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A COMPARATIVE STUDY ON THE METABOLISM OF *ISO*NICOTINIC ACID HYDRAZIDE AND OF *ISO*NICOTINOYL-HYDRAZINOMETHANESULPHONIC ACID IN THE RAT

BY

G. CERIOTTI, A. DEFRANCESCHI, I. DE CARNERI, AND V. ZAMBONI

From the Istituto Carlo Erba per Ricerche Terapeutiche, Laboratorio di Microbiologia, Milan, Italy

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The discovery of the antituberculous activity of *iso*nicotinic acid hydrazide (isoniazid) has stimulated work intended to find new derivatives equally or more active, but less toxic than isoniazid itself. As reported in a previous paper (Ceriotti and Franceschini, 1953), we found that N'-*iso*nicotinoylhydrazinomethanesulphonic acid* (INHM) had these advantages.

During the study of the activity of this drug, a discrepancy was observed between the *in vitro* and the *in vivo* activity (Ceriotti and Franceschini, 1953; Ceriotti, 1953a). The *in vitro* activity of INHM was somewhat lower, the *in vivo* higher, when compared with a corresponding amount of isoniazid.

This interesting feature could be ascribed either to a difference in the mode of action of the two drugs or to a difference of their behaviour when introduced into the organism. Although some difference in the mode of action of the two drugs could not be excluded (Ceriotti, 1953b), the more likely explanation seemed to depend upon some quantitative or qualitative difference in their metabolism.

To compare the fate of isoniazid and INHM in the organism, and to study their physico-chemical properties, the present researches were accordingly planned, in the hope that they might throw light on the immediate problem and have some significance also in the field of general pharmacology.

MATERIAL AND METHODS

The isoniazid and the INHM used throughout our experiments were prepared by the chemical department of our Institute (Table I). For most of the experiments, the Ca salt of INHM was used. When small volumes at high concentration had to be used, for intratracheal and intracranial administration for instance, the Na salt was employed, owing to its higher solubility in water. The amounts of INHM were calculated on the basis of the isoniazid content of this compound.

The concentrations of the drugs were always determined both by chemical and by microbiological methods. It was thus possible to evaluate the degree and the rate of inactivation of the two compounds.

The preparation of samples for assay was different according to the material used and the method applied. No special preparation was required for urine specimens, which were simply diluted on the basis of their presumed content. A series of assay dilutions were made previously and then, from the results of these first determinations, the samples were brought to a concentration resembling as closely as possible that of the standard solution, that is, 2.5 μg./ml., corresponding to the middle part of the average standard curve.

For blood, the determinations were made on serum heated for 10 minutes in a boiling water-bath in centrifuge tubes; the tubes were cooled under running water, some quartz sand added, and they were then centrifuged at 4,000 rev./min. for half an hour. The addition of quartz sand assisted expression of the liquid from the abundant protein precipitate. The clear supernatant fluid was used for the determinations.

Intestine and lungs were carefully freed of fat and connective tissue, minced, mixed with quartz sand, frozen with solid carbon dioxide and triturated to a fine powder. They were then extracted with distilled water by boiling for 10 minutes. The amount of water corresponded to about ten times the weight of the sample used; it could be more or less according to the amount of drug present, giving a lower or higher dilution. An alternative method was also used, which gave similar results. The powdered organs were extracted by repeated washings with distilled water at room temperature, the washes combined and heated for 10 minutes in a boiling water-bath, and then centrifuged again. For intestine, the supernatant fluid was sufficiently free from proteins to allow direct use for the determination.

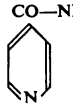
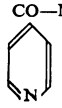
For lungs, boiling for 10 minutes was not enough to precipitate the proteins consistently. As the presence of proteins influences the results of the determination by the microbiological test considerably, additional treatment was required. To four parts of

*See Table I for structural formu'a. This compound has also been called *iso*nicotinic acid hydrazide methanesul honate.

TABLE I

PHYSICO-CHEMICAL PROPERTIES OF ISONIAZID AND INHM

I.T. is the iodometric titre, which is the amount in grammes corresponding to 1 ml. of a 0.1 NI solution

	M.W.	m p.	$E_1^{1\%}$cm. (H_2O)	Log. ε	I.T.		Solubility (20° C.)		
					Theor.	Found	Water	Ethanol	Acetone
 Isoniazid	137·1	169–171°	309 (268·5 mμ)	3·628	0·00343	0·00348	11·5%	2·4%	2%
 INHM Na salt	271·2		158 (263 mμ)	3·631	0·00339	0·00346	12%	0·08%	0·0008%
 INHM Ca salt	536·5	205–210°	159 (263 mμ)	3·630	0·00336	0·00346	2%	0·024%	0·0012%

extract, prepared as described above, by hot or cold procedure, one part of acetate buffer solution 0.01M at *p*H 4.4 was added, the tubes shaken well and kept for 5 minutes at 60° C. The protein precipitate was centrifuged out. To the limpid supernatant fluid, three parts of phosphate buffer 1/15M at *p*H 7 were immediately added. By this treatment the original extract was diluted twofold.

