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Rate of Inactivation of Isoniazid in South Indian Patients with Pulmonary Tuberculosis *

1. Microbiological Assay of Isoniazid in Serum following a Standard Intramuscular Dose

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Since isoniazid is metabolized in man to several derivatives with little or no specific activity against the tubercle bacillus, its rate of inactivation in the body may have an important bearing on its efficacy as an antituberculosis drug. The inactivation rate, though constant in any one person, is known to vary from individual to individual and from race to race. A series of studies on the rate of inactivation of isoniazid in Indian patients with pulmonary tuberculosis has recently been undertaken at the Tuberculosis Chemotherapy Centre, Madras. The present paper describes the first of these studies, in which the concentration of isoniazid in the serum of patients admitted to a controlled comparison of four domiciliary chemotherapeutic regimens was determined by microbiological assay four-and-a-half hours after administration of a standard dose of isoniazid (3 mg/kg body-weight). Patients with serum levels of 0.58 µg/ml or more were classified as slow inactivators of isoniazid and those with levels below 0.58 µg/ml as rapid inactivators. By this definition, 195 (61%) of the 321 patients studied were found to be slow inactivators and 126 (39%) rapid inactivators. A relationship was shown between sex and the rate of inactivation, there being a significantly higher proportion of rapid inactivators among the females than among the males. The observed estimates, of the error of the microbiological assay procedure are discussed and possible ways of reducing the error suggested.

INTRODUCTION

Isoniazid is metabolized in man to several derivatives of little or no antimicrobial activity against the tubercle bacillus (Bernstein et al., 1952 ; Hughes, 1953; Cuthbertson, Ireland & Wolff 1953). The rate of inactivation of isoniazid varies widely from subject to subject (Hughes, Schmidt & Biehl, 1955; Middlebrook & Dressler, 1956), but is constant for a given individual (Hughes, Schmidt & Biehl, 1955; Bell & Riemensnider, 1957b). The catalase activity and the degree of isoniazid-resistance of strains of tubercle bacilli isolated from patients during treatment with isoniazid have been shown to be related to the rate of inactivation of isoniazid (Mandel et al., 1957; Canetti & Grosset, 1958); the isoniazid-resistant strains which emerged during treatment in slow inactivators had a higher degree of resistance and were more frequently catalase-negative than the corresponding strains from rapid inactivators. Mitchell & Bell (1957) have provided suggestive evidence that the rate of inactivation of isoniazid influences the speed with which sputum conversion occurs.

The relationship between the rate of inactivation of isoniazid and the response to treatment has, therefore, been investigated in 321 of the 341 patients admitted to a controlled study of four different chemotherapeutic regimens administered at home, undertaken by the Tuberculosis Chemotherapy Centre (1960). In three of the regimens isoniazid was the sole antimicrobial agent, and in the fourth it was given with PAS. The serum isoniazid concentrations were determined after a

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standard test-dose of 3 mg/kg isoniazid. The test-dose of isoniazid was given by intramuscular injection instead of by mouth to avoid irregularities in absorption from the intestinal tract, as the patients, being treated at home, were not amenable to the dietary restrictions recommended by Bell & Riemensnider (1957b). The serum isoniazid concentrations were determined by the microbiological assay method of Mandel et al. (1956) in preference to chemical methods (Kelly & Poet, 1952; Cuthbertson et al., 1954; Short, 1954; Poole & Meyer, 1958; Berte et al., 1959) as the latter are either too cumbersome, not sufficiently sensitive or not specific for the antimicrobially active free isoniazid and hydrazones.

We report here investigations on the microbiological assay technique and the results of determinations of isoniazid inactivation rates of the patients in the controlled study. Other articles in this issue are concerned with the serum isoniazid concentrations in these patients when they were receiving their prescribed chemotherapy (Gangadharam et al., 1961 [1]) and with the relationship between the rate of inactivation of isoniazid and the response to treatment (Selkon et al., 1961 [2]).

MATERIALS AND METHODS

The 341 patients admitted to the controlled comparison of four regimens of domiciliary chemotherapy (Tuberculosis Chemotherapy Centre, 1960) had newly diagnosed pulmonary tuberculosis, and were aged 12 years or more.

The four prescribed regimens were:

PH. Isoniazid 3.9-5.5 mg/kg body-weight plus PAS (sodium) 0.2-0.3 g/kg daily, divided into two doses, by mouth.

HZ-1. Isoniazid alone, 7.8-9.6 mg/kg daily, in one dose by mouth.

HZ-2. Isoniazid alone, 7.8-9.6 mg/kg daily, divided into two doses, by mouth.

H. Isoniazid alone, 3.9-5.5 mg/kg daily, divided into two doses, by mouth.

Of the 341 patients, three are not considered in this report since they had received more than two weeks of antituberculosis chemotherapy prior to admission to the study; the vast majority (96.2%) of the remaining 338 patients had received none.

The results of the isoniazid inactivation rates of 17 patients were not available for the following reasons: 12 patients had died before the earliest month selected for the test; two had taken their discharge against medical advice; in two patients the tests were contaminated and in one patient the venipuncture was performed after five instead of four-and-a-half hours. The isoniazid inactivation rates of the remaining 321 patients were determined between their sixth and twelfth months of treatment. Additional investigations (see pages 770 and 771) were carried out on 16 newly diagnosed patients who fulfilled the criteria required for admission to the controlled study (Tuberculosis Chemotherapy Centre, 1960).

