FURTHER STUDIES ON THE ISOLATION AND IDENTIFICATION OF ISONICOTINIC ACID HYDRAZIDE AND ITS METABOLIC PRODUCTS

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INTRODUCTION

In a former paper[1] we described the chromatographic methods that we used for the isolation and identification of isonicotinic acid hydrazide (INH) and its metabolic products. On another occasion[2] paper electrophoresis was used for the isolation of the isonicotinyl-hydrazone of pyruvic acid.

The possibility of combining the two techniques led us to study the use of buffered papers for the chromatographic separation of INH metabolites after the electrophoretic run.

Furthermore, new and more sensitive reagents were used for the localization of the spots and more derivatives were studied. In the present paper we report both the new methods and the results that complete our first paper on this subject.

MATERIALS AND METHODS

Deproteinization

The technique of centrifugal ultrafiltration[1], slightly modified[3,4], was always used for the separation of water-soluble compounds from proteins. The residue left after the evaporation of the filtrate (80°/vacuum), however, was now taken up in 1% (w/v) ammonium hydroxide[5]. This permitted a better recuperation of the derivatives after the chromatographic purification.

Chromatographic purification

Descending paper chromatography, as described previously[1], was used for the purification of INH metabolites. The use of ammonia solutions prevented the loss of derivatives such as isonicotinic acid, which have low R_F values when in the form of sodium salts[1]. The absorption region, localized under the U.V. lamp, was cut out and eluted with 1% ammonium hydroxide. Concentration of the eluted material, prior to paper electrophoresis or chromatography, was carried out at 80° under vacuum, and the dry residue was dissolved again in a few microliters of 1% ammonia.

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Paper electrophoresis

The use of paper electrophoresis permits the separation of the acid metabolites of INH, such as isonicotinic acid and the isonicotinyl-hydrazone of pyruvic acid, from the other metabolites. Our first studies[2] were carried out using *M*/15 phosphate buffer, pH 7.0, but more recent experiments have shown that 0.1 *M* TRIS (tris-hydroxymethyl-aminomethane) buffer, pH 7.0, is superior. The samples were spotted on the paper (Macherey-Nagel No. 261), which was then sprayed with the buffer solution, excess buffer being removed by blotting with filter paper. Electrophoresis was then carried out as usual (in our case in a Shandon apparatus), applying a potential of 300 V for a period of 3 hours.

Paper chromatography

Two-dimensional paper chromatography was carried on as described previously[1], with the exception that, for the acid metabolites, ammonium salts were now employed. On the other hand, the use of papers impregnated with 0.1 *M* TRIS buffer, pH 7.0, gave good results and opened up the possibility of combining paper chromatography and paper electrophoresis in two-dimensional separations. In this case the best results were obtained with isoamyl alcohol and with *n*-butyl alcohol, both saturated with 0.1 *M* TRIS buffer, pH 7.0.

Paper electrophoresis combined with paper chromatography

On a sheet of Macherey-Nagel No. 261 filter paper, measuring 20 × 37 cm, a line was drawn at 2.5 cm from the longer edge; another line was drawn in the middle of this line and perpendicular to it, the intersection being the starting point. The samples were spotted and the paper was sprayed with TRIS buffer as described. Paper electrophoresis was then carried out along the longer axis of the paper under the same conditions as before. After the electrophoretic run the paper was dried in the oven (80°) and ascending paper chromatography was carried out along the shorter axis, using one of the buffered solvents quoted above.

Localization of the spots

As was seen before[1], certain compounds, such as the isonicotinyl-hydrazone of pyruvic acid, give only discrete absorption spots under the U.V. lamp and a negative reaction with cyanogen bromide. We found that spraying with 0.1 *N* HCl and drying in the oven at 80° before a second exposure to BrCN made them appear as bluish spots that were easily discernible.

Very good results were also obtained with the reagent recently devised by GREULACH AND HAESLOOP[6] for the identification of hydrazide derivatives (1% w/v aqueous ferric chloride plus an equal volume of 1% w/v aqueous potassium ferricyanide). This reagent has the advantage that it can be used after the reaction with cyanogen bromide. Isonicotinic acid and isonicotinamide do not react.