Several recovery experiments were made to test the validity of these methods of determination and extraction for urine, blood, intestine, and lungs of rats. For serum, intestine, and lungs the recovery is complete by chemical methods, and lies between 80 and 90% with the biological determination. The recovery from urine is complete for isoniazid when urine is diluted tenfold, whereas for INHM only 72% is recovered even at a dilution of 1:200. This is possibly due to the presence of some substances which counteract the action of INHM more than that of isoniazid. It is, of course, a disadvantage in so far as the exactness of the method is concerned; it poses however a very interesting biological problem, which will be discussed later. No special treatment was necessary for chemical determination in urine. For blood and organ extracts the preparation was, for the first part, the same as for the biological determination. However, as complete deproteinization is of the utmost importance, blood and intestine extracts were also always treated with acetate buffer. Both the microbiological and chemical determinations were always performed on the same sample.

Microbiological Determination.—For the microbiological determination, a plate method was worked out, using the strain of *M. tuberculosis* ATCC 607. Sensitivity with this plate method reached 0.5 μg. isoniazid per ml. The method has already been described (Ceriotti, 1952a and b). It has been improved by using an ATCC 607 strain made resistant to 2,000 μg./ml. of streptomycin. This allows the determination of INH and its derivatives in organic liquid extracts in the presence of large amounts of streptomycin.

The micro-organism for inoculation is cultivated in Youmans medium with the addition of 1% Tween 80, to allow diffuse growth, at 37° C. for 24 hr. with shaking. The culture is then diluted to an optical density of 0.050, at 650 mμ, in test-tubes of 11 mm. in diameter, using the Coleman spectrophotometer. Two ml. of the bacterial suspension is thoroughly mixed with 80 ml. of the agar medium, kept liquid at 45° C. and poured on to a supporting layer of 240 ml. of the same medium on a rectangular plate of 20 × 32 cm. The solid medium is the conventional Youmans medium to which 10 g. Tween 80, 20 g. agar, and 7.5 mg. of malachite green per l. are added. After solidification of the agar, 48 holes are made in the plate. At least five holes are used for each dilution sample. A hole containing the standard solution at 2.5 μg./ml. of isoniazid, is alternated with a hole containing the solution under examination. The plates are kept at 37° C. for 20 hours and then the diameters of the inhibition areas are read. The best readings are between 1 and 4 μg./ml.; but 0.5 μg./ml. may often be estimated roughly.

The method described was not sufficiently sensitive for the study of the entire curve of blood levels after oral administration. Hence a dilution method was used, employing the *M. tuberculosis* H37Rv strain as

test micro-organism. The serum extracts, prepared under sterile conditions, were mixed in equal parts with Youmans medium at double the usual concentration; successive dilutions were then made with normal Youmans medium. The test-tubes were then inoculated with 0.01 mg. of the test micro-organism per ml. Controls were made by adding different amounts of the drugs to the serum extracts of untreated animals. Readings were taken after 14 days and the degree of inhibition recorded.

Chemical Determination.—The method used by us for chemical assay (Defranceschi and Zamboni, 1952) was based on the transformation of *iso*nicotinic hydrazide and its metabolic derivatives into *iso*nicotinic acid by alkaline hydrolysis in the presence of permanganate, followed by colorimetric determination of the *iso*nicotinic acid formed, by means of reaction with cyanogen bromide (Konig's pyridine nucleus reaction).

*Iso*nicotinic acid reacts with cyanogen bromide in alkaline medium, forming an intense yellow colour whose absorption maximum is at 427 mμ. The maximum depth of colour is generally obtained 15–25 minutes after addition of cyanogen bromide, when working under the best conditions, e.g., with phosphate buffer at *p*H 11, with cyanogen bromide in a final concentration of 0.5%. The reaction is performed at room temperature (20–25° C.).

The sensitivity is very high. Beer's law is followed well between 0.2 and 5 μg./ml. of final concentration in *iso*nicotinic acid (i.e., the concentration obtained after addition of reagent to the standard solutions).

Animals.—Albino rats of 170–200 g. were used. In single experiments the differences in weight did not exceed 5%. For the excretion experiments the rats were placed separately in glass metabolism cages. About half an hour before administering the drug, each animal was given 3.5 ml. of tap water by a stomach tube. Two and a half ml. more was given each time the urine was collected. The urinary bladder was expressed by massage and the cages washed thoroughly. Five rats were used for each group and their urines pooled.

When studying the blood levels or absorption via the intestine or lungs, the rats were killed at different times after administration of the drug, without giving them water. Blood was collected by puncture of the abdominal aorta, under ether anaesthesia.

A single dosage of 25 mg./kg. was used throughout all these experiments, irrespective of the route of administration. For INHM, this dose was calculated on the basis of isoniazid content of the compound. Only for paper chromatographic study of urines was 100 mg./kg. administered in order to have a sufficiently high concentration.

Results

Intraperitoneal Administration

Excretion.—Elimination in the urine was followed, beginning 30 minutes after the injection, and then every hour until the sixth hour for isoniazid and the seventh hour for INHM (Tables II and III). In these tables the rate of excretion in μg./min. during the selected time is recorded. The ratio of data from biological to that from chemical determinations allows evaluation of the extent of degradation undergone by the drugs.