Procedure

The patient was taken off all drugs for two days before the test. In order to confirm that the treatment had, in fact, been discontinued, a specimen of urine was collected immediately before the injection of isoniazid and examined for the presence of isoniazid by the combined naphthoquinone-mercuric chloride test (Gangadharam et al., 1958). A test-dose of 3 mg/kg body-weight isoniazid [3] was given by intramuscular injection and a specimen of venous blood was collected four-and-a-half hours later. No dietary restrictions were imposed on the patient either before or during the test.

Culture medium

Liquid 7H-10 medium (Cohn, Middlebrook & Russell, 1959), without glycerol, was used in this investigation, with the following modifications:

(a) For growth of the inoculum, the medium contained; in addition to the basic ingredients, 0.5 % bovine albumin fraction V (Armour Laboratories), 0.2% glucose, 0.05 % Tween 80 and 50 µg/ml streptomycin (final concentrations).

(b) For the assay procedure, the medium contained, in addition to the basic ingredients, 0.5% bovine albumin fraction V, 0.2% glucose, 10 µg/ml p-aminobenzoic acid and 50 µg/ml streptomycin (final concentrations).

Assay organism

A culture of *Mycobacterium tuberculosis* strain H37RvSR, resistant to streptomycin, obtained by

[1] **See article on page 793.**
[2] **See article on page 779.**

[3] **The preparations used were Neoteben** (Bayer A.G., **Leverkusen, Germany) and Isonic (Chemidica S.A., Montreux, Switzerland), supplied in ampoules containing 100 mg/ml isoniazid.**

in vitro selection from strain H37Rv, was used as the assay organism. It was maintained by monthly subculture on Löwenstein-Jensen medium containing 1024 µg/ml streptomycin.

Test

A 1/5 dilution of the serum was prepared by adding 2.0 ml of the serum to 8.0 ml of the 7H-10 test medium. Serial twofold dilutions, from 1/10 to 1/80, were then prepared by adding 1.0, 0.5, 0.25 and 0.125 ml of the 1/5 dilution of serum to 7H-10 test medium, to make up the final volume to 2.0 ml. Whenever sufficient serum was available (95% of sera), tests were set up in duplicate from the same 1/5 dilution of the serum. The 2.0-ml volumes of the 1/5 to 1/80 dilutions of serum were inoculated with 0.1 ml of an 8-day-old culture of H37RvSR grown in the Tween-containing 7H-10 liquid medium, and then incubated at 37°C for five days. Smears were prepared by pipetting the unshaken deposit of growth on to slides, with the visual assistance of a concave mirror. The slides were stained in Coplin jars by the Ziehl-Neelsen technique and counterstained with Loeffler's methylene blue.

With each batch of tests, a control series of tubes was set up in duplicate containing 0.00, 0.02, 0.04 and 0.08 µg/ml isoniazid in water, prepared from a stock solution (100 µg/ml isoniazid) sterilized by filtration through sintered glass.

Reading and recording of the results

At least six fields of each smear were examined under x 700 magnification and the proportion of acid-fast bacilli was estimated as 0 %, 25 %, 50 %, 75 % or 100 %. The dilution end-point was defined as the dilution of serum (the mean dilution if tests were set up in duplicate) producing 50% loss of acid-fastness and was determined, where necessary, by interpolation. For example, if none of the bacilli in the 1: 10 dilution, and all the bacilli in the 1: 20 dilution were acid-fast, then the dilution which could have produced 50% loss of acid-fastness was estimated as 1: 15. The isoniazid concentration of each test serum was obtained by dividing the geometric mean of the isoniazid concentrations producing 50% loss of acid-fastness in the controls set up in the study by the dilution end-point of the serum.

RESULTS

The results of the microbiological assays of isoniazid were analysed after transformation to a logarithmic scale in which a twofold decrease in the serum dilution producing 50% loss of acid-fastness, or a twofold increase in isoniazid concentration, was given a value of one working unit., One working unit is, therefore, equivalent to one dilution step in the assay. In consequence, *all* mean isoniazid concentrations quoted are geometric means. Where appropriate, the transformed values were examined by analysis of variance.

Investigation of batch differences

The occurrence of variation between different batches of assays was studied on 399 sera, 321 from the same number of patients (page 766), 30 from further tests carried out on six of these 321 patients (page 770) and 48 from the eight additional patients studied in the investigation reported on page 770. The isoniazid concentrations of the 399 sera were assayed in 29 batches of tests, the number of sera tested in each batch ranging from four to 33. The control dilutions were contaminated in seven batches, leaving 22 batches in which the results on the controls were available. In calculating the isoniazid concentration of a test serum, it is usual (Mandel et al., 1956) to correct for possible variation from batch to batch by dividing the concentration of isoniazid which produces 50% loss of acid-fastness in the control in the particular batch by the dilution of the test serum which produces a similar loss of acid-fastness (the dilution end-point). Such a procedure assumes that factors affecting the results in any batch alter the end-point of the test serum and the control in a similar direction. This assumption was examined in the following way.

First, the isoniazid concentrations of the test sera were calculated by employing the controls set up in in the same batch as the test serum. The averages of the isoniazid concentrations of the test sera in each batch are set out under method I in Table 1. The variation in these averages from batch to batch was found to be significantly greater than the variation in isoniazid concentration from serum to serum within the same batch ($P < 0.005$). The magnitude of this batch variation, expressed as a standard deviation (the square root of the component of variance due to this source in the analysis of variance), was 0.32 dilution step.

