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Microbial transformation of isonicotinic acid hydrazide and isonicotinic acid by *Sarcina* sp*

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Abstract. Metabolism of isonicotinic acid and isoniazid by *Sarcina* sp. led to the formation of two metabolites which were characterised as 2-hydroxyisonicotinic acid and citrazinic acid. The blue pigment formed during fermentation was shown to be derived from the auto-oxidation of citrazinic acid. 2-Oxo-glutarate accumulated as the major keto acid when isonicotinic acid or isonicotinic acid hydrazide metabolism was inhibited by 1 mM sodium arsenite. Isonicotinic acid, 2-hydroxyisonicotinic acid and 2-oxo-glutarate were oxidised by isonicotinic acid hydrazide or isonicotinic acid-grown cells; citrazinic acid was, however, not oxidised. Isoniazid hydrazine hydrolase, isonicotinic acid and 2-hydroxyisonicotinic acid hydroxylases were detected in the cell-free extract of *Sarcina* sp. grown on isonicotinic acid hydrazide or isonicotinic acid.

Keywords. Microbial transformation; isoniazid; isonicotinic acid; *Sarcina*.

Introduction

Metabolism of isonicotinic acid (INA) and N-methylisonicotinic acid has been investigated by Ensign and Rittenberg (1965), Orpin *et al.* (1972) and Wright and Cain (1972). Fishbain *et al.* (1972) reported the hydrolysis of isonicotinic acid hydrazide (INH) to INA and hydrazine by *Mycobacterium smegmatis*, but INA was not metabolised further. Metabolism of INH by *M. tuberculosis* has been reported to yield isonicotinic acid, pyridine-4-aldehyde and pyridine-4-methanol (Krishna Murti, 1974). Cell-free extracts of *M. tuberculosis* also catalyse the formation of the precursor of an yellow pigment in the presence of INH and NAD (Youatt, 1969; Gayathri Devi *et al.*, 1972, 1974).

The isolation and characterisation of a *Sarcina* sp. which metabolises INH, INA and nicotinic acid has been reported by Gupta and Shukla (1978a). This organism metabolises nicotinic acid through the intermediate formation of 6-hydroxynicotinic acid, 2,5-dihydroxypyridine and maleamic acid (Gupta and Shukla, 1978b). The isolation and characterisation of transformation products of INH and INA by *Sarcina* sp. is reported in this paper.

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Abbreviations used : isonicotinic acid hydrazide, INH; isonicotinic acid, INA; dinitrophenyl hydrazone, DNPH; chloramphenicol, CMP.

Materials and methods

Materials

INH was a gift from M/s Indian Drugs and Pharmaceuticals, Limited, Hyderabad. INA was synthesised from γ-picoline by oxidation with potassium permanganate (Vogel, 1966). Citrazinic acid was synthesised by the method of Pan and Wong (1957). The sources of other chemicals have been described earlier (Shukla, 1974; Shukla and Kaul, 1973, 1974).

Microbial and analytical methods

Isolation and characterisation of the organism, composition of phosphate-salts, the medium and methods for the cultivation of organism have been described earlier (Shukla, 1974; Shukla and Kaul, 1973, 1974; Gupta and Shukla, 1978a, c). Disappearance of INH and INA from the media was followed spectrophotometrically at 265 nm; INH, INA and hydrazine were estimated according to the methods of Leifheit and Smith (1959), Rubin *et al.* (1952) and Toida (1962) respectively. Phenolic compounds were estimated by phenol reagent (Spies, 1957), ammonia by Nessler's reagent (Ballentine, 1957), protein by biuret reagent (Gornall *et al*., 1949) and oxygen uptake according to Umbreit *et al.* (1964).

Detection, isolation and characterisation of the intermediates

The accumulation of ultraviolet (UV)-absorbing intermediates in the fermentation broth or incubation mixtures was monitored by recording the UV spectra of broth; the amount of intermediates was estimated on the basis of their molar extinction coefficients. Citrazinic acid was also estimated by phenol reagent. For detection of metabolites, the fermentation broths were lyophilised, extracted with methanol, concentrated and resolved by paper chromatography in butanol, acetic acid, water (4: 1: 1 v/v). The spots were detected under UV or by spraying with alkaline phenol reagent.

