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MODE OF ACTION OF ISONIAZID ON YEAST*

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Abstract—The yeast *Saccharomyces marxianus* (NRRL-Y-1550) was grown in a chemically defined medium in the presence of increasing concentrations of isonicotinic acid hydrazide (INH), 0.4 to 2.4 mg/ml. It was found that the NAD content and the dry weight of the cells decreased with increasing concentrations of INH. Similar results have been reported for *Mycobacterium tuberculosis* H37Ra. The growth inhibition of the yeast by INH was completely reversed by L-tryptophan. When the yeast grew in the presence of INH, but in the absence of L-tryptophan, two growth factors for *Lactobacillus plantarum* (ATCC 8014) were found in the culture medium. One of the growth factors has been identified as anthranilic acid while the other one was the *N*-D-glucosylamine derivative of anthranilic acid, shown to be an artifact of the isolation technique used. These results indicate that the primary mode of action of INH on yeast might be the inhibition of the biosynthesis of 1-(*O*-carboxyphenylamino)-1-deoxyribulose-5-phosphate.

Isonicotinic acid hydrazide (INH), also known as Isoniazid, has been the drug of choice in the chemotherapy of tuberculosis for some 25 years; however, its primary mode of action on *Mycobacterium tuberculosis* is still unknown. The present state of knowledge has been summarized in several recent review articles [1–3].

Very little has been reported on the action of INH on yeast. Neuberg and Forrest [4] showed that INH is not very toxic for yeast and that acetaldehyde accumulated in the fermentation liquor in the form of the isonicotinic acid hydrazone. Scherr and Rafelson [5], using the gradient plate technique, isolated mutants of *Saccharomyces marxianus* resistant to INH that excreted increased amounts of vitamin B_6 in the culture medium. Pardini and Argoudelis [6], using the same technique, isolated a similar INH resistant mutant and identified the members of the vitamin B_6 group which are excreted in the culture medium.

MATERIALS AND METHODS

The media for the microbiological assays were purchased from DIFCO Laboratories, and Isoniazid, NAD and NADP from Sigma Chemical Co.

The yeast *S. marxianus* (NRRL-Y-1550) was grown in a synthetic culture medium consisting of (mg/l) vitamin free acid hydrolyzed casein (Difco), 1000; potassium citrate monohydrate, 1250; citric acid monohydrate, 2500; potassium chloride, 420; calcium chloride dihydrate, 126; magnesium sulfate, 60; ferric chloride hexahydrate, 2.6; manganous sulfate monohydrate, 2.6; ammonium phosphate dibasic, 1000; potassium phosphate monobasic, 560; dextrose, 30,000; and of the following vitamins (μg/liter) thiamine hydrochloride, 260; inositol, 26,000; calcium pantothenate, 2500; biotin, 10; and pyridoxine hydrochloride, 1000. Tap distilled water was used. Cells from a 24-hr wort agar slant culture were suspended in a sterile 0.9% sodium chloride solution, centrifuged and resuspended in a similar sterile solution. Conical flasks containing the culture medium and the added compound were steamed for 10 min, cooled and inoculated all with the same volume of inoculum. 3-Hydroxy-anthranilic acid was dissolved in 0.01 M phosphate buffer, pH 7, sterilized by filtration and added aseptically to the steamed flasks. Isoniazid was dissolved in distilled water (50 mg/ml), steamed for 10 min, and added aseptically to the appropriate flasks 2–3 hr after inoculation. The flasks were incubated at 27° in a model G25 NBS gyrotory shaker at 70 rev/min and removed after 21–25 hr. Growth was determined from a curve relating optical density at 650 nm and dry wt of cells.

The NAD content of the cells was measured according to the method of Bekierkunst and Bricker [7] by the fluorescent technique with methyl ethyl ketone [8]. Protein of yeast cells was determined by the method of Lowry *et al.* [9] after dissolving the trichloroacetic acid precipitate in 1 N sodium hydroxide.

