Metal-Catalysed Auto-oxidation of Isoniazid

BY F. G. WINDER AND JOAN M. DENNENY

Laboratories of the Medical Research Council of Ireland, Trinity College, Dublin

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Isoniazid (isonicotinic acid hydrazide) is stable in the solid state and in concentrated aqueous solutions. In dilute aqueous solutions, however, it breaks down under certain conditions. This breakdown has been shown to require oxygen (and hence may be described as an auto-oxidation) and certain metal ions, either free, such as Mn^{2+} , Cu^{2+} , Fe^{3+} , or combined as haemin; and to be accelerated by increase of pH (Pansy, Koerber, Stander & Donovick, 1953; Bönicke, 1954; Barry, Conalty, Denneny, Gaffney & Winder, 1954; Lewin & Hirsch, 1955; Krüger-Thiemer, 1955; Albert & Rees, 1955).

The breakdown catalysed by haemin has been studied in some detail (Krüger-Thiemer, 1955; Albert & Rees, 1955). The work in the present paper was designed to give further information on the auto-oxidation catalysed by Mn^{2+} and Cu^{2+} ions, both because of its importance in biological and biochemical studies with isoniazid and because these model systems throw some light on the metal-catalysed auto-oxidation of other biologically active substances.

EXPERIMENTAL

Determination of rate of isoniazid breakdown. The required solutions (buffer, metal-salt solutions, chelating agents, peroxidase etc.) were pipetted into 12 cm. $\times$ 1.5 cm. Pyrex test tubes with sufficient water to bring the total volume to 3 ml. Each treatment was usually set up in duplicate. A portion (1 ml.) of a solution of isoniazid or other hydrazide was then added, the tubes were shaken, 1 ml. samples were taken immediately for determination of isoniazid and the tubes incubated in a water bath at the required temperature. After the appropriate period the tubes were removed, rapidly cooled to room temperature and further 1 ml. samples taken for determination of isoniazid.

Velocity constants of isoniazid breakdown were calculated from the formula for a first-order reaction

$$k = \frac{2.3}{t} \log \frac{c_0}{c_t} \text{ (hr.}^{-1}\text{)},$$

where c_0 is the initial concentration of isoniazid and c_t is the concentration of isoniazid after t hr. These constants were normally calculated for a single reaction time (15 or 30 min. for rapid reactions, 60 or 120 min. when the reaction was slower). Isoniazid breakdown deviated considerably from a first-order reaction under many experimental conditions, but in the early stages of the reaction

the deviation was not great (Table 5), and these velocity constants were sufficiently accurate for the purpose of the comparisons made in this paper.

Estimation of isoniazid. Isoniazid solution (1 ml., containing 0.2 μ mole or less), 3 ml. of 2N- H_2SO_4 and 1 ml. of reagent (0.6 g. of dimethylaminobenzaldehyde in 20 ml. of ethanol plus 2 ml. of 10N-HCl) were pipetted into 12 cm. $\times$ 1.5 cm. Pyrex test tubes, which were capped with bubble-caps and placed in a boiling-water bath for 45 min. The solutions were cooled, made up to 6 ml. and the extinctions were read at 450 μ . Appropriate blank and standard determinations were carried out.

This method estimates hydrazine freed by hydrolysis, and hence estimates not only isoniazid but diisonicotinylhydrazine also. The latter gives 20% of the extinction given by an equimolecular amount of isoniazid.

Estimation of hydrogen peroxide. Hydrogen peroxide was estimated by a titanium sulphate method (Winder, 1956).

Products of isoniazid auto-oxidation. Isoniazid (20–200 mm) was shaken with 5 mM- CuSO_4 in 20 mM-borate buffer, pH 7.5, or with 5 mM- MnCl_2 in 10 mM-phosphate buffer, pH 7.5, in an open conical flask in a water bath at 37° for about 6 hr., the pH being readjusted to 7.5 at intervals with 5N-NaOH. Samples of the solutions were then spotted on Whatman no. 1 paper and developed by the ascending technique for 18 hr. at room temperature in propan-2-ol-water (80:20) or in the butanol phase of butanol-acetic acid-water (50:10:40). Isoniazid, isonicotinic acid, diisonicotinylhydrazine and isonicotinamide were identified by their R_F values compared with the authentic substances run as markers, and by the use of the spray reagents of Cuthbertson, Ireland & Wolff (1953). Isonicotinamide was eluted from larger-scale chromatograms and its absorption spectrum in 95% ethanol and in water compared with those of synthetic material. Diisonicotinylhydrazine showed an orange fluorescence on excitation with ultraviolet light of λ 366 μ and gave a pale yellow with cyanogen bromide and benzidine. These helped to distinguish this substance from isonicotinamide, with which its spot on the chromatogram partly overlapped.