Isolation of intermediates from INA

Metabolite-I (2-*hydroxyisonicotinic acid*) : Optimum accumulation of metabollite-I was achieved by incubating resting cells with 0·1 % INA in the presence of KCN (0·5 mM) for 24 h, or without KCN at 30–35° C with occasional shaking for 72–96 h. Formation of metabolite-I was also catalysed by 20–40% ammonium sulphate fraction obtained from the crude extracts of INA-grown cells upon incubation with INA (1 μmol/ml) and methylene blue (0·1 μmol/ml) for 24 h. For isolation of this compound the above reaction mixtures were scaled up, incubated, cells removed by centrifugation or deproteinised by $ZnSO_4$ and $Ba(OH)_2$ (5 ml of each for 100 ml), supernatants recovered by centrifugation at 10000 g far 30 min and lyophilised. The residue was extracted with methanol, dried and purified by chromatography on Dowex-50 column. The eluates containing the λ_{max}310nm absorbing compound were pooled, lyophilised, extracted with ethanol, concentrated, crystallised from ethanol and dried *in vacuo* at 120–130° C.

Metabolite-II : Metabolite-II was isolated from the reaction mixtures consisting of resting cell suspension of INA-grown cells, INA (5 mg/ml), incubated at 30° C

for 48 h with restricted aeration; alternatively by incubating 40% ammonium sulphate fraction of crude extract of INA-grown cells with 2-hydroxy-INA (1 μmol/ml) and methylene blue (0·1 μmol/ml) till all the substrate was completely converted. The reaction mixtures were deproteinised with $ZnSO_4$ and $Ba(OH)_2$, supernatants lyophilised and metabolite purified by Dowex–50 column chromatography. The fractions having λ_{max} at 345 nm and showing positive test with phenol reagent were pooled, lyophilised, residue-extracted with methanol, and methanol extract concentrated *in vacuo* till crystallisation started. The crystals were filtered, recrystallised from hot water and dried *in vacuo* at 100° C to yield metabolite-II as grey powder.

Keto acids : For optimum accumulation of keto acids, resting cells were incubated with INA (1 mg/ml) and sodium arsenite (1 mM) at 30° C with shaking for 4–5 h. The supernatant was recovered by centrifugation, the keto compounds were converted into dinitrophenylhydrazones and separated into acidic and neutral compounds as described earlier (Shukla, 1974). Thin layer chromatography of acidic fraction in petroleum ether, ethylacetate, acetic acid (26 : 14 : 4 v/v) revealed a major component (A) corresponding to 2-oxo-glutarate-dinitrophenylhydrazone and a minor component (B) corresponding to pyruvate-dinitrophenylhydrazone. The two components were separated by column chromatography on silica gel in the same solvent system. The eluates corresponding to compounds A and B were eluted separately, concentrated *in vacuo,* crystallised from ethylacetate and dried *in vacuo.*

Isolation of INH metabolites

The fermentation broths of *Sarcina* sp. growing on isoniazid (0·05%) plus succinate (0·1%) were recovered by centrifugation after 48-72 h growth, lyophilised and applied on Dowex-50 column. INH was adsorbed on the resin, and metabolite-II was eluted with water, followed by metabolite-I. The appropriate fractions were pooled separately, lyophilised, extracted with ethanol, concentrated and crystallised.

The metabolites were identified on the basis of their IR, UV, NMR and mass spectra.

Enzymatic methods

The procedure for the preparation of cell-free extracts and assay of amidase (E.C. 3.5.1.—) activity has been described earlier (Gupta and Shukla, 1975). Isonicotinic acid and 2-hydroxyisonicotmic acid hydroxylases were assayed by measuring the increase in absorbance of reaction mixtures at 310 nm and 345 nm, respectively. Isoniazid hydrazine hydrolase was monitored by following the release of INA and hydrazine from INH.