The compounds which were present in the cells or were excreted in the culture medium of *S. marxianus* and showed nicotinic acid activity for *Lactobacillus plantarum* (ATCC 8014) using Difco's Bacto-Niacin assay medium were identified by paper chromatography in ascending fashion with Whatman No. 3 filter paper in 1 M ammonium acetate–ethanol (3:7) [10] and by bioautography [11]. The chromatogram papers were examined under ultraviolet (u.v.) light to locate the spots of nicotinic acid and its derivatives. Nicotinamide nucleotides were distinguished from nicotinic acid analogues by the method of Kodicek and Reddi [12]. Spots of nicotinic acid and nicotinamide were also

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located by the method of Wang and Kodicek [13].

The compounds which were excreted in the culture medium of *S. marxianus* when it was grown in the presence of INH (2 mg/ml) and showed tryptophan activity for *L. plantarum* (ATCC 8014) using Difco's Bacto-Tryptophan assay medium were identified by paper chromatography in ascending fashion with Whatman No. 3 filter paper in isopropyl alcohol–concentrated ammonium hydroxide–water (8:1:1) and by bioautography [11]. The INH, present in the cell-free culture media, was not inhibitory to *L. plantarum* at the dilutions used for the microbiological assay of niacin or tryptophan. The chromatogram papers were examined under ultraviolet light to locate the spots of anthranilic acid and its derivative. These compounds were also located by spraying the paper chromatogram with the Ehrlich reagent, 2% *p*-dimethylamino benzaldehyde in 2 N HCl in 80% ethanol [14]; a yellow spot appeared immediately at the place where anthranilic acid was and after about 1 hr another faint yellow spot appeared at the place of its derivative. Sugars were visualized by spraying the chromatograms with aniline oxalate [15].

Isolation of anthranilic acid and its derivative. A volume of 50 ml of cell-free culture medium of *S. marxianus* grown for 24 hr in the presence of 2 mg/ml of INH was extracted twice with 20 ml ether. The combined ether extracts were evaporated to dryness under vacuum, 1 ml of ether was added to the residue and approximately 0.5 ml was streaked on Whatman No. 3 filter paper. The paper was developed by the ascending technique for 16 hr in isopropyl alcohol–concentrated ammonium hydroxide–water (8:1:1), dried and the fluorescent area under u.v. light was eluted from the paper with 10 ml of 0.1 N NaOH. The sodium hydroxide extract was acidified with HCl and extracted twice with 10 ml ether. The combined ether extracts were dried with anhydrous Na_2SO_4 and the u.v. absorption spectrum was determined with a Cary model 11M recording spectrophotometer. The ether solution was then evaporated to dryness in a small vial, 0.1 ml *N,O*-bis(trimethylsilyl)acetamide (BSA) was added, kept at 80° for 1 hr, and aliquots were used for gas-liquid chromatography and gas-liquid chromatography–mass spectrometry.

Approximately 3 ml of cell-free culture medium of *S. marxianus* grown for 24 hr in the presence of 2 mg/ml of INH was streaked on 4 in. of Whatman No. 3 filter paper. The paper chromatogram was developed as described above. Small sections of this chromatogram were used for bioautography and for spraying with *p*-dimethylamino benzaldehyde and aniline oxalate. The fluorescent area under u.v. light, which had a smaller R_f value than anthranilic acid and showed activity for *L. plantarum* in Difco's Bacto-Tryptophan assay medium, was cut, eluted from the paper with water and an aliquot was used to study its u.v. absorption spectra under alkaline and acidic conditions. Another aliquot of the eluted compound was made acidic with HCl (pH 1), placed on a steam bath for 30 min, cooled and streaked again on 4 in of Whatman No. 3 filter paper which was developed as described above. Sections of this chromatogram were used for bioautography and for spraying with *p*-dimethylamino benzaldehyde and aniline oxalate.

Synthesis of N-*glycosylamine derivatives.* The *N*-D-glucosylamine and *N*-D-ribosylamine derivatives of anthranilic acid were synthesized according to the method of Doy and Gibson [16]. Their rate of hydrolysis in 0.1 N HCl was compared, under the same conditions, to the rate of hydrolysis of the isolated glycosylamine derivative.