Excretion reaches its maximum during the first 30 minutes after the injection and then decreases very rapidly. About 75% of the drugs administered is excreted by the end of the experiment. Both drugs behave similarly; however, when the respective rates of excretion are compared, the elimination of INHM appears to be much higher than that of isoniazid during the first hour.

Considering now the amount of active drug present in the urine, it may be seen that INHM undergoes less degradation than isoniazid. This seems not only to be related to the higher rate of excretion but to a greater stability of INHM; in fact, for this compound, some activity may still be detected seven hours after administration, whereas for isoniazid no activity is evident after five hours

TABLE II

ISONIAZID EXCRETION IN THE URINE (μG./MINUTE) AFTER ADMINISTRATION OF 25 MG./KG. BY DIFFERENT ROUTES

The data obtained by chemical and biological assays are expressed per kg. body weight

Time after Administration	Intraperitoneal		Intramuscular		Oral		Intratracheal		Intracranial	
	Chem.	Biol.	Chem.	Biol.	Chem.	Biol.	Chem.	Biol.	Chem.	Biol.
30 min.	93·5	35·0	111·0	63·6						
1 hr.	84·0	22·0	141·0	56·0	62·0	23·6	54·0	17·7	106·0	42·2
2 ,,	84·0	9·0	107·0	21·4	142·0	25·0	80·4	12·9	110·0	22·5
3 ,,	70·0	5·0								
4 ,,	50·0	0·4	41·0	3·2	57·0	1·9	56·9	4·5	46·0	3·4
5 ,,	18·0									
6 ,,	11·0		38·0	1·4	25·0		22·3		23·2	
8 ,,			14·3	0·2	10·4		8·7		8·0	
12 ,,			2·7		3·4		3·1		3·4	
24 ,,			0·15		1·1		1·4		1·2	
% recovery ..	77·5	10·2	103·0	26·0	100·0	12·5	81·0	9·5	96·0	17·2

TABLE III

INHM EXCRETION IN THE URINE (μG./MINUTE) AFTER ADMINISTRATION OF 25 MG./KG. BY DIFFERENT ROUTES

The data obtained by chemical and biological assays are expressed per kg. body weight

Time after Administration	Intraperitoneal		Intramuscular		Oral		Intratracheal		Intracranial	
	Chem.	Biol.	Chem.	Biol.	Chem.	Biol.	Chem.	Biol.	Chem.	Biol.
30 min.	191·0	167·0	271·0	220·0						
1 hr.	138·0	83·0	129·0	75·2	4·9	1·6	35·0	21·2	141·0	82·7
2 ,,	55·0	10·3	60·0	22·1	12·7	2·7	45·3	12·9	81·0	23·6
3 ,,	32·6	1·5								
4 ,,	31·0	0·88	30·0	4·1	21·5	1·9	52·0	5·3	40·0	14·0
5 ,,	12·0	0·40								
6 ,,	12·7	0·16	16·8	0·45	70·0	2·4	53·0	4·0	18·4	1·75
7 ,,	1·8	0·16								
8 ,,			5·0	0·10	23·9	0·3	27·2	0·9	9·4	0·83
12 ,,			1·7		9·6		13·7	0·03	3·8	0·36
24 ,,			0·4		4·5		2·6		1·5	0·03
% recovery ..	74·0	33·0	90·0	43·0	80·0	3·2	103·0	13·0	92·0	32·4

even though the total amount excreted at this time is considerable.

Blood Levels.—The blood levels are given in μg./ml. of serum. The decrease is very rapid for both isoniazid and INHM, as anticipated by the data on the rate of excretion ; the blood level of INHM decreases more promptly than that of isoniazid. No activity can be detected two hours after administration with the method used (Fig. 1).

Intramuscular Administration

Excretion.—The rate of excretion was followed from 30 minutes to 24 hours (Tables II and III). A comparison between the two drugs shows the same features as for intraperitoneal administration, that is, a higher rate of excretion and less degradation of INHM. However, if the results with both drugs are compared with the corresponding data after intraperitoneal administration, the rate of excretion appears to be greater after intramuscular administration, both for isoniazid and INHM ; furthermore, isoniazid undergoes less destruction when given this way.

Oral Administration

The drugs were administered by stomach tube in 2.0 ml. water. This route is the most interesting because it is commonly used in clinical treatment and because it has been employed by us for the assay of antituberculous activity of the drugs in mice. Therefore, the rate of absorption from the gastro-intestinal tract was investigated as well as the blood levels.

Excretion.—When compared with corresponding experiments after intraperitoneal and intramuscular injection, the results obtained (Tables II and III) show a very marked reduction of the excretion rate of both drugs. Furthermore, the excretion rates of the drugs are reversed and the excretion of