Results

Microbial transformation of isonicotinic acid

Detection and accumulation of intermediates : Fermentation of INA by *Sarcina* sp. was accompanied by characteristic UV changes in the broth and formation of

a blue pigment. During the early phases of fermentation (9–24 h) a metabolite with λ_{max} around 310 nm (metabolite-I, curve b) accumulated, while at later stages (36 h)another compound (λ_{max} 345 nm,curve c) was also detected (figure 1).

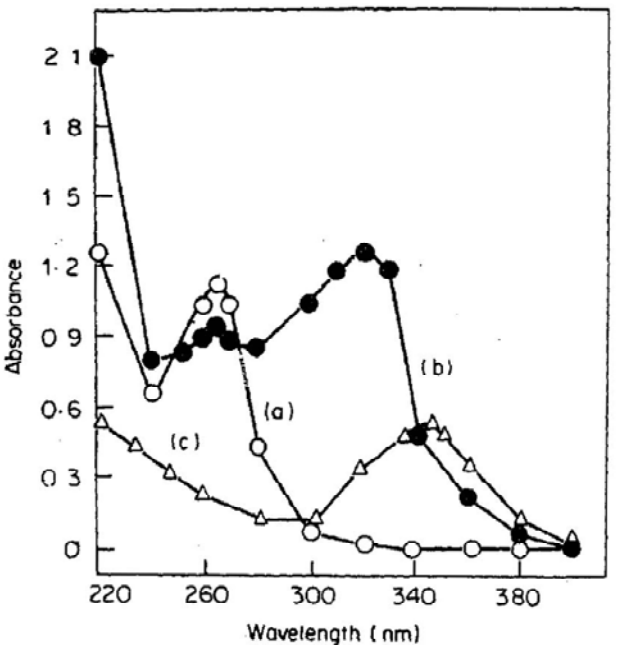

Figure 1. Accumulation of ultraviolet absorbing metabolites at different times during the growth of *Sarcina* sp.

Flasks containing phosphate-salts medium, INA (1 mg/ml) were inoculated and incubated with shaking at 30–32° C. Aliquots were withdrawn at different intervals, deproteinised and UV spectrum of supernatants recorded, **a.** 0 h **b.** 24 h **c.** 48 h.

Incubation of INA with resting cell suspensions gave a similar pattern. In the presence of KCN (0·5 mM), the oxygen uptake was blocked; however, INA slowly disappeared from the medium and metabolite–I accumulated. In the presence of 1 mM sodium arsenite, oxygen uptake and disappearance of substrate were not affected, but there was accumulation of keto acids; higher concentration of sodium arsenite inhibited oxygen uptake, disappearance of INA as well as accumulation of keto acids. Iodoacetate (0·5 mM), sodium azide (5 mM) and several other inorganic salts partially inhibited the oxidation of INA (table 1). Accumulation of both the intermediates was observed in the presence of 10 mM sodium azide. Nickel chloride slightly enhanced accumulation of metabolite–I; while other inhibitors did not significantly alter the pattern of metabolism (data not shown).

Identification of 2-*hydroxyisonicotinic acid* (*metabolite*–I)

The purified metabolite-I had m.p. 315–320° C (with decomposition), an empirical formula of $C_6H_5NO_3$ (C, 51·8%, H, 3·7%, N, 10·07%), λ_{max} 310 nM (ε 4357 M^{-1} cm^{-1}); IR spectrum had absorption bands at 1700 cm^{-1} and 2675 cm^{-1} characteristic for carboxylic acids. A prominent molecular ion at m/e indicated its mole-

Table 1. Efect of inhibitors on the oxidation of INA by resting cell suspension of INA-grown cells of *Sarcina* sp.