Gas–liquid chromatography–mass spectrometry. A Hewlett–Packard model 5710A gas chromatograph and a ⅛ in. × 4 ft stainless steel column packed with 3% OV-17 coated on 80/100 mesh Chromosorb W were used. The column was conditioned by several injections of 10 μl BSA at 85° and heated to 250° after each injection. Critical g.l.c. operating conditions were: injector and detector temperatures 150° and 300°, respectively; flow rate 30 ml/min; carrier gas N_2; and the column temperature was kept at 85° for 2 min and then programmed to 250° at 16°/min. Mass spectra were determined with a Perkin Elmer Hitachi RMU6E single focusing mass spectrometer equipped with a gas chromatographic inlet system. The helium separator was maintained at 250° as was the ion source and the heated transfer line from the g.l.c.; the ionization potential was 20 eV. For gas-liquid chromatography–mass spectrometry analysis, the rate of temperature programming was reduced from 16°/min to 10°/min.

RESULTS AND DISCUSSION

The yeast *S. marxianus* excretes in the culture medium compounds that are growth factors for *L. plantarum* growing in Bacto-Niacin assay medium. These growth factors have been identified by paper chromatography and bioautography as NAD and nicotinic acid (Table 1). The cells of the yeast contain, in addition, NADP and nicotinamide. In the culture medium and in the cells, a compound is present that has not been identified; it might be nicotinamide riboside since it fluoresces when the paper chromatogram is exposed to a methyl ethyl ketone–ammonium hydroxide mixture [12]. When the yeast grows in the presence of INH (> 1.5 mg/ml) nicotinic acid derivatives are not excreted in the culture medium and the addition of nicotinic acid into the medium reverses, to some extent, the effect of INH (Table 3). It has been shown that the *M. tuberculosis* excreted also nicotinic acid and its derivatives in the culture medium [17]. Bekierkunst and Bricket [7] reported that the effect of INH on tubercle bacilli is to decrease their NAD content. The data in Table 2 show that it has a similar effect on the yeast *S. marxianus*. The effect of various precursors of NAD in yeast in reversing the action of INH is shown in Table 3. The data of the table show that nicotinic acid and quinolinic acid reverse, to some extent, the action of INH. However, the cause of death of the yeast cells is not due to the reduction of their NAD content, since it remains constant while their dry wt decreases. The effect of increasing concentrations of NAD precursors in reversing the action of a certain amount (2 mg/ml) of INH is shown in Table 4. Only L-tryptophan could

Table 1. Paper chromatography of authentic compounds and bioautography of compounds in the cells or excreted in the culture medium of *S. marxianus* (NRRL-Y-1550)

Compound	Paper chromatography (R_f)	Bioautography (cell extract) (R_f)	Bioautography (culture medium) (R_f)
NAD	0.09	0.09	0.09
NADP	0.015	0.015	
Nicotinic acid	0.71	0.71	0.71
Nicotinamide	0.81	0.81	
Niacin ribonucleoside	0.59 (8)*		
Nicotinamide ribonucleoside	0.62 (8)		
Unknown		0.56	0.56

* The number in parentheses indicates

Table 2. Effect of Isoniazid (INH) on the growth and on the nicotinamide-adenine dinucleotide (NAD) content of the cells of the yeast *S. marxianus* (NRRL-Y-1550)

INH concn (mg/ml)	Growth (dry wt) (mg*)	NAD (μg/mg protein*)
0	115	2.77
0.4	117	3.60
0.8	86	3.08
1.2	42	2.26
1.6	25	1.75
2.0	7	1.36
2.4	2	1.23

* Average of two determinations.

prevent almost completely the growth inhibition of INH.

Since all these data seemed to indicate that, in *S. marxianus*, INH inhibited the biosynthesis of L-tryptophan, we next examined the culture medium in which the yeast was growing (in the presence of INH) for the possible accumulation of tryptophan precursors. Indeed, a diluted aliquot from the yeast culture medium showed tryptophan activity for the test organism *L. plantarum* growing in Bacto-Tryptophan assay medium. The yeast culture medium showed no tryptophan activity when the yeast grew in the absence of INH. Bioautography [11] revealed that two active compounds were secreted in the yeast culture medium (Table 5). The compound with the larger R_f value which showed tryptophan activity for *L. plantarum* was identified by paper chromatography (Table 5), ultraviolet absorption spectroscopy (Fig. 1c), gas–liquid chromatography (Fig. 2) and mass spectrometry, as anthranilic acid. An ether extract of the culture medium of the yeast, growing in the presence of INH, had two maxima (Fig. 1a), one at 335 nm and the other one at 250 nm. The maximum at 250 nm had a much higher absorptivity than if it were due to only anthranilic acid, which was explained as being due to the small amount of INH extracted from the culture medium. A similar u.v.