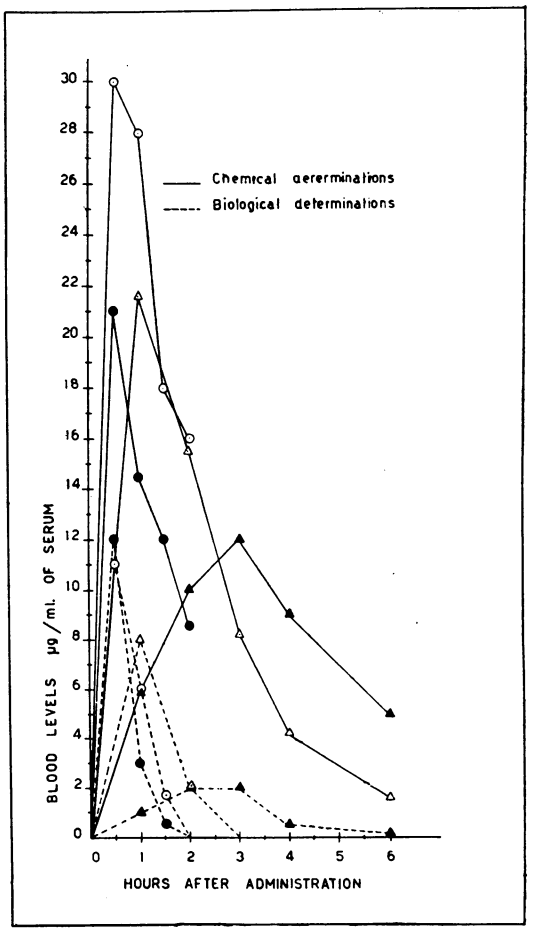


FIG. 1.—Blood levels after administration of isoniazid and INHM by different routes, in μg./ml.; chemical and biological determinations. ○ Isoniazid intraperitoneal route; △ isoniazid oral route; ● INHM intraperitoneal route; ▲ INHM oral route

isoniazid is now much more rapid than that of INHM. The data from the biological and chemical determinations must be considered separately. The chemical data show a clear displacement of the peak excretion rate from 30 minutes (parenteral administration) to the second hour for isoniazid and to the sixth hour for INHM. The peak excretion rate cannot be determined from the microbiological determinations.

For isoniazid these data are similar to those obtained after intraperitoneal injection ; for INHM they are very much lower. Some active substance is still present at the sixth hour after INHM and at the fourth hour after isoniazid. The total amount of active drug recovered after administration of INHM is lower than that after isoniazid, probably because the former remains longer within the organism.

Blood Levels.—In order to follow as completely as possible the curve of the blood levels, besides the plate method with ATCC 607, the dilution method with strain H37Rv was also used.

From both the microbiological and chemical determinations, the different behaviour of the two drugs appears very clearly. The maximum blood level of isoniazid is reached at the first hour and then decreases very rapidly, although there is a rather large amount of inactive compound still present. The levels of INHM are never so high as those of isoniazid. However, they are more constant and prolonged ; a small amount of active substance is still present after six hours, and, as seen from the chemical determinations, a maximum is reached at the third hour.

Absorption.—An analysis of the data on excretion and blood levels after administration of the two drugs by different routes induced us to believe that the differences found could only be ascribed to a different rate of absorption. Therefore, absorption from the gastro-intestinal tract was followed by determining the amount of the drugs still present, at different times after administration. The stomach and small intestine, and the large intestine, were studied separately.

A most striking difference (Tables IV and V) between the two compounds was revealed by these experiments. Isoniazid was absorbed very rapidly. During the first 30 minutes more than two-thirds disappeared from the intestine ; after four hours the drug had been almost completely absorbed before reaching the large intestine. Furthermore, the amount present in the intestine was largely inactivated. INHM, on the contrary, was absorbed at a very much slower rate ; after six hours, one-fourth of the total drug administered was still present, mostly in the large intestine. The amount of inactivated drug was also much smaller than after isoniazid ; inactivation seemed to take place more in the small than in the large intestine.

TABLE IV

PERCENTAGE RECOVERY OF ISONIAZID FROM THE INTESTINE AT DIFFERENT TIMES AFTER ORAL ADMINISTRATION OF 25 MG./KG. BODY WEIGHT

Time after Administration	Biological Assay	Chemical Assay	Biol. × 100 / Chem.
30 min.	12·2	31	39
1 hr.	8	23	35
2 ,,	4	15	27
3 ,,	1·8	7·7	23
4 ,,	1	6·6	15

TABLE V

PERCENTAGE RECOVERY OF INHM FROM THE INTESTINE AT DIFFERENT TIMES AFTER ORAL ADMINISTRATION OF 25 MG./KG. BODY WEIGHT, CALCULATED AS ISONIAZID

a = stomach + small intestine; b = large intestine

Time after Administration	Biological Assay	Chemical Assay	Biol. × 100 / Chem.
30 min. a	96	93	103
1 hr. a	77	90	85
2 ,, a	34 } 51·6	45·3 } 69·3	75
2 ,, b	17·6 }	24 }	73
3 ,, a	6·4 } 40·8	13 } 59·5	49
3 ,, b	36·4 }	46·5 }	78
4 ,, a	5·5 } 5·7	11·2 } 45·7	49
4 ,, b	20·2 }	34·5 }	59·5
6 ,, a	—	5·1 } 24·1	
6 ,, b	13·8	19 }	72·5

An apparent discrepancy arises when comparing the inactivation rate of the two drugs within the intestine and the respective amounts of active substance found in the urine. This may easily be explained by the rapid absorption and, consequently, rapid excretion of isoniazid before inactivation could be completed by the organism ; in fact *in vitro* studies (De Carneri, 1952, unpublished) show that the time required for inactivation is rather long even in organs as active as liver, at optimal concentrations.