Inhibitors (mM)		Uptake of oxygen (μl in 2 h)	Inhibition (%)
None		250	0
Sodium arsenite	(1·0)	253	0
Sodium arsenite	(5·0)	56	78
Iodoacetate	(0·5)	210	16
KCN	(0·5)	0	100
Semicarbazide	(5·0)	251	0
Sodium azide	(10·0)	100	60
$CoSO_4$	(10·0)	110	56
$NiCl_2$	(10·0)	120	52
$ZnSO_4$	(10·0)	120	52
Sodium fluoride	(10·0)	150	40

Reaction mixtures contained in a total volume of 2·8 ml; phosphate buffer (pH 7·0), 100 μmol; INA-grown cells, 3·54 mg dry wt; INA, 6 μmol and inhibitors.

cular weight to be 139. This suggested the incorporation of one oxygen atom in INA molecule (123 + 16). There were other prominent peaks at m/e 122 (loss of OH) and 111 (loss of carbonyl group). The NMR spectrum had proton signals at 2·07 τ (doublet, J = 6 cps, of one proton intensity corresponding to α- proton of pyridine ring) and at 3·19 τ (two proton intensity corresponding to β protons). Isonicotinic acid has two α and two β protons. The reduction in α-proton in the metabolite suggested that the oxygen atom has been introduced at α-position of pyridine ring. The compound was thus identified as 2-hydroxyisonicotinic acid (2-hydroxy-INA) (Gupta and Shukla, 1978c).

Identification of metabolite-II as citrazinic acid

The purified metabolite–II had m. p. 290–300° C (with decomposition). The UV spectrum had a λ_{max} at 240 nm ($_{\epsilon}$ 7500 M^{-1} cm^{-1}), 345 nm ($_{\epsilon}$5010 M^{-1} cm^{-1}); fluorescence spectrum of the compound had emission maximum at 445 nm (excitation wavelength at 355 nm). The m.p. of the metabolite was not depressed by mixing authentic citrazinic acid (290–300° C). The IR spectra of isolated compound and citrazinic acid were superimposable, thereby confirming that metabolite-II was citrazinic acid. When the fermentation broths were further incubated for 48–72 h at room temperature, a blue pigment (λ_{max} 625 nm) was formed. This was purified by DEAE-cellulose chromatography and found to be identical with the pigment formed by auto-oxidation of citrazinic acid.

Keto acid products of INA metabolism

The major keto acid dinitrophenylhydrazone (component A) had empirical formula of $C_{11}H_{10}O_8N_4$ (C, 40·53%, H, 2·98%, N, 17·05%), and the same mobility as 2-oxo-glutaric acid-DNP (R_f, 0·25) on thin layer (TLC) and paper chromatograms in butanol, ethanol, water (7:1:2) (R_f, 0·50) and superimposable IR spectra. The minor compound (B), m.p. 208–210° C (undepressed on mixing with authentic sample of pyruvate-DNP) had the same mobility on TLC (R_f 0·50) and IR spectrum identical to that of authentic pyruvate-DNP. During initial stages of INA metabolism by resting cells (0–4 h), 2-oxo-glutarate was the only keto acid detected but on further incubation (5–12 h) smaller amounts of pyruvate also accumulated. Maximum accumulation of 2-oxo-glutarate corresponded to 12% of INA metabolised (table 2).

Table 2. Accumulation of keto acids during metabolism of isonicotinic acid by isonicotinic acid-grown cells of *Sarcina* sp.

Incubation period	Concentration of dinitrophenyl hydrazone (DNPH) keto acids in reaction mixture		
	Total DNPH-keto acid (μg/ml)	DNPH-pyruvic acid (μg/ml)	DNPH-keto glutaric acid (μg/ml)
0	0	0	0
1	100	0	100
2	200	0	200
3	234	0	234
4	250	0	250
5	302	5	297
6	278	7	271
7	270	9	261
12	162	2	160
24	0	0	0

Reaction mixtures containing resting cell suspension of isonicotinic acid-grown cells, isonicotinic acid, (1 mg/m^1) and sodium arsenite (1 mM) were incubated with shaking at 30–32° C. Aliquots were withdrawn at diferent intervals, deproteinised, centrifuged and supernatant taken for estimation of keto acid.