The complete procedure for the localization of INH metabolites is then as follows: (1) observation under the U.V. lamp; (2) treatment with cyanogen bromide for 1 h followed by observation under the U.V. lamp; (3) exposure to ammonia vapours and observation in daylight and under the U.V. lamp; (4) spraying with 0.1 *N* HCl and drying in the oven at 80°, followed by treatment with BrCN and

observation under the U.V. lamp (in some cases the second exposure to BrCN is not necessary, depending on the degree of impregnation after the first one); (5) spraying with the GREULACH-HAESLOOP reagent.

RESULTS AND DISCUSSION

Great improvements on our former techniques for the chromatographic purification of INH metabolites were obtained by using 1% ammonium hydroxide as solvent. In Table I the R_F values are given for the metabolites that were most affected by the change—isonicotinic acid (INAcid), the isonicotinyl-hydrazone of pyruvic acid (Py.INHzone) and the isonicotinyl-hydrazone of acetaldehyde (Ac.INHzone)—besides those obtained for the derivatives that have not been studied before—isonicotinamide (INAmide) and di-isonicotinic acid hydrazide (di-INH).

TABLE I

R_F-VALUES OF SOME INH-METABOLITES ON MACHEREY-NAGEL NO. 261 FILTER PAPER

Sample	*R_F value in solvent**		
	1	*2*	*3*
Py-INHzone**	0.41–0.22	0.30–0.17	0.56
Ac-INHzone	0.40	0.29	0.70
INAcid**	0.29	0.27	0.61
INAmide	0.66	0.93	0.72
Di-INH	0.37	0.85	0.67

* 1 = Isopropanol–1% NH_4OH (20:3) (ascending); 2 = Butanol saturated with 1% NH_4OH (ascending); 3 = Propanol–NH_4OH (70:30) (descending).
** As ammonium salts.

As can be seen, the use of 1% ammonia as solvent prevents the loss of isonicotinic acid, which occurred when the metabolites were purified as their sodium salts[1], since now the R_F values in propanol-ammonia are all over 0.5.

On the other hand, the isonicotinyl-hydrazone of pyruvic acid, as its ammonium salt, gave double spots in isopropanol–ammonia and in ammonia-saturated butanol. The spots were eluted and the amount of pyruvic acid in each one of them was calculated as described previously[2]. 56.2% of the hydrazone was found in the spot with the higher R_F value and 43.8% of it was found in the slowest spot. It was assumed that the spots were due to isomers of the same derivatives.

The values given in Table I, together with the ones already found for INH and its metabolites[1] were plotted in the map shown in Fig. 1, which represents a two-dimensional chromatogram run by the ascending technique with (a) isopropanol–1% ammonia (20:3) and (b) ammonia (1%)–saturated butanol.

In Fig. 1 we can see that one of the spots of the ammonium salt of the isonicotinyl-hydrazone of pyruvic acid is partially superimposed by the spot of the isonicotinyl-hydrazone of acetaldehyde. In this respect the sodium salt is superior, since it has a lower R_F value[1] and superposition is avoided.

The results obtained by paper chromatography of INH metabolites on TRIS-buffered papers are shown in Table II. As can be seen, *n*-butanol saturated with

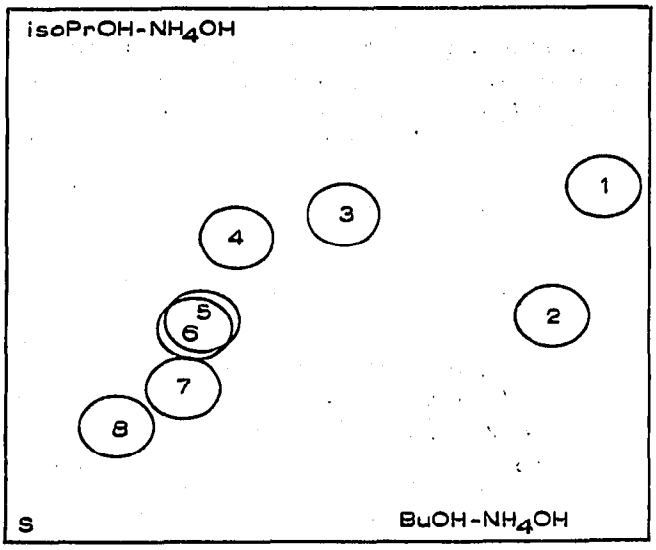

Fig. 1. Map of a two-dimensional paper chromatogram of INH metabolites (Macherey-Nagel No. 261 filter paper, unbuffered) run with (a) isopropanol–NH_4OH and (b) butanol–NH_4OH, by the ascending technique. (1) INAmide; (2) di-INH; (3) INH; (4) acetyl-INH; (5) Py-INHzone (56.2% of the ammonium salt); (6) Ac-INHzone; (7) INAcid (ammonium salt); (8) Py-INHzone (47.7% of the ammonium salt).