Intratracheal Instillation

Administration by this route was studied for two reasons ; firstly, to see if absorption through the bronchial mucosa, like the intestinal, could also reveal differences of behaviour between isoniazid and INHM and, secondly, because it would be interesting, from a practical point of view, to see whether it was possible to obtain a high concentration of these two drugs in the lungs, which was unobtainable by other routes.

Direct instillation was preferred to administration by aerosol in order to have more accurate

control of the amount introduced. Therefore, after incision of the skin of the throat under local anaesthesia, the salivary glands were separated in the mid line and the trachea reached through the pretracheal muscles and freed for about half a centimetre. The solution (0.1 ml.) was then instilled slowly into the tracheal lumen, using a very fine needle.

Excretion.—The results (Tables II and III), are very similar to those obtained by oral administration. The excretion peak, as seen from the data on chemical determinations, is reached at the fourth hour after isoniazid and at the sixth hour after INHM. However, when the results of the microbiological determinations are considered, the differences between the two drugs are not so striking. In fact, after both isoniazid and INHM, the maximum amount of active substance is excreted in the urine during the first hour. The difference between isoniazid and INHM consists chiefly in the fact that the active substance is excreted in higher quantity and over a longer period after the latter drug.

Absorption—(Table VI).—Absorption is very rapid. After isoniazid the drug cannot be detected in the lungs as early as 30 minutes after instillation. INHM is present in active form until the second hour but only to about 1% of the amount injected. However, if the ratio of drug to weight of pulmonary tissue is examined, the concentration appears to be high, and worth considering for therapeutic purposes.

Intracranial Administration

Intracranial administration is also interesting both from a theoretical and a practical point of view. It would, in fact, be worth knowing whether the meninges behave similarly to the intestinal and bronchial or to the peritoneal membrane. Furthermore, as streptomycin is usually preferred to dihydrostreptomycin in the treatment of meningeal tuberculosis, and its toxicity is increased by isoniazid, though not by INHM (Ceriotti, 1953, c and d), it was important to learn whether the latter could be advantageously substituted for the former.

The drugs were injected intracranially in 0.05 ml. of solution by the transorbital route. A short period of convulsions sometimes followed the injection; the animals, however, usually showed no important disturbances. Only the excretion was studied.

Excretion.—Excretion after intracranial administration (Tables II and III) does not show great differences between isoniazid and INHM. The maximum is attained during the first hour after INHM, a little later after isoniazid; there is then a sharp decrease in the excretion rate. However, it may be also observed here that the degradation of INHM proceeds at a lower rate than that of isoniazid. In fact, some active substance is still detectable in the urine at the twelfth and sometimes at the twenty-fourth hour after INHM administration; after isoniazid on the other hand no activity can be found after the fourth hour.

Chromatographic Analysis

In order to learn how isoniazid and INHM were degraded during their passage through the body, a paper chromatographic method for examination of the urine was devised; a more detailed description will be published elsewhere (Defranceschi, 1953). The dose of both compounds administered during this experiment was 100 mg./kg. intraperitoneally in order to give rather high concentrations in the urine.

The urine content of isoniazid and INHM was determined both chemically and microbiologically, and the concentration was then adjusted so as to give 20 μg. in 10 μl. as determined chemically. This was not possible for urine samples collected after 120 minutes, because they were already too diluted; a larger amount of urine was therefore used without further dilution. Standard solutions

TABLE VI

ISONIAZID AND INHM, RECOVERY FROM LUNGS AT DIFFERENT TIMES AFTER INTRATRACHEAL INSTILLATION OF 25 MG./KG. BODY WEIGHT

Time after Administration	Isoniazid				INHM				
	Biological		Chemical		Biological		Chemical		Biol. × 100 / Chem.
	mg.	μg./g. of Lung	mg.	% of Drug Administered	mg.	μg /g. of Lung	mg.	% of Drug Administered	
30 min.	—	—	2·25	0·9	0·260	52·0	0·385	1·5	68
1 hr.					0·164	32·8	0·328	1·3	50
2 ,,					0·168	33·6	0·328	1·3	51
3 ,,							0·236	0·94	
4 ,,							0·215	0·86	

containing 20, 10, and 5 μg. in 10 μl., were developed at the same time. Several samples of each urine were chromatographed.

Strips of Whatman paper No. 1 (1.5 × 37 cm.) were used. The urine samples were applied 6 cm. from the edge, which was immersed in the solvent. Irrigation was carried out with the following solvent mixture: isoamyl alcohol (50), water (50), acetic acid (1.5). Only the organic (upper) layer was used. After development, the spots were detected both chemically and biologically.

For chemical detection the dried paper strips were suspended in a closed glass jar, saturated with cyanogen bromide vapours. After 20 minutes, 20 ml. of 3% aqueous ammonia was poured out on the bottom of the jar. After another 15 minutes, pyridine derivatives (namely, *iso*nicotinic acid, nicotinic acid, nicotinamide) gave an intense yellow colour; isoniazid and INHM gave brownish-yellow spots and were better detected under ultra-violet light (mineral light lamp), giving an intense bluish fluorescence. The Rf found after chromatography with the solvent mixture used are as follows: isoniazid (0.80), INHM (0.01), *iso*nicotinic acid (0.42), nicotinic acid (0.51), nicotinamide (0.48), and compound X (0.26).