Keto acid accumulation during maleate metabolism

Incubation of maleate-grown cells of *Sarcina* sp. with maleate in the presence of 1 mM sodium arsenite led to the accumulation of pyruvic acid as the major keto acid, only minor quantities (less than 5% of total keto acids) of 2-oxo-glutarate were detected (table 3).

Microbial transformation of INH

Isolation and characterisation of metabolites : When *Sarcina* sp. was grown in media containing 0·05% INH, metabolite–I (λ_{max} 310 nm) appeared in early stages

Table 3. Accumulation of keto acids during metabolism of maleic acid by maleic acid grown cells of *Sarcina* sp[a]

Incubation period	Concentration of DNPH–keto acids		
	Total DNPH–keto acids (μg/ml)	DNPH–pyruvic acid (μg/ml)	DNPH–keto-glutaric acid (μg/ml)
0	0	0	0
2	100	98	2
4	160	153	6
6	80	74	5
12	0	0	0

a Conditions of the experiment are same as given in table 1 except maleic acid and maleic acid-grown cells were used instead of isonicotinic acid and isonicotinate-grown cells.

followed by metabolite-II. When succinate (0·1%) was added to the medium, growth as well as accumulation of metabolites was enhanced. INA and hydrazine were not detected during any stage of incubation. The metabolite-I derived from fermentation of INH was shown to be 2-hydroxy-INA on the basis of m.p. (315–320° C), UV and IR spectra. Metabolite-II was similarly identified as citrazinic acid on the basis of m.p. (295–300° C), UV and IR spectra. Formation of blue pigment was also observed when INH fermentation broths, after removal of cells, were incubated at room temperature or in the cold. This pigment was identical with that of the pigment derived from citrazinic acid.

keto acid inter mediates of INH metabolism

Resting cell suspensions of INH-grown cells when incubated with INH (0·05%) and sodium arsenite (1 mM) at 30° C for 24 h gave maximum accumulation of keto acids. 2-Oxo-glutarate accounted for 95% of keto acid fraction while pyruvate represented only a minor fraction. 2-Oxo-glutarate accumulated exclusively during initial phase of INH metabolism.

Oxidation of substrate by INA and INH grown-cells

Isoniazid was oxidised slowly by INH-grown cells but faster by INA-grown cells; INA was oxidised rapidly in both the cases. 2-Hydroxy-INA was also oxidized by INH as well as INA-grown cells; citrazinic acid, however, was not oxidised. 2, 5-, 2, 6-, 2, 3-dihydroxypyridines and maleamic acid were not oxidised. Fumaramate 2-oxo-glutarate, fumarate, citrate, *cis*-aconitate and pyruvate were oxidized at slower rates (figures 2 and 3).

Enzymatic studies The various enzymes implicated in INH and INA metabolism have been assayed in the cell-free extracts of induced and noninduced.