Secondly, the isoniazid concentrations of the test sera were calculated by dividing the mean of the isoniazid concentrations which produced 50% loss of acid-fastness in the controls in the 22 batches (0.0344 µg/ml isoniazid) by the dilution end-point

TABLE 1

DISTRIBUTION OF THE MEAN CONCENTRATIONS OF ISONIAZID IN THE SERA IN DIFFERENT BATCHES OF TESTS, CALCULATED BY TWO METHODS

Mean concentration of isoniazid (µg/ml)	Test batches			
	Calculated from control in each batch (method I)		Calculated from the mean of controls in all batches (method II)	
	No.	%	No.	%
< 0.35	0	*(0)* [a]	1	*3*
0.35–	3	*(14)*	1	*3*
0.44–	6	*(27)*	14	*48*
0.56–	6	*(27)*	3	*10*
0.70–	5	*(23)*	5	*17*
0.88–	1	*(5)*	5	*17*
> 1.11	1	*(5)*	0	*0*
Total	22	*101*	29	*98*

[a] The parentheses indicate percentages based on fewer than 25 observations.

of the serum. The averages of the isoniazid concentrations in the 29 batches expressed in this way are set out under method II in Table 1. Again, the variation of these averages from batch to batch was significantly greater than the variation from serum to serum within the same batch (P = 0.01). The magnitude of the batch variation, also expressed as a standard deviation, was 0.26 dilution step, an estimate lower than the corresponding estimate of 0.32 obtained by the first method. Thus, there is no evidence from our results that the factors responsible for batch variation altered the end-points of the test serum and the control in the same direction in each batch. In calculating the isoniazid concentrations of the test sera the second method was therefore employed.

The concentrations of isoniazid which produced 50 % loss of acid-fastness in the controls set up in the 22 batches are presented in Table 2. The distribution of these concentrations has a mean of 0.0344 µg/ml and a range of 0.030-0.060 µg/ml. The skewness of this distribution suggests that a single factor, such as the ability of the test organisms to grow in the medium, may have been the principal cause of variation in the control end-points. It will be appreciated that the dilutions of the test sera, but not those of the controls, contained human serum and that the presence of this human serum might have promoted the growth of the test organism. Thus, the failure of the controls to diminish batch variation might be due to this difference.

Effect of streptomycin

In the early stages of the use of the microbiological assay procedure in this laboratory, 39 (7.5%) of 522 tests set up were contaminated. Streptomycin 50 µg/ml (final concentration) was therefore added to both the medium used for growing the test organism and that used for microbiological assay. This alteration in the technique was followed by a reduction of the contamination rate to 0.5% of the 1005 subsequent tests. In order to be certain, however, that the addition of streptomycin did not influence the microbiological assay of isoniazid, the following investigation was conducted. The isoniazid concentrations of seven sera were determined by three different methods. In method A, a medium containing 50 µg/ml streptomycin was inoculated with a culture of H37RvSR grown in a medium containing the same concentration of streptomycin. In method B, a medium not containing streptomycin was inoculated with the culture of H37RvSR grown in a medium containing 50 µg/ml streptomycin. In

TABLE 2

DISTRIBUTION OF THE CONTROL CONCENTRATIONS OF ISONIAZID WHICH PRODUCED 50% LOSS OF ACID-FASTNESS IN DIFFERENT BATCHES OF TESTS

Concentration of isoniazid which produced 50 % loss of acid-fastness [a] (µg/ml)	Control batches	
	No.	%
0.030	13	*(59)* [b]
0.035	2	*(9)*
0.037	2	*(9)*
0.040	2	*(9)*
0.046	1	*(5)*
0.053	1	*(5)*
0.060	1	*(5)*
Total	22	*101*

[a] Mean concentration which produced 50% loss of acid-fastness = 0.0344 µg/ml.

[b] The parentheses indicate percentages based on fewer than 25 observations.

method C, neither the medium used for the assay nor that used for growing the inoculum contained streptomycin. The smears prepared from these tests were examined in a random order. The mean concentration of isoniazid for the seven sera was estimated as 0.32 µg/ml with method A, 0.46 µg/ml with method B, and 0.36 µg/ml with method C. As the differences are neither in the same direction nor statistically significant, it can be concluded that the addition of streptomycin did not interfere with the assay of isoniazid.

Stability of isoniazid in serum during storage in the deep-freeze

As sera were generally stored in the deep-freeze at -20°C, sometimes for many weeks, before their isoniazid concentrations were estimated, the stability of isoniazid in serum during storage was investigated. The isoniazid concentrations of 10 sera were assayed within 12 hours of collection of the blood from the patients and then again at the end of two, four, six and eight weeks of storage in the deep-freeze. The smears prepared from these tests were heat-fixed and then kept for staining until all the smears were available. They were stained in one batch and the slides were read in a random order. The results of this experiment are presented in Tables 3 and 4. The concentration of isoniazid decreased from week to week and there was a linear relationship between the log concentration and the duration of storage (Table 4, term c, $P < 0.001$). The fall in the isoniazid concentration during storage is estimated as 3.7% per week. This estimate has been used to correct the results obtained with sera which had been stored for more than seven days before being assayed. In contrast to these findings with serum, a solution of 100 µg/ml isoniazid in water, stored at 4°C and

TABLE 3
STABILITY OF ISONIAZID IN SERUM DURING STORAGE AT -20°C

Serum number	Serum concentration of isoniazid (µg/ml)				
	Weeks of storage at -10°C				
	0	2	4	6	8
1	0.29	0.29	0.29	0.26	0.26
2	1.04	1.04	1.04	0.93	0.69
3	0.29	0.29	0.29	0.26	0.23 [a]
4	1.04	1.14	1.04	1.04	1.04
5	1.04	1.04	1.04	1.04	0.69
6	1.04	1.14	1.04	0.93	0.69
7	0.26	0.26	0.23	0.17	0.17
6	2.07	1.37	1.14	1.04	1.04
9	1.04	1.04	1.04	0.92	1.04
10	1.04	1.04	1.04	0.69	0.92
Mean (µg/ml)	0.75	0.74	0.70	0.61	0.57

[a] One missing value has been estimated using standard statistical techniques.