Table 3. Effect of various compounds in reversing the action of Isoniazid (INH) on the yeast *S. marxianus* (NRRL-Y-1550)

Compound added (20 μg/ml)	INH concn (mg/ml)	Growth (dry wt) (mg*)	NAD (μg/mg protein*)
Nothing	0	117	2.72
	1.0	48	3.12
	1.5	10	2.07
	2.0	3	1.32
L-Tryptophan	0	113	2.65
	1.0	104	2.62
	1.5	102	2.60
	2.0	104	2.60
Nicotinic acid	0	115	4.45
	1.0	95	4.15
	1.5	76	4.32
	2.0	35	4.45
Quinolinic acid	0	117	4.02
	1.0	98	4.28
	1.5	78	4.12
	2.0	38	3.94
3-Hydroxy-anthranilic acid	0	104	4.20
	1.0	90	4.36
	1.5	51	3.52
	2.0	20	2.65

* Average of two determinations.

Table 4. Effect of increasing concentration of various compounds in reversing the action of Isoniazid (INH, 2 mg/ml) on the yeast *S. marxianus* (NRRL-Y-1550)

Added compound	Concn of added compound (μg/ml)	Growth (dry wt) (mg*)	NAD (μg/mg protein*)
− INH		112	2.67
+ INH		6	1.34
3-Hydroxy-anthranilic acid	3.0	7	0.60
	6.0	12	0.72
	12.0	18	0.88
	24.0	26	1.16
Quinolinic acid	2.5	26	3.98
	5.0	30	4.14
	10.0	30	4.22
	20.0	30	4.33
Nicotinic acid	2.5	25	4.03
	5.0	25	3.96
	10.0	35	4.31
	20.0	30	4.25
L-Tryptophan	0.2	30	1.18
	0.4	51	1.39
	0.8	82	1.88
	1.6	105	2.52

* Average of two determinations.

absorption spectrum was obtained (Fig. 1b) when an aqueous solution (pH 4) of a mixture of anthranilic acid and INH was extracted with ether. From the absorbance data of the ether extract it is estimated that the amount of anthranilic acid excreted in the culture media is approximately 20 μg/ml. The g.l.c. chromatogram of the TMS derivative of the isolated anthranilic acid (Fig. 2) had two peaks; the first one was identified by mass spectrometry as being due to TMS ester of anthranilic acid m/e: 209 (M^+, 98%); 194 ($M^+ - 15$, 96%); 150 [$M^+ - (15 + 44)$, 30%]; 119 ($M^+ - 90$, 100%), and the second one as being due to the di-TMS derivative of anthranilic acid m/e: 281 (M^+, 21%); 266 ($M^+ - 15$, 100%). Similar data for the g.l.c. of the TMS derivatives of anthranilic acid have been reported by Dalgliesh *et al.* [18].

The other compound that showed tryptophan activity for *L. plantarum* was not ether extractable. Sections of a paper chromatogram of cell-free culture medium of the yeast, were sprayed (a) with aniline oxalate giving a brown spot and (b) with *p*-dimethylamino benzaldehyde giving, after about 1 hr, a faint yellow spot at the corresponding places where bioautography of another section had revealed tryptophan activity. The compound was eluted from the paper chromatogram with water and

Table 5. Paper chromatography and bioautography of authentic compounds and of compounds present in the culture medium of *S. marxianus* (NRRL-Y-1550) growing in the presence of isonicotinic acid hydrazide