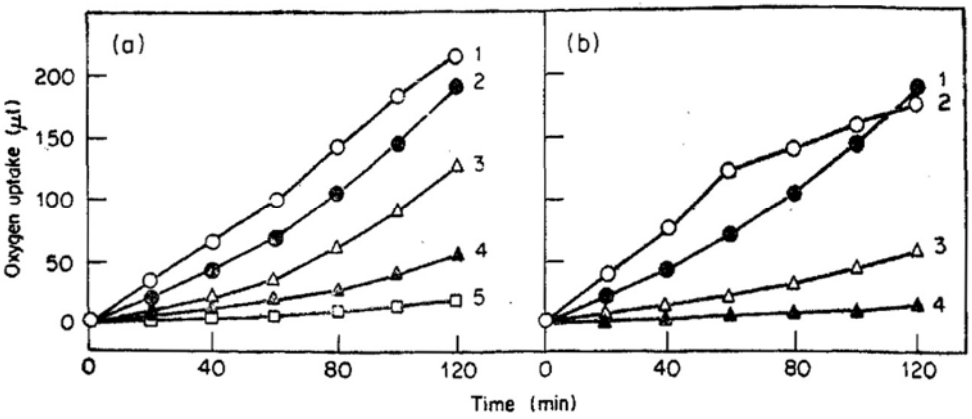

Figure 2. Oxygen uptake with different substrates by INH-grown cells [Warburg flasks (15 ml capacity) contained in total volume of 2·8 ml; cells 3·54 mg dry wt., substrates 5 μmol, Kreb's cycle intermediates 10 μmol; chloramphenicol (CMP) 100 μg/ml where indicated; 0·2 ml of 20% KOH was added in the central well and flasks incubated with shaking at 37° C].

(a) (1) Succinic acid or succinic acid + CMP; (2) INA or INA + CMP; (3) Fumaric acid or fumaric acid + CMP; 2-oxo-glutarate or 2-oxo-glutarate + CMP; (4) The presence of one of the following: INH fumaramate, citrate, isocitrate, *cis*-aconitate, (5) endogenous, 2, 5-, 2, 6- and 2, 3-dihydroxypyridine, maleamic acid or maleic acid.

(b) (1) INA; (2) 2-OH-INA or 2-OH-INA + CMP; (3) citrazinic acid isolated from broth or citrazinic acid (synthetic); (4) endogenous.

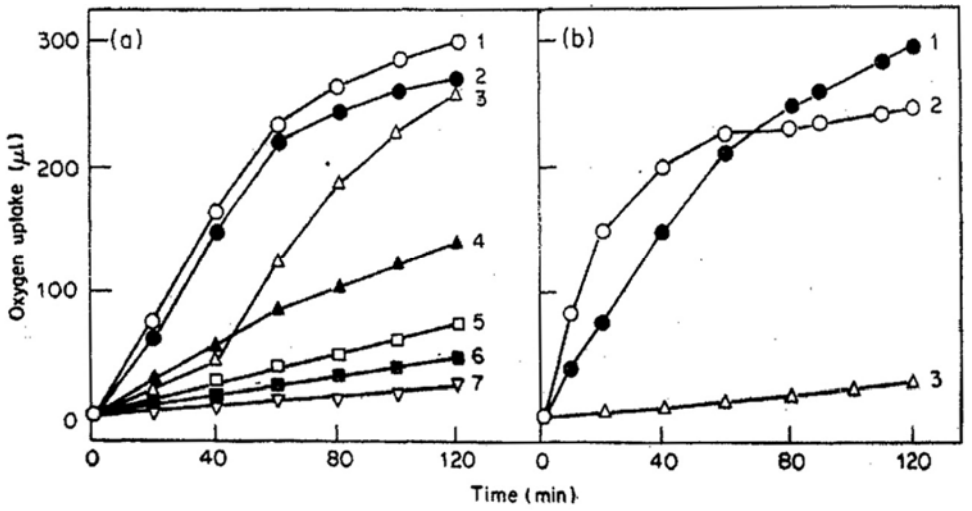

Figure 3. Oxygen uptake with different substrate by INA-grown cells of *Sarcina* sp.
(a) (1) Succinic acid; (2) fumaric acid, (3) 2-oxo-glutarate or 2-oxo-glutarate + CMP; (4) *cis*-aconitic acid or *cis*-aconitic acid + CMP; (5) pyruvate fumaramate; (6) citrate or maleamate or isocitrate; (7) endogenous, 2, 5-, 2, 6-, 2, 3-dihydroxypyridine or maleate.

(b) (1) INA; (2) 2-OH–INA or2-OH-INA+CMP; (3) Endogenous or citrazinic acid.

cells. An enzyme cleaving INH to yield equimolar amounts of INA and hydrazine has been detected in extracts of INH or INA-grown cells. Succinate-grown cells lacked this enzyme (table 4). INA and 2-hydroxy INA hydroxylases responsible for conversion of INA to citrazinic acid were similarly detected in induced cells but not in succinate-grown cells (table 5). An enzyme preferentially hydrolysing fumaramic acid was also detected in induced cells; activity with maleamic acid as the substrate was rather weak (table 6). The role of this enzyme in INA or INH metabolism is not clear.

