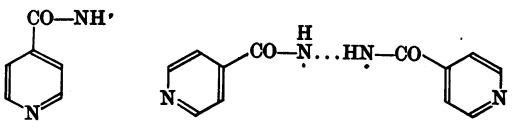
* With reference to isoniazid originally present.

† See 'Experimental' for discussion of this figure.

‡ Corrected by factor 94/105 as discussed in 'Experimental'.

slowly in its absence). It had already been shown that, in the usual tubercle bacillus culture media, isoniazid was slowly oxidized to *iso*nicotinic acid (Bönicke & Reif, 1953), and the presence of phosphate was believed necessary for this effect.

It was found that (II) does not arise from the chemical combination of (I) and (III) under the reaction conditions used here, and hence it must be an intermediate in the conversion of (I) into (III). (It is possible that there may be another route from (I) to (III) not passing through (II).) The symmetrical nature of (II), together with the probable participation of hydrogen peroxide in its formation (see below), suggests that it may be formed by a free-radical reaction, i.e. the combination of two equivalents of the supposed oxidation product (IV). Although free-radical reactions are not common in dilute aqueous solution, the reaction under consideration may be facilitated by the ease of hydrogen-bonding to a dimer (V), which could give di*iso*nicotinoylhydrazine by a simple rearrangement of bonds.



(IV) (V)

It is known that haemoproteins (e.g. methaemoglobin and cytochrome *c*) catalyse the oxidation of some heterocyclic substances by hydrogen peroxide (Albert & Falk, 1949), such oxidations producing further quantities of hydrogen peroxide. Hence an attempt was made to find if hydrogen peroxide played a part in the present reactions. It can be seen from Fig. 2 that the addition of a trace of hydrogen peroxide removed an apparent lag, whereas additions of catalase somewhat decreased the rate of oxidation. Thus it would appear that hydrogen peroxide as well as oxygen plays a part in the haemin-catalysed destruction of isoniazid.

The antagonistic action of haemin on isoniazid in the presence of tubercle bacilli probably takes the same form as in their absence, namely oxidative destruction of the isoniazid. Hence it is unnecessary to assume with Fisher (1954) that isoniazid injures mycobacteria by interfering with their porphyrin metabolism and that haemin reverses this interference because of its structural resemblance to these porphyrins.

Susceptible strains of the bacillus contain haemin, whereas resistant strains lack it (Fisher, 1954), so it is tempting to assume that the true toxic agent is not isoniazid but a substance generated by its oxidation. However, the known degradation products, di*iso*nicotinoylhydrazine (II) has only about one-tenth of the toxic action of isoniazid for this bacillus (Offe, Siefken & Domagk, 1952) and *iso*nicotinic acid is inactive. Isoniazid has been shown to have a high avidity for the cations of biologically important heavy metals (Albert, 1953), but our further studies have revealed no quantitative correlation between avidity for metals and antitubercular activity among hydrazides. Thus the mode of action of isoniazid remains unknown.

Since this work was done, we have seen a brief communication which claims (*a*) that the oxidation in air of isoniazid in the presence of haemin leads to (II) and (III), and (*b*) that the di*iso*nicotinoylhydrazine arises by the condensation of isoniazid with *iso*nicotinic acid (Krüger-Thiemer, 1955). We agree with (*a*), but believe that (*b*) is refuted by data presented here.

SUMMARY

1. Haemin destroys isoniazid by catalysing its oxidation in air at pH 7·5 even in the absence of tubercle bacilli. The oxidation takes place in the absence of haemin, but is slower.

2. The products are firstly, 1:2-di*iso*nicotinoylhydrazine and, after further oxidation, *iso*nicotinic acid.

3. An improved synthesis of di*iso*nicotinoylhydrazine is given.

4. Chemical aspects of the mode of action of isoniazid are discussed.

We wish to thank Professor Robert Knox for suggesting this problem, and Dr J. E. Falk for helpful discussions and the assay of haemin.

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