Polymerization of styrene. Borate buffer (4 ml.; 0.2M; pH 8.0), 4 ml. of 20 mM- CuSO_4 , 4 ml. of styrene and 4 ml. of 100 mM-isoniazid were pipetted into a 100 ml. conical flask, which was then plugged with cotton wool and shaken at 37° for 3 hr. Similar mixtures, but with the copper or the isoniazid omitted, were also shaken. After incubation, 1 ml. of conc. HCl was added to each flask to dissolve any precipitated copper salts. The contents of the flasks were well shaken, transferred to centrifuge tubes and centrifuged at 3000 g for 10 min. A thin layer of white polymer-coated styrene droplets was observed between the water and styrene layers from the flask containing isoniazid and copper, but not from the flasks containing isoniazid or

copper alone. Because of difficulties introduced by evaporation of the styrene, satisfactory quantitative estimates of the amount of polymer formed were not obtained.

Effect of isoniazid auto-oxidation on aromatic compounds. Borate buffer (1 ml.; 0.1M; pH 7.5) 1 ml. of 10 mM-substrate, 1 ml. of mM-isoniazid and 1 ml. of 0.2 mM-CuSO₄ were pipetted into a 25 ml. conical flask, which was shaken at 37°. Similar mixtures without isoniazid were also incubated. Absorption curves were obtained after various intervals. Substrates used were phenol, cresol, catechol and aniline. Benzene was also used as a substrate, but with ten times the above-mentioned concentrations of isoniazid and copper.

Materials. *iso*Nicotinamide and diisonicotinylhydrazine were prepared by Dr Peter Mitchell, of these Laboratories, by the methods of Ainsworth (1954) and Albert & Rees (1955) respectively. The hydrazides of acids other than isonicotinic acid were also gifts from Dr Mitchell. Isoniazid was obtained from the Nepera Chemical Co., and isonicotinic acid from L. Light and Co. Ltd.

Peroxidase (horse-radish, P.Z. 170) and catalase (ox-liver, crude) were purchased from Nutritional Biochemicals Corp. Haemin was obtained from L. Light and Co. Ltd.

Styrene (L. Light and Co. Ltd.) was washed with water and distilled. Benzene was purified by shaking with conc. H₂SO₄ for several hours, leaving with metallic sodium for 18 hr. and distilling.

The other chemicals used were of A.R. quality. Manganese was used as MnCl₂, copper as CuSO₄ and iron as FeCl₃.

No special precautions were taken to eliminate trace metals. Glass-distilled water was used. Glassware was cleaned in chromic acid and thoroughly rinsed in tap water and distilled water.

Buffers were prepared by dissolving potassium dihydrogen phosphate, sodium bicarbonate, boric acid, sodium diethylbarbiturate, glycine, sodium citrate or 2-amino-2-hydroxymethylpropane-1:3-diol (tris) in water, adjusting to the required pH with 10N-NaOH or 10N-HCl, making up to the required volume and re-checking the pH.

All pH adjustments were made with a Radiometer pH meter 21, and extinction readings were made with a Beckman model DU spectrophotometer.

RESULTS

Table 1 shows the effect of trace amounts of Mn²⁺, Cu²⁺, Fe³⁺ ions and haemin on the auto-oxidation

of isoniazid in various buffers, pH 7.5, at 37°. Slow and variable oxidation took place in some of the buffers in the absence of added metals, which was presumably due to traces of metals in the solutions, and could be completely eliminated by the use of chelating agents. The catalytic effect of Cu²⁺ and Mn²⁺ ions was strong in some buffers and almost absent in others. The effect of Mn²⁺ ions was detectable at a concentration as low as 0.05 μM in 25 mM-phosphate buffer.

There was considerable variation in oxidation rates under apparently similar conditions from experiment to experiment, as may be seen by comparing the results in the different tables, but agreement between replicate determinations in the same experiment was good, and the pattern of results within an experiment was always reproducible. The causes of inter-experimental variation have not been discovered.

Manganese dioxide and Cu²⁺ ion oxidized isoniazid in the absence of oxygen in phosphate buffer, pH 7.5, at 37°, becoming reduced to Mn²⁺ and Cu⁺ ions. This may therefore be assumed to take place in the auto-oxidation of dilute solutions of isoniazid in the presence of traces of these metals. Cu⁺ ion is reoxidized by oxygen to Cu²⁺, explaining the catalytic effect. On the other hand, Mn²⁺ ion is not appreciably oxidized to MnO₂ by oxygen under the above conditions. Small traces of MnO₂ may, however, be formed and these may be sufficient to explain the catalytic effect. It is possible, on the other hand, that oxygen reacts with a metal-isoniazid chelate, and that oxidation of the isoniazid takes place by electron transfer within the complex without a change in valency of the free metal ions.

Co²⁺, Ni²⁺, Zn²⁺, Pb²⁺, Al³⁺ and molybdate ions had no appreciable effect on isoniazid in phosphate buffer, pH 7.5, at 37°.