TABLE 4
ANALYSIS OF VARIANCE OF DATA IN TABLE 3

Term	Source of variation	Sum of squares	Degrees of freedom	Mean square	F	P
a	Sera	43.6575	9	4.8508		
b	Weeks of storage	1.2536	4	0.3134	9.44	<0.001
c	Linear regression	1.1653	1	1.1653	35.09	<0.001
d	Deviation from regression	0.0883	3	0.0294	–	N S[a]
e	Residual	1.1631	35 [b]	0.0332		
	Total	46.0742	48 [b]			

[a] NS indicates that the variance ratio is less than 1.0.
[b] One missing value has been estimated using standard statistical techniques.

TABLE 5
CONSISTENCY OF INDIVIDUAL ISONIAZID INACTIVATION RATES

Treatment regimen	Number of patients	Mean serum concentration of isoniazid (µg/ml)					
		Before treatment	Duration of treatment				
			7 days	3 month	6 months	9 months	12 months
PH	6	0.58	0.53	0.56	0.62	0.65	0.54
HI-1	3	0.31	0.36	0.25	0.43	0.41	0.29
HI-2	2	0.84	0.84	0.84	1.04	1.09	1.14
H	3	0.26	0.31	0.43	0.42	0.50	0.44
All regimens	14	0.45	0.46	0.47	0.57	0.60	0.54

tested concurrently with the sera, showed no alteration in the concentration of isoniazid during six weeks of storage.

Consistency of individual isoniazid inactivation rates

The consistency of individual rates of inactivation of isoniazid was investigated in 14 patients (6 PH, 3 HI-1, 2 HI-2, 3H), six of whom were part of the main population of 321 patients under study (page 766). Serum concentrations of isoniazid were determined four-and-a-half hours after an intramuscular test-dose of 3 mg/kg isoniazid, just before treatment was started, and then again after seven days and after three, six, nine and 12 months of treatment. The results are set out in Table 5. Considering all treatment groups, there was a very slight suggestion of an increase in the mean concentration of isoniazid with time. The trend (linear regression), however, is not statistically significant ($0.1 < P < 0.2$). This applied to all four regimens.

The mean weight of the 14 patients was 82.6 lb. (range, 59-109 lb.) on admission to treatment, 88.4 lb. at six months and 89.8 lb. at 12 months.

Accuracy of the test

The error of the test procedure for determining the isoniazid inactivation rate on different occasions in a patient is composed of the error of the microbiological assay and of other procedures in the test, such as inadvertent variation in the test-dose of isoniazid injected and variation in its rate of absorption. During the course of the study, a number of estimates were made of the variation from different sources that contributed to the error of the test procedure. These are set out in Table 6.

Error of the assay procedure. Estimates of the variation from assay to assay, carried out on the same test serum in the same batch of assays, were obtained from *(a)* the experiment on the stability of isoniazid in serum during storage in the deep-freeze (Table 6, term a), and *(b)* the untabulated results of duplicate tests on 65 sera, which were set up and read in a random order (Table 6, term b). Combining these estimates, the standard deviation was 0.15 dilution step (Table 6, term c). Since this variation was between assays in the *same* batch, and since differences from batch to batch were found to be significant (page 767), an estimate of the total error of the assay is provided by adding to the former, the variation from batch to batch (Table 6, term d, 0.26 dilution step). Thus calculated, the total error was 0.30 dilution step when expressed as a standard deviation (Table 6, term e). As an example of the interpretation of this estimate in terms of isoniazid concentrations, if 20 assays, randomly distributed in different batches, were carried out on the same serum with an isoniazid concentration of 1.0 µg/ml, then in 19 of these assays the estimated concentration would be expected to be between 1.50 and 0.67 µg/ml.

Error of the test procedure. The error of the test procedure–that is, the variation in the determinations of the serum isoniazid concentration on different occasions on the same patient–was estimated from *(a)* the results of multiple tests on the same patient in the experiment on the consistency of individual isoniazid inactivation rates (Table 6, term f), and *(b)* the results of duplicate tests (not tabulated here) carried out on 43 patients, at six and 12 months

TABLE 6

ESTIMATES OF ERROR DUE TO DIFFERENT SOURCES IN THE DETERMINATION OF SERUM CONCENTRATION OF ISONIAZID IN PATIENTS FOUR-AND-A-HALF HOURS AFTER AN INTRAMUSCULAR TEST-DOSE OF 3 mg/kg ISONIAZID

Source of error	Estimate of error			
	Term	Source of estimate	Degrees of freedom	Standard deviation (dilution steps)
Assay procedure:				
Between assays on same serum in same batch	a	Table 4, term e [a]	35	0.18
	b	Page 770 [b]	65	0.14
	c	Combined estimate (a and b)	100	0.15
Between batches of tests	d	Page 770 ; Table 1 [c]	28	0.26
Between assays on same serum	e	c + d		0.30
Test procedure :				
Between tests on same patient	f	Table 5 [d]	67	0.60
	g	Page 770 [e]	42	0.44
	h	Combined estimate (f and g)	109	0.54
Test procedures other than in the assay		h-e		0.45

[a] Residual from experiment on the effect of storage of sera at –10°C.
[b] Between duplicate tests on the same serum.
[c] Between batches of tests.
[d] Between tests on the same patient from experiment on the stability of individual isoniazid inactivation rates.
[e] Between patients with test results at six and 12 months.