The intensity of single spots could be estimated roughly by this method and microbiologically-inert compounds, containing the pyridine group, detected. More quantitative evaluation was obtained by the biological method, which estimates the active material. After development and drying, the strips were sterilized by ethylene oxide, in order to avoid contamination. They were then placed on large agar plates, inoculated with ATCC 607, as for the cup method. After 23 hours of incubation at 37° C., the diameters of the inhibition were recorded and the areas calculated by the ellipse formula. The areas of inhibition of standard samples, when plotted against the logarithm of the concentration, gave a straight line, which was taken as a basis for calculating the concentration of the active substances in samples of urine (Fig. 2).

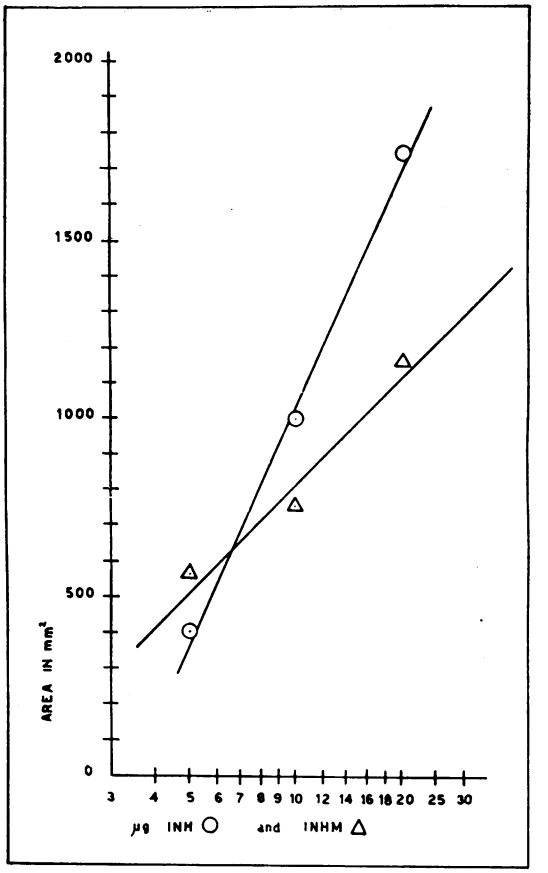


FIG. 2.—Filter paper chromatography of isoniazid and INHM. Detection on germ-agar plate (ATCC 607). Inhibition areas plotted against the log of the amount of drugs in μg.

This method is not strictly quantitative because the variations are still rather large. However, it allows a good semi-quantitative estimation of the amounts of active substances present. Furthermore, separation and identification of different active compounds is made possible. This is particularly important here, because it demonstrates that INHM gives rise to a moderate amount of isoniazid. The results obtained both by chemical and microbiological detection are summarized in Table VII. As may be seen from these data, the

TABLE VII

PAPER CHROMATOGRAPHY EXPERIMENTS ON URINES OF RATS, GIVEN 100 MG./KG. OF ISONIAZID AND INHM INTRAPERITONEALLY

The percentages of the biologically active substance found by direct micro-determination and after chromatography are compared

Time after Injection (min.)	Isoniazid				INHM				
	Micro-deter.	Chromatographic Data			Micro-deter.	Chromatographic Data			
	Biological Assay	Biological Assay	Isonicotinic Acid	Compound X	Biological Assay	Biological Assay INHM	As Isoniazid	Isonicotinic Acid	Compound X
30	94	80	+++	−	82	105	−	±	−
60	65	71	++++	+	64	94	±	++	±
120	43	40	++++	++	42	41	24	+++	++

percentages of biologically-active substance determined by chromatographic and plate-cup method agree rather well for isoniazid. For INHM, however, the plate gives consistently lower values. These results agree with those of the recovery experiments already discussed ; it seems that urine contains a substance or substances which inhibit the action of INHM more than that of isoniazid, and are dissociated from the active compound by the chromatographic procedure. *Iso*nicotinic acid is formed from both compounds, but at a much higher rate from isoniazid. Another not yet identified compound, which reacts with CNBr and has a lower Rf than *iso*nicotinic acid, is released more promptly by isoniazid. In conclusion, the paper chromatographic experiments show a more rapid degradation of isoniazid as compared with INHM ; some isoniazid is also formed from the latter.

Determination of Ionization Constants

Although the difference in the degree of dissociation of isoniazid and INHM arises directly from a consideration of their formulae, and needs no special demonstration, yet to obtain more detailed information and quantitative data, the ionization constants of the two compounds were determined. The determination for INHM was made by direct titration ; for isoniazid the spectrophotometrical method had to be used instead. Several related compounds were also studied by this method, and the results will be referred to elsewhere. For the SO_3 group of INHM, the pK_a was found to be 3.75 ; for isoniazid the hydrazine group gave a value of $pK_{b_1} = 10.3$, and the pyridine nitrogen of $pK_{b_2} = 12.2$.

It can be stated on the basis of these results that, at physiological *p*H, INHM is completely dissociated and isoniazid completely undissociated.