Effect of pH. The rate of auto-oxidation of isoniazid in the presence of Cu²⁺ or Mn²⁺ ions in 25 mM-phosphate buffer was increased by raising

Table 1. *Effect of metals on the velocity constant of the auto-oxidation of isoniazid in various buffers*

A total volume of 4 ml. was incubated at 37° in open unshaken tubes. Isoniazid was initially present at 0.2 mM, and the buffers and metals were as given in the table. pH was 7.5. Isoniazid was determined in samples taken at 0 and 120 min. when haemin was present, and at 0 and 30 min. in the other cases, and the first-order velocity constant of its disappearance was calculated.

Buffer	Metal catalyst			
	0.045 mM-Mn ²⁺ <i>k</i> (hr. ⁻¹)	0.045 mM-Cu ²⁺ <i>k</i> (hr. ⁻¹)	0.045 mM-Fe ³⁺ <i>k</i> (hr. ⁻¹)	0.045 mM-Haemin <i>k</i> (hr. ⁻¹)
25 mM-Phosphate	1.2	0.29	0.01	0.46
5 mM-Phosphate	1.8	0.06	0.06	0.43
25 mM-Bicarbonate	2.9	1.7	0.10	0.57
25 mM-Borate	0.03	3.4	0.06	0.43
5 mM-Veronal	0.00	2.5	0.03	0.29
25 mM-Glycine	0.09	0.00	0.05	0.31
25 mM-Citrate	0.02	0.00	—	0.44
25 mM-Tris	0.09	0.02	—	0.26

the pH over the range studied (Table 2). Oxidation by Mn^{2+} ion was more strongly affected by pH changes than oxidation by Cu^{2+} ion.

Effect of temperature. Over the range 30–40° a 5° increase in temperature resulted in a 1.6-fold increase in rate of auto-oxidation of isoniazid, whether this was catalysed by Cu^{2+} or Mn^{2+} ions.

Effect of chelating agents. The effects of a number of chelating agents on the Mn^{2+} -catalysed auto-oxidation of isoniazid are shown in Table 3. Ethylenediaminetetra-acetate, diethyldithiocarbamate and $\alpha\alpha'$ -dipyridyl inhibited the oxidation when present at concentrations higher than equimolar to that of the Mn^{2+} ions, but they stimulated when present at lower concentrations. These facts can be explained most simply on the assumption that formation of 1:1 complexes with these agents increased the effectiveness of Mn^{2+} ion as a catalyst under the conditions of this experiment, whereas the formation of 1:2 complexes inactivated the Mn^{2+} ion.

o-Phenanthroline and 8-hydroxyquinoline showed rather different behaviour. The former inhibited the auto-oxidation at a molar concentration one-tenth of that of the Mn^{2+} ; the latter did not inhibit until present at a concentration more than twice that of the Mn^{2+} ion.

Effect of hydrogen peroxide. Hydrogen peroxide markedly inhibited the auto-oxidation catalysed by Mn^{2+} ion (Table 4). Presumably the hydrogen peroxide reduced the oxidized form of the catalyst (MnO_2 or its equivalent), so that less was available for the oxidation of isoniazid. Conversely, catalase stimulated this auto-oxidation by removing hydrogen peroxide, which was normally a reaction product.

On the other hand, hydrogen peroxide strongly stimulated auto-oxidation of isoniazid catalysed by Cu^{2+} ion. In this case hydrogen peroxide did not react with the oxidized form of the catalyst, but presumably oxidized the reduced form and thereby made more Cu^{2+} ion available for isoniazid oxidation. The reaction of Cu^+ ion with hydrogen peroxide probably produced free OH radicals (Haber & Weiss, 1934; Baxendale, Evans & Park, 1946; Waters, 1945), which may also oxidize isoniazid.

Effect of concentration of isoniazid. The auto-oxidation of isoniazid catalysed by Mn^{2+} or Cu^{2+} ions followed a first-order course under certain conditions, and hence unimolecular velocity 'constants' have been calculated throughout this paper. However, the 'constant' when Mn^{2+} ion was catalyst in fact decreased during the course of the reaction, particularly towards the end of the reaction, and with lower concentrations of isoniazid (Table 5). One cause of the decrease of the reaction 'constant' during the reaction was the

Table 2. *Effect of pH on the velocity constant of the auto-oxidation of isoniazid in phosphate buffer*

A total volume of 4 ml. was incubated at 37° in open unshaken tubes. Concentrations were: isoniazid, 0.2 mM; phosphate, 25 mM. Concentration of the metal ions are given in the table. Isoniazid was determined in samples taken at 0 and 30 min. and the first-order velocity constant of its disappearance was calculated.

pH	No metal catalyst k (hr. ⁻¹)	0.045 mM- Cu^{2+} k (hr. ⁻¹)	0.045 mM- Mn^{2+} k (hr. ⁻¹)
7.5	0.005	0.18	1.2
7.0	0.002	0.14	0.60
6.5	0.000	0.11	0.18
6.0	0.000	0.05	0.11
5.5	0.000	0.02	0.002

Table 3. *Effect of chelating agents on the velocity constant of auto-oxidation of isoniazid in phosphate buffer catalysed by Mn^{2+} ion*

A total volume of 4 ml. was incubated at 37° in open unshaken tubes. Concentrations were: isoniazid, 0.2 mM; Mn^{2+} , 0.045 mM; phosphate, 25 mM; pH 7.5. Isoniazid was determined in samples taken at 0 and 30 min., and the first-order velocity constant of its disappearance was calculated.