Table 4. Isoniazid hydrolysis in crude extracts of *Sarcina* sp.

Component omitted	INH disappeared (μmol) or INA, hydrazine formed (μmol)								
	INH-grown cells			INA-grown cells			Succinate-grown cells		
	INH	INA	Hydrazine	INH	INA	Hydrazine	INH	INA	Hydrazine
Substrate (INH)	0·03	0·01	0·008	0·021	0	0	0·06	0	0
Enzyme	0	0	0	0	0	0	0·045	0	0
None	3·12	3·0	2·65	4·12	4·0	3·96	0·105	0	0

Reaction mixtures for assay of enzyme contained in 6 ml: potassium phosphate buffer (pH 7·0) 100 μmol, substrate 10 μmol, enzyme protein 8–10 mg; incubated at 37° C for 2 h, deproteinised by $ZnSO_4$, centrifuged and supernatants used for estimation of INA and hydrazine.

Table 5. NA and 2-hydroxyisonicotinic acid hydroxylases in cell-free extracts of *Sarcina* sp. grown on INH, INA and succinate.

Enzymes	Specific activity (units/mg)		
	INA-grown cells	INH-grown cells	Succinate-grown cell
INA-hydroxylase	1·60	1·32	0
2-OH-INA hydroxylase	0·65	0·612	0

Reaction mixtures in the total volume of 3 ml contained: potassium phosphate buffer (pH 7·0) 100 μmol, methylene blue 0·2 μmol, enzyme protein 1–3 mg, substrate (0·3 μmol INA or 0·2 μmol 2-OH-INA). One unit is defined as the amount of enzyme causing the formation of one nmol of product per min. Specific activity is defined as enzyme units/mg protein.

Discussion

The isolation and characterisation of *Sarcina* sp. degrading INH has been reported earlier, INA and nicotinic acid were also metabolised by this organism (Gupta

Table 6. Maleamic and fumaramic deamidase activity in INH–, INA– and succinate grown cells of *Sarcina* sp.

Substrate	Ammonia released (μmol in 2 h)		
	INH–grown cells	INA-grown cells	Succinate-grown cells
Maleamic acid	0·01	0·91	0·01
Fumaramic acid	6·25	7·3	0·01

Reaction mixtures in a total volume of 4 ml had potassium phosphate buffer (pH 7·0) 100 μmol, enzyme protein 20–26 mg and substrate 50 μmol The reaction mixtures were incubated at 37° C for 2 h, deproteinised, centrifuged and supernatant used for estimation of ammonia.