TRIS separates the two isomers of the isonicotinyl-hydrazone of pyruvic acid, but this does not happen with isoamyl alcohol saturated with TRIS.

The use of 1% ammonia as solvent is essential when buffered papers, such as those described are used. We found that sodium carbonate affects the displacement of the metabolites by interfering with the buffer. This does not happen when they are

TABLE II

R_F-VALUES OF INH AND SOME OF ITS METABOLITES ON MACHEREY-NAGEL NO. 261 FILTER PAPER IMPREGNATED WITH 0.1 *M* TRIS BUFFER, pH 7.0

Sample	*R_F value in solvent**	
	1	*2*
INH	0.40	0.62
Acetyl-INH	0.39	0.57
Py-INHzone**	0.05	0.06–0.33
Ac-INHzone	0.47	0.63
INAcid**	0.08	0.23
INAmide	0.51	0.62
Di-INH	0.45	0.63

* 1 = Isoamyl alcohol saturated with TRIS buffer (ascending); 2 = Butanol saturated with TRIS buffer (ascending).
** As ammonium salts.

dissolved in ammonia (Table III), since no residue is left after drying the spotted samples.

Attention must be paid to the fact that the ammonium salt of isonicotinic acid sublimes at rather low temperatures, and that some loss is bound to occur when the papers are dried in the oven at 110°.

TABLE III

EFFECT OF THE SOLVENT UPON THE R_F-VALUE ON BUFFERED PAPERS

Macherey-Nagel No. 261 filter paper impregnated with 0.1 *M* TRIS buffer, pH 7.0. Solvent system: isoamyl alcohol saturated with 0.1 *M* TRIS buffer, pH 7.0. Sample: isonicotinyl-hydrazone of acetaldehyde, 0.1 % (w/v).

Sample (µg)	*Solvent*	R_F	*Interfering substance (µg Na_2CO_3)*
2	1 % NH_4OH	0.69	—
4	1 % NH_4OH	0.68	—
6	1 % NH_4OH	0.68	—
2	10 % Na_2CO_3	0.64	20
4	10 % Na_2CO_3	0.58	40
6	10 % Na_2CO_3	0.42	60

The electrophoretic mobilities found for the various metabolites of INH on Macherey-Nagel filter paper impregnated with 0.1 *M* TRIS buffer, pH 7.0, after application of a potential of 300 V during 3 hours, are shown in Table IV.

As was noticed before, paper electrophoresis is specially useful for the separation of the acid metabolites, such as isonicotinic acid and the isonicotinyl-hydrazone of pyruvic acid, especially when combined with ascending paper chromatography, as shown in Fig. 2.

Table V summarises the results obtained with the sequential procedure for the localization of INH and its metabolites. It must be noted that the colours produced by the reactions involving cyanogen bromide are frequently affected by traces of

TABLE IV

ELECTROPHORETIC MOBILITY OF INH AND ITS METABOLITES

Macherey-Nagel No. 261 filter paper impregnated with 0.1 *M* TRIS buffer, pH 7.0; potential applied 300 V for 3 hours

Sample	*Displacement*	
	pole	*cm*
INH	—	1.4
Acetyl-INH	—	1.4
Py-INHzone	+	5.8
Ac-INHzone	+	1.6
INAmide	—	1.4
INAcid	+	8.5
Di-INH	+	1.6

ammonia in the paper, by the concentration of BrCN within the developing chamber and by the time of contact. A simpler procedure giving more consistent results, consists in observing the chromatogram under the U.V. lamp and placing it in an atmosphere of BrCN plus ammonia, thus omitting steps 2 and 3.