Additions	k (hr. ⁻¹)
Control	1.08
0.003 mM-Ethylenediaminetetra-acetate	2.18
0.09 mM-Ethylenediaminetetra-acetate	0.05
0.008 mM-Diethyldithiocarbamate	1.96
0.09 mM-Diethyldithiocarbamate	0.06
0.008 mM- $\alpha\alpha'$ -Dipyridyl	1.75
0.09 mM- $\alpha\alpha'$ -Dipyridyl	0.06
0.006 mM- <i>o</i> -Phenanthroline	0.74
0.09 mM- <i>o</i> -Phenanthroline	0.62
0.017 mM-8-Hydroxyquinoline	2.28
0.09 mM-8-Hydroxyquinoline	1.77
0.20 mM-8-Hydroxyquinoline	0.12

Table 4. *Effect of hydrogen peroxide and of catalase on the velocity constant of isoniazid auto-oxidation in phosphate buffer*

A total volume of 4 ml. was incubated at 37° in open unshaken tubes. Concentrations were: isoniazid, 0.2 mM; Cu^{2+} , 0.045 mM; Mn^{2+} , 0.045 mM; phosphate, 25 mM; H_2O_2 , 0.37 mM; crude catalase, 12.5 μ g./ml.; pH 7.5. Samples were taken at 0 and 15 min., and the first-order velocity constant of isoniazid disappearance was calculated.

Additions	k (hr. ⁻¹)
Cu^{2+}	0.21
Cu^{2+} + H_2O_2	3.00
Cu^{2+} + catalase	0.12
Mn^{2+}	2.42
Mn^{2+} + H_2O_2	0.24
Mn^{2+} + catalase	3.86
H_2O_2	0.04
Catalase	0.00

accumulation of hydrogen peroxide, which inhibited the Mn^{2+} -catalysed auto-oxidation. In addition, the metal ions may become converted into an inactive form.

When the initial concentration of isoniazid was increased (with constant concentration of metal ion), the amount of isoniazid destroyed in a short time increased also, though not exactly proportionately to the initial concentration of isoniazid (Table 6). Up to a certain concentration of isoniazid (which depended on the metal catalyst and on the complexing agent present) the amount destroyed increased slightly more rapidly than the initial concentration. Above this concentration the amount destroyed increased considerably more slowly. These results suggest that at low concentrations of isoniazid the metal ions do not attain their full activity, and that at high concentrations of isoniazid the metal-ion catalyst tends towards 'saturation' and the reaction towards zero order. This last-named effect may be due to the rate of re-oxidation of the metal ions limiting the rate of the reaction, or to formation of a more stable chelate when the ratio of isoniazid to metal is high.

Products of auto-oxidation of isoniazid. The auto-oxidation products were studied in solutions more concentrated than those used for the other studies, and hence the results may not be exactly comparable. The previously recorded products of isoniazid auto-oxidation, *isonicotinic acid* and *diisonicotinyl hydrazine* (Krüger-Thiemer, 1955; Albert & Rees 1955) were found when either Mn^{2+} or Cu^{2+} ion was the catalyst. However, particularly with Mn^{2+} ion as catalyst, substantial quantities of a third substance were found. This was identified as *isonicotinamide* by chromatography against synthetic *isonicotinamide* in propan-2-ol-water and in butanol-acetic acid-water, by the blue colour of its spot with cyanogen bromide and benzidine and by its absorption spectrum after elution from chromatograms (Table 7).

The formation of *isonicotinamide* was presumably due to reduction of isoniazid or an intermediate oxidation product [perhaps the radical 4-*isonicotinamido* ($4-NC_5H_4 \cdot CO \cdot NH \cdot$) suggested by Albert & Rees (1955)] by the reduced form of the metal-ion catalyst.

During isoniazid auto-oxidation catalysed by Mn^{2+} ion, hydrogen peroxide accumulated in amounts rather more than equimolar to those of the isoniazid destroyed. The reactions involved in the auto-oxidation may be assumed to be something as follows:

Table 5. *Change in the first-order velocity constant during the course of the auto-oxidation of isoniazid in phosphate buffer catalysed by Mn^{2+} ion*

A total volume of 4 ml. was incubated at 37° in open unshaken tubes. Concentrations were: Mn^{2+} , 0.045 mM; phosphate, 25 mM; isoniazid as indicated in the table; pH 7.5. Isoniazid was determined in samples taken at 0 min. and at the indicated times, and first-order velocity constants of isoniazid disappearance were calculated for each successive interval, i.e. 0–15 min.; 15–30 min., etc.