(Table 6, term g). The combined estimate (Table 6, term h) of this variation, expressed as a standard deviation, was 0.54 dilution step. If 20 test procedures were carried out on different occasions on the same patient, and the mean of the serum isoniazid concentrations at four-and-a-half hours was 1.0 µg/ml, then in 19 of these tests the isoniazid concentration would be expected to be between 2.08 and 0.48 µg/ml.

Error of the test procedures apart from the assay. The error of the test procedures apart from the assay was calculated by subtracting the error of the microbiological assay from the total error of the test procedures. Expressed as a standard deviation, this error was equal to 0.45 dilution step (Table 6, term i).

Serial serum concentrations from half an hour to five-and-a-half hours

Serial isoniazid serum concentrations were determined in eight newly diagnosed, previously untreated patients half an hour, one hour, and two, four-and-a-half and five-and-a-half hours after an intramuscular injection of 3 mg/kg isoniazid. The results are presented in Table 7 and in Fig. 1. At four-and-a-half hours, five of the eight patients had serum concentrations above 0.58 µg/ml and three had serum concentrations below 0.58 µg/ml. For reasons given in the next section, the former have been classified as slow inactivators and the latter as rapid inactivators of isoniazid.

The highest mean serum concentration observed for the five slow inactivators was 2.60 µg/ml and that for the three rapid inactivators was 2.18 µg/ml.

The serum concentrations at half an hour varied considerably between patients and there was no clear difference between rapid and slow inactivators. At one hour, a difference was discernible, but there was still considerable variation between patients in the same inactivation group and slight overlapping between the groups. The variation between patients in the same inactivation group was markedly less at two, four-and-a-half and five-and-a-half hours and there was a clear distinction between the mean concentrations of the slow and rapid inactivators.

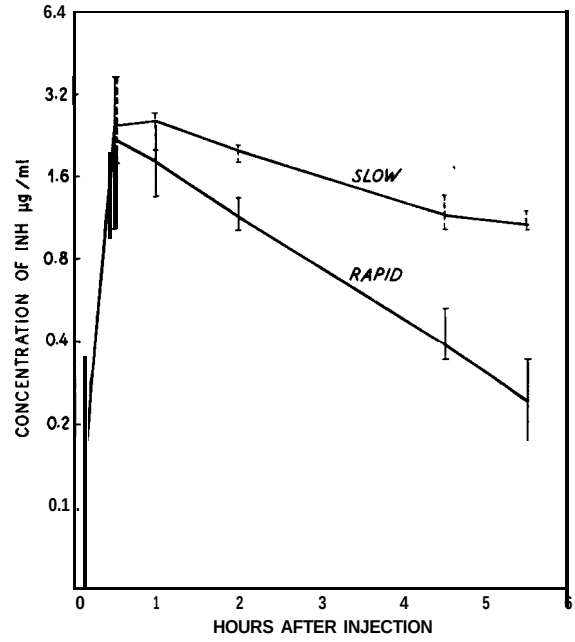
TABLE 7

SERUM CONCENTRATIONS OF ISONIAZID AT DIFFERENT INTERVALS AFTER INTRAMUSCULAR INJECTION OF 3 mg/kg ISONIAZID

Rate of inactivation of isoniazid	Patient number	Serum concentration of isoniazid (µg/ml)				
		Hours after administration of test dose				
		½	1	2	4 ½	5 ½
Slow	1	1.82	2.75	2.06	1.03	1.03
	2	3.68	2.75	2.06	1.17	1.03
	3	2.06	2.06	2.06	1.03	1.03
	4	2.75	2.75	1.82	1.38	1.17
	5	2.30	2.75	2.06	1.11	1.03
	Mean	2.44	2.60	2.01	1.15	1.06
Rapid	1	3.68	2.06	1.03	0.34	–
	2	2.75	1.38	1.38	0.34	0.17
	3	1.03	2.06	1.03	0.52	0.34
	Mean	2.18	1.80	1.14	0.39	0.24

FIG. 1

SERIAL MEAN SERUM CONCENTRATIONS OF ISONIAZID (INH) IN FIVE SLOW AND THREE RAPID INACTIVATORS AFTER AN INTRAMUSCULAR DOSE OF 3 mg/kg BODY-WEIGHT



The dotted and solid vertical lines indicate the range of the observations for the slow and rapid inactivators, respectively.

The mean concentration of the slow inactivators was 1.8 times that of the rapid inactivators at two hours, 2.9 times at four-and-a-half hours and 4.4 times at five-and-a-half hours.