Discussion

Before discussing the results obtained, the methods of determination deserve some consideration. Recovery is practically complete, both by microbiological and by chemical determination, so far as intestine and lungs are concerned.

From serum, only 80% recovery is possible microbiologically. From urine isoniazid is recovered almost completely when urine samples are diluted 1 to 10 ; but INHM is recovered only partially even at a dilution of 1 to 200. This phenomenon may be explained by assuming that some substances which inhibit INHM are present in urine, and that these substances have only a very low inhibitory action towards isoniazid. This supposition is confirmed when the results obtained by the cup-plate method are compared with the microbiological data from chromatographic experiments. The ratio of biologically-active substances to the total determined chemically is much higher after chromatography. This means that the supposed antagonist substance or substances move along the paper strips at a different rate to INHM and the latter is therefore no longer inhibited and can exert its action completely. These facts suggest that the mode of action of the two substances may be partly different. This conclusion is supported also by the observation (Ceriotti, 1953b) that an ATCC 607 strain, made dependent upon INHM, does not grow in the presence of isoniazid. The difference is only partial, because some isoniazid is recovered in urine after the administration of INHM ; and this means that the latter certainly acts also through its degradation to isoniazid. The biological problem arising from the preceding results, and involving the mode of action of the two drugs, is now under examination.

Although the interference of unknown inhibitory substances does not allow an absolute evaluation of isoniazid by the microbiological cup-plate method in certain experiments, this does not seem to alter the general conclusions drawn from the results obtained, as far as the problems under study are concerned. Some of these conclusions may also be emphasized. In fact, a phenomenon generally observed in the majority of our experiments was the lower degradation rate of INHM as compared with isoniazid. This phenomenon is partially concealed by the antagonistic action of the urine, and it appears even more evident when the latter is excluded by chromatography. It must therefore be concluded that the difference in degradation rate between isoniazid and INHM is also greater than that one disclosed by the microbiological determinations. The lower degradation rate of isoniazid may be seen clearly from the results of experiments on absorption from the intestine as well as from the excretion data. In these experiments, the values from the microbiological cup-plate method can also be considered absolute, as shown by the recovery experiments. Isoniazid appears to be broken down considerably in the intestine before being absorbed, whereas INHM remains unaltered for a fairly long time.

The experiments with oral administration received particular attention because they are more intimately connected with *in vivo* tests for activity ; they were conducted by the diet–drug method. When the two drugs are administered by mouth, besides being rather rapidly destroyed, isoniazid is also absorbed at a much higher rate

than INHM and is therefore excreted more quickly. The blood levels attain a higher maximum with isoniazid, but they also fall rapidly. With INHM, on the contrary, as a result of the slow rate of absorption, the blood levels are not so high, but are maintained longer. Owing to the extreme sensitivity of *M. tuberculosis*. to these drugs, this behaviour seems to be more favourable to therapeutic action. In fact, if the concentration required for drug activity is low, it is conceivable that high, but transient, levels of drug concentration exert a lesser effect than low, but stable levels, provided that the latter exceeds the minimum required for effective action. Beyond these limits an increase in concentration may not be paralleled by a corresponding increase in activity. Both the lower degradation rate and slower absorption and, consequently, the more prolonged blood levels of INHM, administered orally, may account for the better protection afforded by this drug in experimental mouse tuberculosis.

Another important difference between isoniazid and INHM may be observed in relation to the various routes of administration. The data of excretion rates, as summarized in Figs. 3 and 4, show this difference clearly. After administration of isoniazid, the excretion maxima lie between half an hour for intraperitoneal, and two hours for oral administration. With INHM, on the other hand,

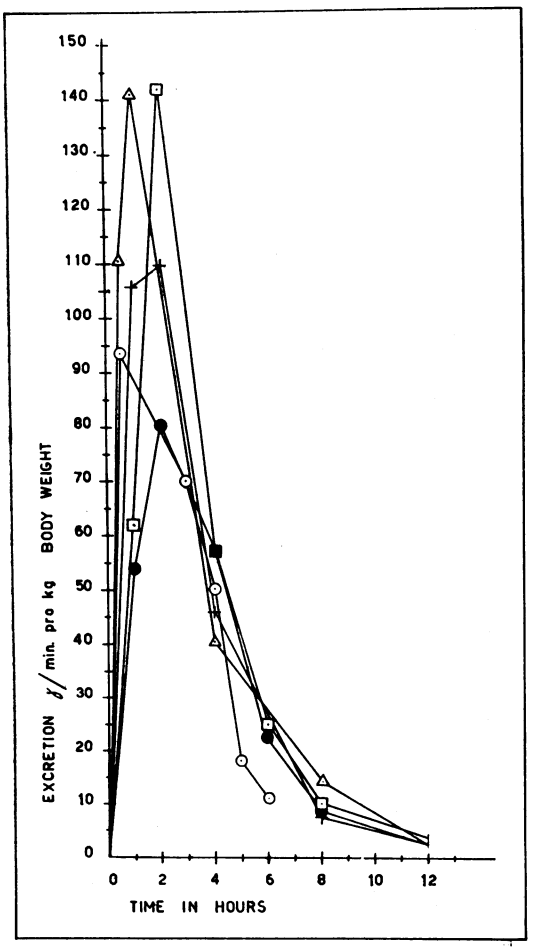


FIG. 3.—Excretion curves of isoniazid after administration by different routes. Results of chemical determinations in μg. per min. per kg. body weight. ⊙ Intraperitoneal route; △ intramuscular route; □ oral route; ● intratracheal route; × intracranial route.