Time from start of reaction (min.)	Initial concn. of isoniazid	
	1.8 mM k (hr. ⁻¹)	0.18 mM k (hr. ⁻¹)
15	0.27	2.3
30	0.43	1.6
60	0.40	1.2
120	0.32	0.54
240	0.22	0.33

Table 6. *Effect of the concentration of isoniazid on its auto-oxidation in phosphate buffer*

A total volume of 4 ml. was incubated at 37° in open unshaken tubes. Concentrations were: Mn^{2+} , 0.045 mM; Cu^{2+} , 0.045 mM; ethylenediaminetetra-acetate (EDTA), 0.003 mM; phosphate, 25 mM; pH 7.5.

Initial concn. of isoniazid (μmoles/l.)	Catalyst		
	Mn^{2+}	Mn^{2+} + EDTA	Cu^{2+}
	Incubation period (min.)		
	30	30	60
Isoniazid destroyed (μmoles/l.)			
18.2	—	15.3	1.8
45.6	17.2	39.4	5.4
91.3	36.5	77.4	12.1
182	88.4	136	30.9
365	169	—	80.1
913	192	—	136

Table 7. *Absorption maxima of isonicotinamide eluted from the chromatogram*

	Absorption maximum (mμ)	
	95% Ethanol	Water
Isoniazid	264	263
<i>iso</i> Nicotinic acid	272	263
<i>Diisonicotinylhydrazine</i>	263	266
<i>iso</i> Nicotinamide	270	268
' <i>iso</i> Nicotinamide' from auto-oxidation	270	268

The exact amount of hydrogen peroxide formed thus depends on the amounts of the different breakdown products of isoniazid. Hydrogen peroxide accumulated in this system because Mn^{2+} ion did not reduce hydrogen peroxide under the conditions of our experiments, though under other conditions Mn^{2+} ion can transfer single electrons to hydrogen peroxide to form free OH radicals (Baxendale *et al.* 1946; Waters, 1945).

Peroxide did not accumulate when Cu^{2+} ion was the auto-oxidation catalyst. It has been mentioned already that Cu^{2+} ion probably accepts single electrons from hydrogen peroxide to form free OH radicals in this system, as has been shown to take place under other conditions (Baxendale *et al.* 1946; Waters, 1945). It is probable that the reoxidation of Cu^+ by oxygen takes place by a succession of single electron transfers with successive formation of HO_2^* , H_2O_2 , HO^* and water (Weiss, 1958). The formation of free radicals during the Cu^{2+} -catalysed auto-oxidation of isoniazid is supported by the fact that this system stimulates polymerization of styrene. Further, the isoniazid- Cu^{2+} -oxygen system gave coloured reaction products on incubation with benzene, aniline and monohydroxy- and dihydroxy-phenols, whereas the Cu^{2+} -oxygen system produced much less colour or none. This suggests formation of OH radicals, which attacked the aromatic rings, but the presence of isoniazid in the system introduced a certain ambiguity into the results. Short-lived reaction intermediates, such as the free radicals $4\text{-NC}_5\text{H}_4\cdot\text{CO}\cdot\text{NH}^*$ or $4\text{-NC}_5\text{H}_4\cdot\text{CO}^*$, may be formed from isoniazid itself and these may be responsible for some of the effects. This ambiguity can be removed only by further characterization of the reaction products. The isoniazid- Cu^{2+} -oxygen system also irreversibly decolorized certain dyes, e.g. methylene blue, Evans blue and crystal violet.

Effect of peroxidase on isoniazid. It has been claimed that peroxidase oxidizes isoniazid (Krüger-Thierner, 1958; Tirunaryanan & Vischer, 1959). The results in Table 8, however, show that peroxidase had little effect on isoniazid, even when present at very high concentrations. Old solutions of peroxidase had more effect, which may be due to the liberation of some free haem derivatives from the peroxidase, and the results of the above-mentioned workers may be explicable along these lines.

When hydrogen peroxide or phenol was added to the peroxidase-isoniazid system little effect on the isoniazid was observed, but when both were added together the isoniazid was rapidly destroyed. Hence it would appear that peroxidase does not oxidize isoniazid to isonicotinic acid, as has been suggested (Krüger-Thierner, 1958), but that quinone intermediates in the oxidation of phenols

by peroxidase react with isoniazid. For example, when peroxidase, quinol, hydrogen peroxide and isoniazid were incubated together a dark-brown insoluble product resulted, similar to that obtained when isoniazid was incubated under the same conditions with *p*-quinone. The product of both reactions on chromatography in propan-2-ol-water gave the same spot, with R_f 0.88, which gave a pink some hours after exposure to cyanogen bromide and benzidine.