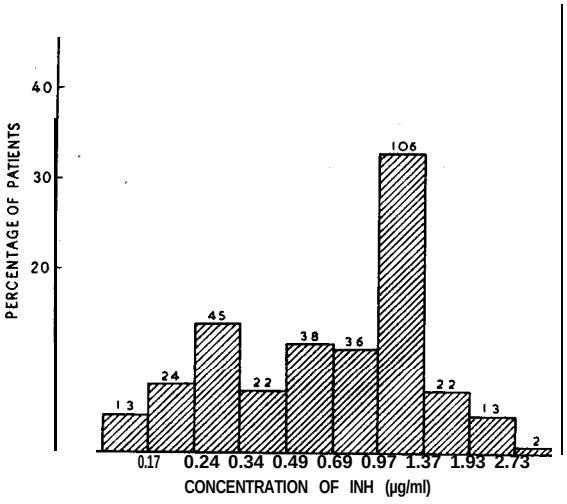
Distribution of serum concentrations of isoniazid of South Indian patients

The distribution of the serum concentrations of isoniazid of the 321 South Indian patients (page 766) four-and-a-half hours after an intramuscular injection of 3 mg/kg of isoniazid is set out in Fig. 2. The distribution is bimodal and appears to consist of two normal distributions which overlap, particularly in the 0.49-0.69 µg/ml class interval. The means of the two distributions are approximately 0.30 and 1.10 µg/ml. In order to determine whether the 38 patients in the 0.49-0.69 µg/ml class interval really did belong to one or other of these distributions and were not a group with an intermediate rate of inactivation, the rate of inactivation of isoniazid of 37 of them was retested. Of the 37 patients who were retested, 17 (46%) had serum concentrations above 0.69 µg/rnl and 9 (24 %) below 0.49 µg/ml. Although this evidence does not exclude the possibility of there being an intermediate group, it does suggest that at least the majority of patients in this class interval do belong to one or other of the main distributions. It was therefore decided to separate the two distributions arbitrarily at the mid-point (0.58 µg/ml) of this class interval and to classify those with serum concentrations at or above 0.58 µg/ml as slow inactivators of isoniazid and those with serum concentrations below 0.58 µg/ml as rapid inactivators. According to this classification, 195 (61%) of the 321 patients were slow inactivators and 126 (39%) were rapid inactivators.

The standard deviation of the serum concentrations of isoniazid for the slow inactivators was estimated to be 0.54 dilution step. It was shown on page 771 that the error of the test procedure was also estimated as 0.54 dilution step. Thus, all of the variation among the slow inactivators is likely to be due to the error of the test procedure. The corresponding standard deviation for the rapid inactivators could not be estimated as a relatively large proportion

FIG. 2

DISTRIBUTION OF SERUM CONCENTRATIONS OF ISONIAZID (INH) FOUR-AND-A-HALF HOURS AFTER AN INTRAMUSCULAR DOSE OF 3 mg/kg BODY-WEIGHT



The number above each block indicates the number of patients on whom determinations of serum concentrations of isoniazid were made.

of the patients had serum concentrations of isoniazid below the level of sensitivity of the assay.

Relationship between sex, body-weight, age and rate of inactivation of isoniazid

There was no evidence. of an association between the serum concentration of isoniazid and body-weight either among the 195 slow inactivators (correlation coefficient *(r)* = 0.11, P = 0.1-0.2) or among the 126 rapid inactivators *(r* = 0.17, P= 0.07). The distribution of weights for the slow and rapid inactivators is presented, separately for males and females, in Table 8. Considering the males, the weight distributions for the slow and rapid inactivators are very similar, the means being 98.3 and 100.4 lb., respectively. Among females, there was a slight suggestion that the rapid inactivators weighed less than the slow inactivators; thus, 10 (18% of the 55 rapid inactivators weighed 60-69 lb. as compared with four (7%) of the 54 slow inactivators (P ~ 0.2). The mean weight for the rapid inactivators was 81.2 lb. as compared with 85.9 lb. for the slow inactivators (P ~ 0.06).

Of 212 male patients, 141 (67%) were slow inactivators as compared with 54 (50%) of 109

TABLE 8

DISTRIBUTION OF WEIGHTS ACCORDING TO RATE OF INACTIVATION OF ISONIAZID AND SEX OF PATIENTS

Weight (lb.[a])	Males				Females			
	Slow inactivators		Rapid inactivators		Slow inactivators		Rapid inactivators	
	No.	%	No.	%	No.	%	No.	%
60-69	2	1	1	1	4	7	10	18
70-79	4	3	4	6	14	26	16	29
80-89	26	18	9	13	17	31	16	29
90-99	56	40	21	30	13	24	7	13
100-109	26	78	20	28	3	6	6	11
110-119	18	13	8	11	3	6	0	0
120 or above	9	6	8	11	0	0	0	0
Total	141	99	71	100	54	100	55	100
Mean	98.3		100.4		85.9		81.2	

[a] 1 lb.= 0.45 kg.

females, the difference being highly significant (P < 0.01). A sub-analysis was undertaken to see if this difference was evident in the various weight-groups and the findings are presented in Table 9. It can be seen that the proportion of slow inactivators among the males is uniformly higher in all the weight-groups than the corresponding proportion for the

TABLE 9

PROPORTION OF SLOW INACTIVATORS AMONG MALE AND FEMALE PATIENTS ACCORDING TO BODY-WEIGHT

Weight (lb.[a])	Males		Females	
	Total	Percentage of slow inactivators	Total	Percentage of slow inactivators
60-79	11	(55)*	44	41
80-89	35	74	33	52
90-99	77	73	20	(65)
100 or above	89	60	12	(50)
Total	212	67	109	50

[a] I lb. = 0.45 kg.

[b] The parentheses indicate percentages based on fewer than 25 observations.

females. It may therefore be concluded that the difference between the sexes in respect of the rate of inactivation of isoniazid cannot be attributed to differences in body-weight and is presumably a genuine sex difference. An analysis (not tabulated here) revealed that there was no association between the rate of inactivation of isoniazid and the age of the patient.