Compound	R_f values		
Indole	0.91		
Isoniazid	0.47–0.66*		
D-Ribose	0.48		
D-Glucose	0.33		
Anthranilic acid	0.38		
L-Tryptophan	0.32		
N-O-carboxyphenyl-D-glucosylamine	0.21		
N-O-carboxyphenyl-D-ribosylamine	0.21		
Yeast cell-free medium	0.47–0.66*	0.38	0.21
Bioautography of yeast cell-free medium		0.38	0.21
Ether extract of yeast cell-free medium	0.47–0.66*	0.38	
Bioautography of ether extract of yeast cell-free medium		0.38	
Acid hydrolyzed compound with R_f 0.21	0.38	0.33	0.21
Mixture of anthranilic acid and D-glucose dissolved in water	0.38	0.33	0.21

* Streaked.

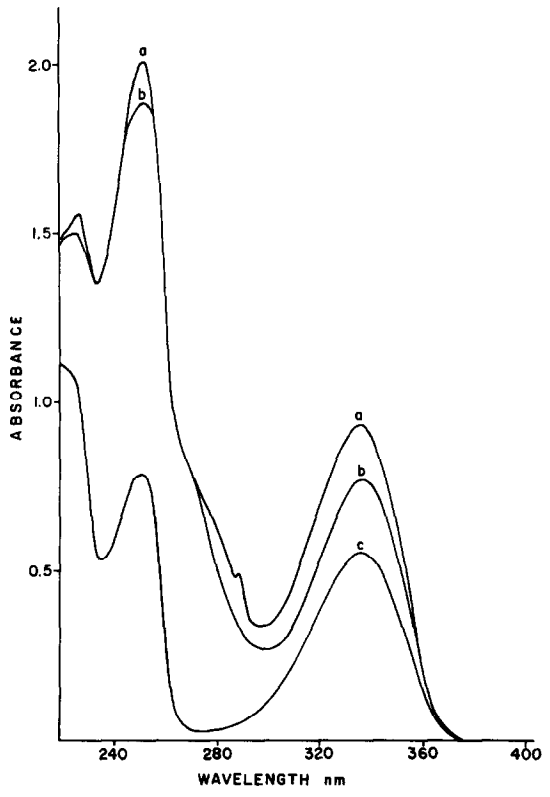

Fig. 1. Ultraviolet absorption spectra of: (a) the ether extract of the culture medium of *S. marxianus* (NRRL-Y-1550) growing in the presence of isonicotinic acid hydrazide; (b) the ether extract of an aqueous solution of a synthetic mixture of anthranilic acid and isonicotinic acid hydrazide; and (c) anthranilic acid in ether isolated, through paper chromatography of the ether extract of (a).

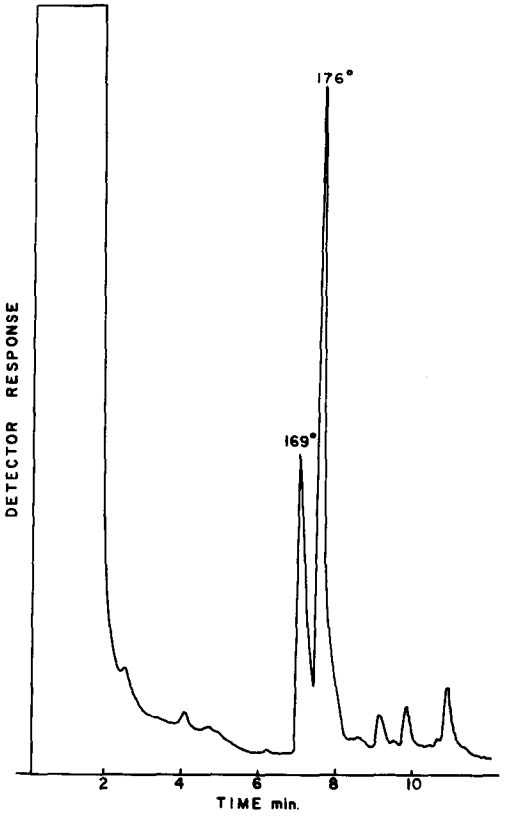

Fig. 2. Gas–liquid chromatogram of the TMS derivatives of anthranilic acid isolated through paper chromatography of the ether extract of the culture medium of *S. marxianus* (NRRL-Y-1550) growing in the presence of isonicotinic acid hydrazide.