TABLE V

RESULTS OBTAINED WITH THE SEQUENTIAL PROCEDURE FOR THE LOCALIZATION OF INH AND SOME OF ITS METABOLITES ON PAPER CHROMATOGRAMS

(The values given are the amounts in μg)

Step	*Description*	*INH*	*Acetyl-INH*	*Py-INHzone*	*Ac-INHzone*	*Di-INH*	*INAcid*	*INAmide*
1	U.V.	brownish	brownish	brownish	slate	slate	brownish	brownish
		5	15	2	1	2	15	10
2	BrCN/visible	—	brownish	brownish	brownish	brownish	—	—
		—	15	4	1	2	—	—
3	BrCN/U.V.	brownish	yellow	brownish	brownish	brownish	brownish	brownish
		5	1	2	0.6	0.6	10	10
4	NH_4OH/visible	yellow	brownish	brownish	brownish	brownish	yellow	yellow
		0.4	1	4	0.8	0.6	0.5	0.5
5	NH_4OH/U.V.	yellow	yellow	brownish	brownish	brownish	yellow	yellow
		1	0.5	2	0.2	0.2	0.5	0.5
6	HCl/BrCN/U.V.	—	yellow	bluish	brownish	bluish	—	—
		—	1	2	0.6	2	—	—
7	GREULACH-HAESLOOP	deep blue	deep blue	deep blue	deep blue	deep blue	—	—
		1	0.2	0.6	0.2	0.2	—	—

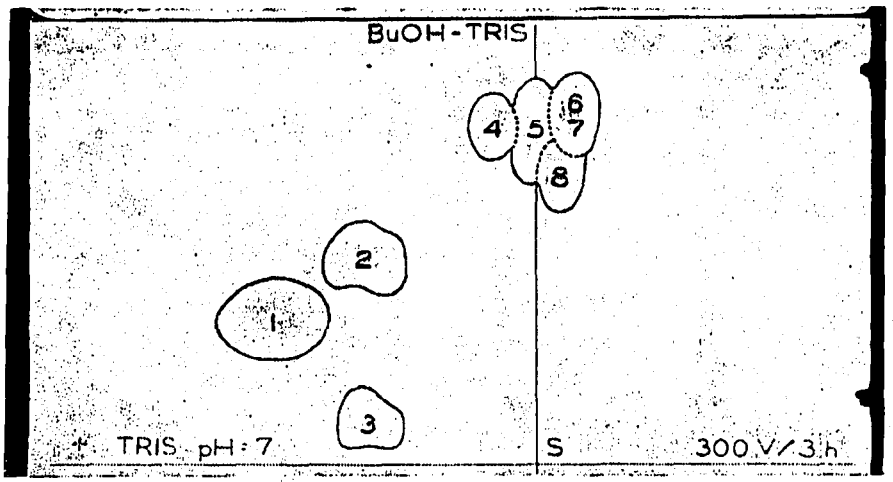

Fig. 2. Two-dimensional paper electrophoresis (0.1 *M* TRIS buffer, pH 7.0, 300 V, 3 h) and ascending paper chromatography (*n*-butanol satd. with TRIS buffer) of INH and some of its metabolic products: (1) INAcid; (2) and (3) Py-INHzone; (4) di-INH; (5) Ac-INH-zone; (6) INH; (7) INAmide; (8) acetyl-INH.

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SUMMARY

Proceeding with former studies[1] on the isolation and identification of some of the metabolic products of isonicotinic acid hydrazide, the authors combined paper electrophoresis and paper chromatography in two-dimensional separations, which they found to be specially useful for the study of acid metabolites, such as isonicotinic acid and the isonicotinyl-hydrazone of pyruvic acid. Better separations of the non-acid metabolites were obtained as before[1], by means of two-dimensional paper chromatography on unbuffered papers.

Dissolution of the metabolites in 1% ammonium hydroxide instead of in 10% sodium carbonate as usual, gave better results in the sense that isonicotinic acid was not lost during the chromatographic purification and that the R_F values on the buffered papers were not affected by the amount of solution spotted.

The technique for the localization of the spots was greatly improved by the use of a new and very sensitive reaction for hydrazide derivatives, carried out at the end of a sequential procedure devised to reveal all the metabolic derivatives in the range of 1 to 0.2 μg/sq. cm.

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