DISCUSSION

The results of determinations of the rate of inactivation of isoniazid reported here have demonstrated that South Indian patients can be divided into two groups–namely, the rapid and the slow inactivators – as has been previously reported for North American patients (Middlebrook & Dressler, 1956; Price Evans, 1959). The success of a method for determining the rate of inactivation of isoniazid can best be judged by its ability to distinguish these two groups of patients. In this study, 38 (12%) of the 321 patients had serum concentrations between 0.49 and 0.69 µg/ml, the class interval in which the distributions of the slow and rapid inactivators overlapped, and thus could not be assigned with certainty to either of these two groups. This percentage of indeterminate results may be compared broadly with the estimates of other workers (Mandel et al., 1959; Price Evans, 1959), although there were differences between the three studies, not only in the procedures employed, but also in the class intervals used for grouping the isoniazid assay results. The results of the above-mentioned workers are rather more satisfactory than those reported here; thus, 1.5 % of the 254 patients studied by Mandel et al. (1959) and 8 % of the 123 patients reported by Price Evans (1959) had indeterminate serum concentrations of isoniazid. It is therefore necessary to consider modifications of the technique used at the Tuberculosis Chemotherapy Centre which could eliminate or decrease the proportion of patients with indeterminate results.

Increased precision in classifying patients as rapid or slow inactivators can be obtained in two ways: (a) by increasing the difference between the means of the distributions for the slow and rapid inactivators, and ***(b)*** by decreasing the variation within each of these distributions. Considering the first approach, the results of serial assays on patients indicate that the longer the period between the peak serum concentration and the collection of blood for assay, the greater is the difference in the mean serum concentrations of isoniazid between the rapid and the slow inactivators. Middlebrook & Dressler (1956) recommended six hours as the optimum period after oral administration of a 4 mg/kg test-dose. In this study, an interval of four-and-a-half hours between the administration of the test-dose and the collection of blood was chosen, because it was thought that the difference between the times when peak serum concentrations are attained following intramuscular and oral administration would be about one-and-a-half hours. Peak serum isoniazid concentrations were, however, achieved after about 30 minutes following the intramuscular injection of the test-dose, and after about 70 minutes following oral administration of a dose of about the same size (Gangadharam et al., 1961 [1]), a difference of only 40 minutes. The original estimate of one-and-a-half hours was thus 50 minutes too long. However, if the period between administering the test-dose and collecting the blood had been prolonged beyond four-and-a-half hours, a larger proportion of sera would have contained concentrations of isoniazid that were below the limit of sensitivity of the assay method, and this would have impaired the statistical analysis of the results. A longer interval than four-and-a-half hours could, however, be employed if a larger test-dose of isoniazid than 3 mg/kg were used. In future studies at the Tuberculosis Chemotherapy Centre it is proposed to administer an intramuscular test-dose of 6 mg/kg isoniazid and to collect blood six hours later.

Considering the second approach, a decrease in the variation from patient to patient within each of the two inactivation groups would also result in greater precision in the classification of the patients. Owing to the presence of the patients who could not be classified as slow or rapid inactivators with certainty, and because a proportion of assay results lay below or above the range of the method, exact estimates of the standard deviations of the distributions of rapid and slow inactivators could not be calculated. Nevertheless, it appears probable that the error of the test procedure-that is, the variation from test to test on the same patient-accounted for the variation from patient to patient in the group of slow inactivators, which is the better defined of the two distributions (Fig. 2).

In considering the error of the test procedure, it is important to realize that it is derived from two sources. The source yielding the greater part of the error (standard deviation of 0.45 dilution step) con-

[1] **See article on page 793.**

sists of the effects of factors other than the microbiological assay, such as possible inaccuracies in the measurement of the test-dose or variations in its rate of absorption. These two sources of error might be controlled by injecting a carefully measured dose in a large volume by the intravenous route. Jenne (1960), however, using the intravenous route, obtained a similar proportion of indeterminate results to that encountered in our study. The second source is the error of the assay itself, which has a slightly smaller standard deviation (0.30 dilution step). A considerable portion of this error was due to batch variation, and it is a disturbing comment on the present method that the controls set up to eliminate this variation entirely failed to do so. While it has not been possible to define and estimate all the causes of error in the test procedure, it is believed that the estimates obtained of certain variations which contributed to it have been helpful in indicating which aspects of the test should receive most attention in future attempts to reduce the error. In the meantime, until a procedure is available which completely separates the distributions of slow and rapid inactivators, it would seem advisable to carry out repeat tests on those patients whose first results are indeterminate, and classify them on the basis of the mean of the two tests.

Mandel et al. (1956) had previously reported that the microbiological assay of isoniazid was unaffected by the addition of 100 µg/ml streptomycin to undiluted human serum. However, these authors did not report the concentration of streptomycin present in the serum dilution which produced 50% loss of acid-fastness. The present investigation has shown that 50 µg/ml streptomycin added to the assay medium probably did not interfere with the assay, and was responsible for a marked reduction in contamination rates.

The finding in the present study that isoniazid in serum was destroyed at a rate of 3.7% per week for eight weeks during storage at –20°C is at variance with the findings of Bell & Riemensnider (1957a), who reported that there was no loss of isoniazid on up to 21 weeks' storage in the deep-freeze.