Peroxidase plus phenol increased the rate of oxidation of isoniazid by Mn^{2+} ion, presumably because the hydrogen peroxide formed in the Mn^{2+} -catalysed oxidation was used by the peroxidase-phenol system for the removal of further isoniazid. The reaction rate is apparently increased four times, but first-order velocity constants give a false picture in this case. The figures for the amount of isoniazid destroyed per unit time are in keeping with the formation of rather more than one molecule of hydrogen peroxide for each molecule of isoniazid auto-oxidized, and the use of each molecule of hydrogen peroxide for the destruction of a further molecule of isoniazid by peroxidase-phenol.

Hydrogen peroxide formed in this way is more rapid and efficient in the destruction of isoniazid than is hydrogen peroxide added in bulk. Addition of hydrogen peroxide actually slows down the destruction of isoniazid by Mn^{2+} -peroxidase-phenol. Excess of hydrogen peroxide either partially inhibited the peroxidase action, or changed the nature or amount of the quinone

Table 8. *Effect of peroxidase on isoniazid*

A total volume of 4 ml. was incubated at 37° in open unshaken tubes. Concentrations were: isoniazid, 0.2 mM; phosphate, 25 mM; H_2O_2 , 0.4 mM; phenol, 0.5 mM; Mn^{2+} , 0.045 mM; NaN_3 , 0.4 mM; peroxidase, 5 $\mu\text{g./ml.}$ except where specified otherwise; crude catalase, 25 $\mu\text{g./ml.}$; pH 7.5. Isoniazid was determined in samples taken at 0 and 15 min., and first-order velocity constants of isoniazid disappearance were calculated.

Additions	k (hr. ⁻¹)
Control	0
Peroxidase	0
Phenol	0
Peroxidase + phenol	0.15
Peroxidase + H_2O_2	0.10
Peroxidase + phenol + H_2O_2	3.0
Peroxidase + phenol + H_2O_2 + NaN_3	3.9
Peroxidase + phenol + H_2O_2 + catalase	2.0
Mn^{2+}	2.6
Mn^{2+} + peroxidase + phenol	10.0
Mn^{2+} + peroxidase + phenol + NaN_3	14.0
Mn^{2+} + peroxidase + phenol + catalase	12.0
Peroxidase (50 $\mu\text{g./ml.}$)	0.06
Peroxidase (50 $\mu\text{g./ml.}$; stored for 4 months in solution)	0.20

Table 9. *Comparison of the velocity constants of the auto-oxidation of hydrazides with their growth-inhibitory activity for Mycobacterium tuberculosis*

A total volume of 4 ml. was incubated at 37° in open unshaken tubes. The hydrazides were initially present at 0.2 mM. pH 7.5. Samples were taken at 0 and 120 min. for haemin, at 0 and 30 min. for the others, and the first-order velocity constants of the disappearance of the hydrazides were calculated. (a) Bernstein, Lott, Steinberg & Yale (1952) and Bernstein *et al.* (1953): Ravenel strain in modified Kirchner medium. (b) R. Knox (Albert, 1956): H 37 Rv strain in semi-solid Dubos medium. (c) Winder & Denny: BCG strain in Proskauer & Beck medium with 2.5% of serum.

Hydrazide of	0.045 mM-Mn ²⁺ 25 mM-phosphate	0.045 mM-Cu ²⁺ 5 mM-veronal	0.01 mM-Haemin 25 mM-phosphate	Minimum inhibitory concn. for <i>M. tuberculosis</i> (μg./ml.)		
	<i>k</i> (hr. ⁻¹)	<i>k</i> (hr. ⁻¹)	<i>k</i> (hr. ⁻¹)	(a)	(b)	(c)
isoNicotinic acid	1.5	2.4	0.013	0.02	0.025	0.03
Picolinic acid	—	2.2	0.079	0.15	1.5	1.5
Nicotinic acid	—	3.0	0.011	2.0	25.0	> 10.0
Furoic acid	—	3.5	0.036	0.06	—	1.0
Thiophencarboxylic acid	2.2	9.0	0.022	8.0	—	1.0
Benzoic acid	1.6	4.6	0.092	3.0	12.5	> 100
o-Chlorobenzoic acid	—	2.5	0.005	—	—	> 100
p-Hydroxybenzoic acid	0.25	15.5	0.18	12.0	—	—
p-Aminobenzoic acid	—	6.9	0.13	6.0	—	—
Pyrrolidonecarboxylic acid	0.53	1.9	0.009	—	—	> 100
Maleic acid	—	10.0	0.31	—	—	—
Cyanacetic acid	1.0	1.0	0.23	—	3.0	> 100

products formed. The stimulation by subinhibitory levels of azide of destruction of isoniazid by these systems also requires explanation. These levels of azide may modify the products of peroxidase action on phenols.

Catalase inhibited the destruction of isoniazid by peroxidase-phenol-peroxide, for obvious reasons. However, it increased the destructive effect of peroxidase-phenol-Mn²⁺.