and Shukla, 1978a). Nicotinic acid was metabolised *via* 6-hydroxynicotinic acid and 2,5-dihydroxypyridine as intermediates (Gupta and Shukla, 1978b). Studies on the oxidation and catabolism of substrates with resting cells indicated that INH and INA were probably metabolised by the same pathway while nicotinate was metabolised differently. Studies presented in this paper substantiate this conclusion. The two metabolites which were detected during INA or INH metabolism have been isolated and characterised as 2-hydroxyisonicotinic acid and 2,6-dihydroxyisonicotinic acid (citrazinic acid). The enzymes responsible for conversion of INH to INA and hydroxylation of INA to citrazinic acid *via* 2-hydroxyisonicotinic acid have also been demonstrated in cell-free extracts of induced cells. 2-Oxo-glutarate was detected as the only keto acid accumulating during the early stages of INA/INH metabolism in the presence of 1 mM sodium arsenite. Pyruvate was detected only in minor quantities and on longer incubations when the INA was almost completely used up. Cleavage of pyridine ring in many pyridine derivatives like nicotinic acid (Behrman and Stanier, 1957), picolinic acid (Shukla and Kaul, 1973), 2- and 3-hydroxypyridine (Gupta and Shukla, 1975; Khanna and Shukla, 1977) lead to maleamic acid which on further metabolism yields pyruvate. Accumulation of pyruvate has also been observed when *Sarcina* sp. metabolises nicotinic acid (Gupta and Shukla, 1978b) or maleic acid in the presence of 1 mM sodium arsenite. In the case of INA metabolism, this pattern is apparently not followed. The oxidation of INA by INH-grown cells and oxidation of 2-hydroxy-INA by INA- and INH-grown cells of *Sarcina* sp. readily suggest their role as intermediates. Citrazinic acid is, however, not oxidised when supplied in the medium. This may indicate that citrazinic acid is either not an intermediate or the cells are impermeable to this compound. Citrazinic acid has been shown to be intermediate in INA metabolism of a *Pseudomonas* sp. (Ensign and Rittenberg, 1965). It is thus plausible that INH and INA may be metabolised by *Sarcina* sp. through 2-hydroxy-INA and citrazinic acid. The following observations suggest that maleamate pathway is not involved in INA degradation by *Sarcina* sp.: (1) accumulation of 2-oxo-glutarate rather than pyruvate, (2) failure of cells to oxidise maleamic acid and maleic acid at appreciable rates, and (3) the absence of relevant enzymes in cells. An alternative

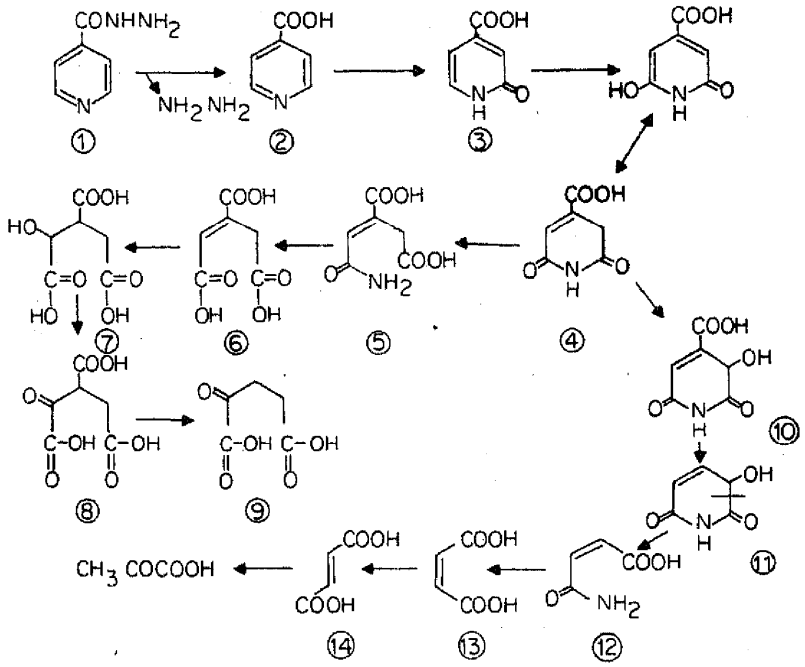

Figure 4. Tentative pathway(s) of INH metabolism in *Sarcina* sp. (1) isoniazid; (2) isonicotinic acid; (3) 2-hydroxyisonicotinic acid; (4) citrazinic acid, (5) *cis*-aconitamide; (6) *cis*-aconitic acid; (8) isocitric acid, (9) 2-oxoglutaric acid, (10) 2, 3, 6 trihydroxyisonicotinic acid; (11) 2, 3, 6-trihydroxypyridine; (12) maleamic acid; (13) maleic acid; (14) fumaric acid.

pathway involving ring cleavage of citrazinic acid between C_2 and N to yield *cis*-aconitamide and *cis*-aconitic acid may explain the accumulation of 2-oxo-glutarate (figure 4). However, further work is required on INA metabolism by *Sarcina* sp. as well as other organisms to establish this pattern of pyridine ring cleavage.

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