The rate of inactivation of isoniazid has been shown to be determined genetically, slow inactivation being a simple Mendelian recessive trait (Knight, Selin & Harris, 1959; Price Evans, 1959) which occurs with different frequencies in different racial groups (Harris, Knight & Selin, 1958; Armstrong & Peart, 1959). Harris, Knight & Selin (1958) found that 44% of Americans of Caucasian ancestry were slow inactivators, as compared with 12% of Americans of Japanese descent, and Armstrong & Peart (1959) found that only 5% of Eskimos were slow inactivators as compared with 56% of non-Eskimos. Price Evans (1959) found that 52 % of Americans of Caucasian ancestry were slow inactivators and the remainder rapid inactivators. Our finding that the proportion of slow inactivators in Indian patients was 61% is thus similar to the proportion of this group in Americans of Caucasian descent.

The relationship shown in this study between the rate of inactivation of isoniazid and sex is different from the findings of Price Evans (1959). In our investigation, the proportion of rapid inactivators among female patients was significantly higher than that among male patients and this difference could not be accounted for by differences in body-weight. Price Evans (1959), on the other hand, demonstrated a relationship between the rate of inactivation of isoniazid and body-weight, but not between the rate of inactivation and sex. It is difficult to explain this discrepancy. It may be due to the fact that his population contained a larger proportion of children.

We have no definite information on the effect of malnutrition on the rate of inactivation of isoniazid. Suggestive evidence is, however, afforded by the serial determinations of the inactivation rates of 14 patients that were done on admission to treatment and after three, six, nine and 12 months of treatment. Although the patients were malnourished on admission to treatment and gained, on the average, 7 lb. in weight during the 12 months of treatment, there was no corresponding alteration in their rates of inactivation of isoniazid.

SUMMARY

1. The serum isoniazid concentrations were determined four-and-a-half hours after an intramuscular injection of 3 mg/kg isoniazid in 321 of 341 patients participating in a controlled chemotherapy study.

2. The addition of streptomycin in a final concentration of 50 µg/ml was found to have no effect on the assay.

3. Isoniazid in serum was destroyed at a rate of 3.7% per week for eight weeks during storage at – 20°C.

4. Estimates were obtained of the error of the procedure for determining the rate of inactivation of isoniazid (0.54 dilution step) and for the method

of microbiological assay of isoniazid in serum (0.30 dilution step). The error due to factors other than that of the microbiological assay method was estimated as 0.45 dilution step.

5. A defect of the microbiological assay method was the presence of batch variation, which was not corrected for by the use of isoniazid controls. The error introduced by batch variation was estimated as 0.26 dilution step.

6. Patients with serum isoniazid concentrations of 0.58 µg/ml or more were classified as slow inactivators and those with less than 0.58 µg/ml as rapid inactivators. Of the 321 patients studied, 195 (61%) were classified as slow inactivators and 126 (39 %) as rapid inactivators.

7. The rate of inactivation of isoniazid in individual patients was found to remain constant during 12 months of treatment.

8. A relationship was shown between sex and the rate of inactivation of isoniazid, there being a significantly higher proportion of rapid inactivators among the females as compared with the males.

RÉSUMÉ

Dans le cadre des essais de chimiothérapie antituberculeuse organisés dans l'Inde, le Centre de Madras a étudié l'inactivation de l'isoniazide, métabolisé dans l'organisme en substances sans action antimicrobienne appréciable.

On sait d'après des études antérieures que la vitesse d'inactivation varie d'un sujet à l'autre, mais elle est constante pour le même individu. Elle semble être en relation avec la résistance à l'isoniazide des bacilles tuberculeux isolés des malades, et avec l'activité de la catalase. Les bacilles provenant d'inactivateurs lents étaient plus fortement résistants à l'isoniazide. Il est possible aussi que la vitesse d'inactivation ait une influence sur la négativation des crachats.

La vitesse d'inactivation a été suivie chez 321 des 341 malades participant aux études de chimiothérapie comparée, et traités à domicile, à Madras. L'isoniazide a été administré aux malades en doses standard de 3 mg/kg de poids corporel, à différents moments au cours d'une année (7 jours, puis 3, 6, 9, 12 mois après le début du traitement). Avant le test, les malades ne recevaient aucun médicament pendant 2 jours. L'administration d'isoniazide a été faite par voie intramusculaire, afin de supprimer la variable que représente l'absorption intestinale. Le sérum a été prélevé 4½ heures après l'injection. L'addition de streptomycine, à la concentration finale de 50 µg/ml, n'a pas eu d'influence sur les résultats. Le test a été effectué par la méthode bactériologique, avec une souche de bacille tuberculeux résistante à la streptomycine (H37Rv.SR).

L'isoniazide est détruit dans le sérum conservé à −20°C, à raison de 3,9 % par semaine pendant 8 semaines. La distribution des sujets selon le temps d'inactivation individuel donna une courbe bimodale, qui parut consister en deux courbes normales se recouvrant dans la zone de 0,49-0,69 µg/ml. On a décidé arbitrairement de considérer comme inactivateurs rapides les sujets chez lesquels la concentration d'isoniazide était inférieure à 0,58 µg/ml, et comme inactivateurs lents ceux qui présentaient des concentrations égales ou supérieures à 0,58 µg/ml. Selon ce critère, le premier groupe comprenait 39 % des sujets, le second 61 %. La proportion de ceux dont les chiffres de concentration se trouvaient dans la zone de chevauchement —0,49-0,69 — était de 12 %. D'autres auteurs, dans d'autres études, ont obtenu des chiffres nettement inférieurs (1,5 % sur 254 malades, 8 % sur 123 malades). Or la valeur de ce chiffre est un indice de la précision de la méthode. Plus cette dernière est fine, plus ce chiffre est bas. Les auteurs de l'article estiment donc qu'il y a lieu d'améliorer la technique de test qu'ils ont appliquée et discutent les moyens d'y parvenir.

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