Auto-oxidation of some other hydrazides. The auto-oxidation of a number of other hydrazides was also studied. Although all of those investigated were auto-oxidized, the rates varied considerably, as did the effects of the different catalysts (Table 9). The series was too limited to permit generalizations on the effect of structure on the rate of auto-oxidation. No correlation was observed between activity *in vitro* against *Mycobacterium tuberculosis* and auto-oxidation rate.

DISCUSSION

The observations in this paper show that isoniazid and other hydrazides may be quite rapidly auto-oxidized under conditions prevailing in bacterial and enzyme systems. Thus at pH 7.5 and 37°, with 2 μg. of Mn²⁺ ion/ml. present, isoniazid (100 μg./ml.) may be reduced to half its concentration in less than 30 min. The oxidation rate is governed by the metal ions present, the pH and the anions present, and may also be affected by chelating agents and by hydrogen peroxide. Hence, studies on the effect of isoniazid on bacterial and biochemical systems

should be accompanied by some examination of the rate of breakdown of isoniazid under the exact conditions used, both because it will affect the concentration of isoniazid present and because the products of auto-oxidation may be responsible for some of the effects attributed to isoniazid itself.

Suggestions have been made that breakdown of isoniazid is involved in its action on *Mycobacterium tuberculosis* (Albert & Rees, 1955; Winder, 1956, 1959; Krüger-Thiemer, 1958). However, the evidence in favour of these hypotheses is indirect, and there are a number of difficulties in their way. First, no correlation has been found between the auto-oxidation rates of hydrazides and their activity against *M. tuberculosis in vitro*, so that other properties of the molecules must be assumed to play a large part in determining their activity. Secondly, the stable products of isoniazid auto-oxidation (isonicotinic acid, diisonicotinyl hydrazine, isonicotinamide and hydrogen peroxide) are much less toxic for *M. tuberculosis* than is isoniazid, although, as has been pointed out (Winder, 1956; Krüger-Thiemer, 1958), their toxicity might be considerably greater if they are formed within the mycobacterial cell.

However, in addition to the above-mentioned products, the isoniazid-Cu²⁺-oxygen and isoniazid-haemin-oxygen systems form free radicals under suitable conditions. These radicals may be of two types: radicals derived from the reduction of oxygen, such as HO·, and the reaction intermediates derived from the oxidation of isoniazid, which may be short-lived free radicals such as

4-NC₅H₄·CO·NH⁺ or 4-NC₅H₄·CO⁺. The former type of radicals show oxidizing and hydroxylating activity and the latter would be expected to attack certain chemical groupings. Therefore, in the presence of isoniazid and oxygen, Cu²⁺ ion or haem derivatives in the cell may lead to chemical attacks on other cell components. A hypothesis that this is responsible for the action of isoniazid on *M. tuberculosis* is outlined elsewhere (Winder, 1959).

Many other readily oxidizable substances have chemical and biological effects which may be attributed to their auto-oxidation. For example, the metal-catalysed auto-oxidation of ascorbic acid oxidizes fatty acids (Geyer, Kydd & Ryan, 1957) and benzene (Ekman, 1944), destroys vitamin B₁₂ (Rosenberg, 1956) and inactivates β-glucuronidase (Levy & Marsh, 1957). Ascorbic acid is toxic to a wide range of organisms (Lwoff & Morel, 1942*a, b*; Strangeways, 1937), particularly when Cu²⁺ ion and hydrogen peroxide are added (Ericsson & Lundbeck, 1955), which would increase HO⁺ formation. The toxicity of certain sulphur compounds is probably due to their auto-oxidation (Woiwod, 1954; Taylor, 1957). These chemical and biological effects of auto-oxidation are presumably due to the formation of free radicals and other reaction intermediates.

Certain of the biological effects of metal ions such as Cu²⁺ and Fe³⁺ appears to be due to their ability to catalyse auto-oxidation. These two metals in particular increase the toxicity of a number of chelating agents for many micro-organisms (Sorkin, Roth & Erlenmeyer, 1952; Albert, Rees & Tomlinson, 1956; White, 1955). The chelating agents may facilitate auto-oxidation by these ions, or may themselves be auto-oxidized, or may facilitate penetration of the ions into the organisms where oxidation may be more destructive.

Characteristically, many auto-oxidizable substances are toxic under certain conditions and growth-promoters under others. If these substances can produce free radicals or other toxic auto-oxidation intermediates, and yet are capable of destroying free radicals produced otherwise (e.g. by ionizing radiation), this situation can be understood. However, interpretation of the results in any instance is difficult. Whether such a substance will function as a net producer or destroyer of free radicals will depend on many factors: the concentration of radicals produced otherwise, the concentration of the auto-oxidizable substance, the oxygen concentration, the metal ions present, the pH, the temperature, etc. This is also probably the explanation of why some of these substances may function either as mutagenic or anti-mutagenic agents.