its u.v. absorption spectrum was similar to that of anthranilic acid. The eluted compound was not stable in acid solution, but it was very stable in alkaline solution. A water extract of the compound from a paper chromatogram was next hydrolyzed with hydrochloric acid and the sugar moiety of the compound was identified as glucose and not, as expected, ribose (Table 5). The other part of the compound was identified as anthranilic acid. We then synthesized both the *N*-D-glucosylamine and the *N*-D-ribosylamine derivatives of anthranilic acid by the method of Doy and Gibson [16], and their rate of hydrolysis in 0.1 N hydrochloric acid was compared. The isolated compound from the yeast culture medium had a rate of hydrolysis similar to the synthetic *N*-D-glucosylamine derivative of anthranilic acid and not to that of *N*-D-ribosylamine derivative which is hydrolyzed much faster than the *N*-D-glucosylamine derivative. A search in the literature revealed a paper by Parks and Douglas [19] in which they reported the isolation of an *N*-fructosyl derivative of anthranilic acid from cultures of *Saccharomyces*, and they considered this compound as a possible intermediate in the biosynthesis of indole and tryptophan in yeast.

Although a hexose and not a pentose derivative of anthranilic acid had been speculated as an intermediate in the biosynthesis of tryptophan in *Saccharomyces*, the fact that a paper chromatogram of the product of the acidic hydrolysis of the isolated compound gave a spot (Table 5) at a similar place to the unhydrolyzed compound seemed a little strange. Thus, equal amounts of D-glucose and anthranilic acid were dissolved in water and an aliquot of this solution was streaked on Whatman No. 3 filter paper with the aid of warm air. After development of the chromatogram, an *N*-D-glucosylamine derivative of anthranilic acid, identical to that isolated from the yeast culture medium, was eluted from the paper chromatogram. It is believed that the isolated compound from the yeast culture medium which has a smaller R_f value than anthranilic acid and shows tryptophan activity for *L. plantarum* is an artifact of the isolation technique used.

During these studies on the mode of action of INH on yeast, it was observed that after about 24 hr the yeast was able to grow in the culture medium that contained no precursor of NAD or L-tryptophan, even in the presence of 2 mg/ml of INH. It was found that, by that time, almost all of the INH was present in the form of its hydrazone with acetaldehyde as has been reported [4]. It seems that yeast is using acetaldehyde as a detoxifying agent for INH. The large amount of the hydrazone of INH with acetaldehyde found in the supernatant of cultures to which L-tryptophan or nicotinic acid had been added indicates that the NAD precursors do not block the entrance of INH into the cell. The fact that the concentration of INH that inhibits yeast is

many orders of magnitude higher than that required to inhibit sensitive tubercle bacilli (0.02 to 0.06 μg/ml) could be due to the mechanism that is available in the yeast to detoxify INH very easily.

The results presented here seem to indicate that the primary mode of action of INH on the yeast *S. marxianus* (NRRL-Y-1550) is to inhibit the biosynthesis of L-tryptophan. The effect of INH on the NAD content of the yeast cells is of a secondary nature. Apparently, L-tryptophan and NAD are essential for the synthesis of enzyme(s) which in turn will produce the compound(s) necessary for the detoxification of INH. The specific step at which INH inhibits the biosynthesis of L-tryptophan in yeast could be at the Amadori rearrangement where a carbonyl-containing compound is formed which conceivably could react with INH. If this is true, one could expect the accumulation of anthranilic acid ribotide in the yeast culture medium. However, *N*-(5′-phosphoribosyl) anthranilic acid or its dephosphorylated form is very unstable [20] at the low pH (4.0–4.5) of the yeast culture medium. Consequently anthranilic acid would be the compound that accumulates. The fact that no anthranilic acid accumulates in the yeast culture medium when the yeast grows in the presence of both L-tryptophan and INH is due most probably to feedback inhibition by tryptophan. It has been reported that L-tryptophan inhibits anthranilate synthetase in *S. cerevisiae* [21, 22].

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