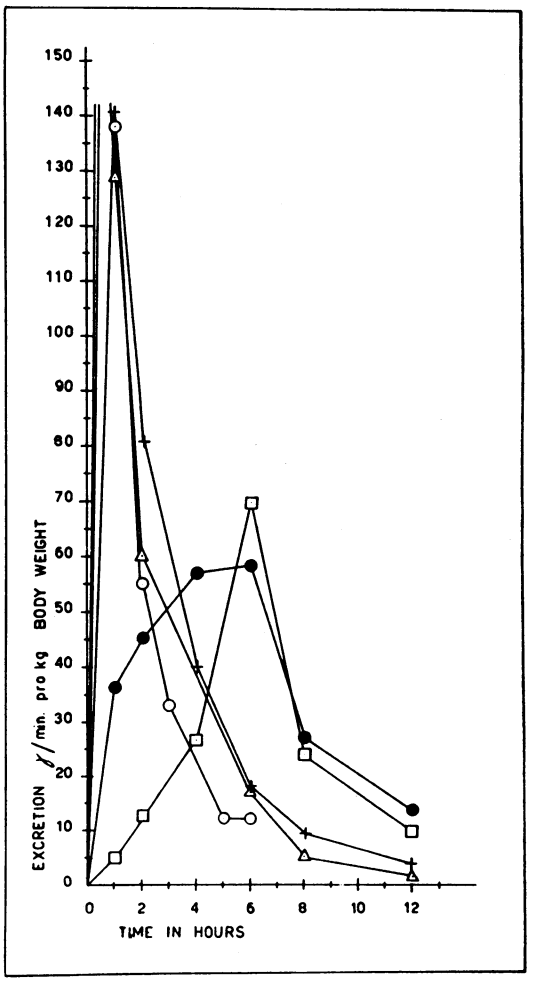


FIG. 4.—Excretion curves of INHM after administration by different routes. Results of chemical determinations in μg. per min. per kg. body weight. ⊙ Intraperitoneal route; △ intramuscular route; □ oral route; ● intratracheal route; × intracranial route.

there is a much larger variation. After intraperitoneal and intramuscular administration, excretion is almost immediate and reaches a very high peak after half an hour. After oral and intratracheal administration the maximum is shifted towards the sixth hour. These results are to be correlated with the different rates of absorption. For isoniazid, passage through the intestinal mucosa does not differ much from passage through the peritoneal serosa or the capillary walls, whereas INHM passes slowly through the mucosae. Recovery from the intestine gives direct evidence of this assumption, as does that from lungs, though this is not so striking.

The physico-chemical properties of the two drugs may supply some explanation of these findings. Isoniazid is soluble both in water and in organic solvents. INHM as the Na or Ca salt is soluble practically only in water; at *p*H 7–8, the former is completely undissociated, and the latter completely dissociated. It is well known that substances which are soluble both in water and organic solvents generally penetrate the intestinal wall most rapidly; moreover, the rate of absorption is dependent on the degree of dissociation, since it is higher for less dissociated compounds, as has been demonstrated for alkaloids and a large series of sulphonamide drugs (Travell, 1940; Ellison, and Richardson, 1938; Poth and Knotts, 1942; Poth, Knotts, Lee, and Jami, 1942; Fisher, Troast, Waterhouse, and Shamon, 1943). The rate of excretion, however, does not show a similar relationship (Fisher, Troast, Waterhouse, and Shamon, 1943). The present experiments agree well with the observations on sulphonamides, in so far as absorption through the intestinal wall is concerned. However, INHM readily crosses the blood–brain barrier, as shown by the results of intracranial administration.

In summing up the experiments described in this paper, some differences between isoniazid and INHM may be pointed out: INHM is absorbed at a much lower rate than isoniazid through the intestinal wall; it is also much less degraded and thus creates more stable active blood levels. This behaviour finds its basis in the physico-chemical properties of the two drugs. INHM seems to act both directly and through its transformation to isoniazid.

Summary

1. Absorption, blood levels and excretion rate were studied after administration of *iso*nicotinic acid hydrazide (isoniazid) and of sodium and calcium salts of N'-*iso*nicotinoylhydrazinomethanesulphonic acid (INHM) by different routes.

2. Degradation products were detected in the urine by paper chromatography.

3. Some physico-chemical properties of the two drugs were determined.

4. No great difference between isoniazid and INHM could be observed when intraperitoneal, intramuscular, or intracranial routes of administration were used. However, the highly-dissociated INHM was absorbed slowly through the intestinal wall. Absorption and excretion were also less after intratracheal administration and INHM was degraded at a lower rate than isoniazid.

5. *Iso*nicotinic acid and an unknown compound, reacting with BrCN, are formed both from isoniazid and INHM; however, this formation is less rapid after INHM, which also gives rise to some isoniazid.

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