SUMMARY

1. Mn²⁺ and Cu²⁺ ions were the most active catalysts found for isoniazid auto-oxidation at pH 7.5 and 37°. The velocity constant of the oxidation of 0.2 mM-isoniazid was 2.9 hr.⁻¹ when catalysed by 0.045 mM-Mn²⁺ in bicarbonate buffer, and 3.4 hr.⁻¹ by 0.045 mM-Cu²⁺ in borate buffer. Mn²⁺ ion was active down to 0.05 μM.

2. The activity of the catalysts depended on the buffer. Mn²⁺ ion was most active in phosphate or bicarbonate and Cu²⁺ ion in bicarbonate, borate or veronal.

3. Certain chelating agents increased the catalytic activity of Mn²⁺ ion when they were present at a lower molar concentration than it, but inhibited its activity at higher concentrations.

4. Hydrogen peroxide inhibited isoniazid auto-oxidation catalysed by Mn²⁺ ion, but stimulated that catalysed by Cu²⁺ ion.

5. Isoniazid auto-oxidation followed an approximately unimolecular course under certain conditions.

6. Hydrazides of a number of other acids also auto-oxidized.

7. *iso*Nicotinic acid, diisonicotinylhydrazine and isonicotinamide were products of auto-oxidation of isoniazid catalysed by either metal. Hydrogen peroxide resulted from auto-oxidation catalysed by Mn²⁺ ion. Free radicals, probably OH⁺ and radicals derived from isoniazid, were formed during auto-oxidation catalysed by Cu²⁺ ion.

8. Peroxidase did not affect isoniazid unless hydrogen peroxide and a phenol were present, when condensation products derived from isoniazid and the phenol were formed.

9. It is suggested that free-radical formation during auto-oxidation is a major factor in the toxicity of isoniazid and other auto-oxidizable substances.

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The Trisaccharide Fraction of Some Monocotyledons

By J. S. D. BACON

Department of Biochemistry, The Macaulay Institute for Soil Research, Craigiebuckler, Aberdeen

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In those plants which accumulate fructosans (chiefly members of the Compositae and Campanulaceae, and the monocotyledons), oligosaccharide fractions of a similar character are always present. Relatively little is yet known about these fractions, despite the increased attention which has been given in recent years to the structure of the polysaccharides.

Dedonder (1952) and Bacon & Edelman (1951) suggested that the trisaccharide fraction of the tubers of *Helianthus tuberosus* contained *O*- α -D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*- β -D-fructofuranosyl-(1 $\rightarrow$ 2) β -fructofuranoside (1^F- β -fructosylsucrose). Later work (Allen & Bacon, 1956) confirmed this, and also demonstrated the virtual absence of other trisaccharides. In unpublished work, the author has recently shown that the rampion (*Campanula rapunculus* L.) resembles the composite in this respect.

The use of paper chromatography has shown the presence of fructose-containing oligosaccharides in various monocotyledons: for example, in the leaves and stems of wheat (Bradfield & Flood, 1950; Lopatecki, Longair & Farstad, 1957), in onion bulbs (Egyptian 'Silvers'; Dr J. Edelman, personal communication, 1951), in leaves and stems of barley (Porter & Edelman, 1952) and in the seeds of cereals (MacLeod, 1951; White & Secor, 1953), but in very few cases have the substances been identified. Schlubach, Lübbers & Borowski (1955) have reported that *O*- α -D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*- β -D-fructofuranosyl-(6 $\rightarrow$ 2) β -D-fructofuranoside

(6^F- β -fructosylsucrose; 'kestose') is the main trisaccharide of perennial ryegrass (*Lolium perenne* L.).

It seemed that the use of chromatographic methods which have permitted the isolation of the products of fructose transfer by invertases (Edelman, 1956; Bacon, 1954) might help to characterize the trisaccharide fraction of monocotyledons. These trisaccharides are of interest not only because they serve as substrates for digestive enzymes, and for microbial attack above and below the ground, but also because their structures may act as pointers to the mechanism of fructosan biosynthesis (cf. Feingold, Avigad & Hestrin, 1956).

In the experiments reported here these methods were applied to the onion, *Allium cepa* L. (a preliminary report has appeared: Bacon, 1957), the leek, *A. porrum* L., and two grasses: Italian ryegrass (*Lolium multiflorum* Lam.) and tall oatgrass (*Arrhenatherum elatius* L., J. and C. Presl, var. *bulbosum*, 'onion couch').

Two trisaccharides, 1^F- β -fructosylsucrose and *O*- β -D-fructofuranosyl-(2 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 2) β -D-fructofuranoside (6^a- β -fructosylsucrose) have been identified in all four species; 6^F- β -fructosylsucrose is probably present, but in smaller amounts, in both grasses.

MATERIALS

Onions and leeks. These were grown in 1956-57 in the gardens of this Institute, the varieties of onion being 'Crosslings' and 'Golden Mammoth', and of leek 'Scottish