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BIOCHEMICAL AND GENETICAL STUDIES
ON INH METABOLISM

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Biochemical and Genetical Studies on INH Metabolism

Semi-Annual Report

30 November 1964

Shigeichi Sunahara

Tokyo National Chest Hospital

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PART I

Studies on Isoniazid Inactivation in Monkeys

INTRODUCTION

It was established by us beyond question that the inactivation of isoniazid in humans was a genetic trait and there was not only individual but also racial difference. The frequency distribution curve of the biologically active isoniazid serum levels 6 hours after the oral administration of 4 mg/kg of body weight of isoniazid is trimodal, corresponding to the three phenotypes of isoniazid metabolism--rapid, intermediate and slow inactivators. On the other hand, we observed in vitro that there was very remarkable species and drug differences in the acetylation of isoniazid, sulfonamide and PABA by the liver homogenate of various kinds of animals, and the most active site of acetylation was the liver.

The purpose of the present research is to examine the correlation between the blood concentration and the capacity of tissue to acetylate isoniazid using *Cynomoldus* monkeys as animals of experiment.

METHODS & MATERIALS

The blood level of the biologically active isoniazid was measured by the vertical diffusion method, and the capacity of acetylation of the tissue homogenate by Short's method as indicated in our previous reports. The *Cynomoldus* monkeys (*Macaca irus*) imported from Malaya, Cambodia and Viet Nam were used in the following experiments.

RESULTS

Fig. 1, 2 & 3 indicate the relationship between 4 and 6 hour levels of the biologically active isoniazid plasma levels after the test doses of 4 mg/kg, 8 mg/kg or 16 mg/kg of isoniazid. Fig. 4 & 5 demonstrate the frequency distribution curve of the values. If we take the concentration 4 hours after 4 mg/kg of INH or 6 hours after 8 mg/kg of INH, we have bimodal curves, but it seems difficult to establish a trimodality as in the case of the humans. There is no marked difference in the inactivation of isoniazid among the monkeys imported from three different countries as indicated in Fig. 4. In Fig. 6 is shown the acetylating capacity of isoniazid of the liver, kidney and spleen of the monkeys. Also in the case of monkeys, the liver is the most active organ concerning isoniazid inactivation. The kidney and spleen show sometimes the activity to a certain degree. It remains obscure whether there is any difference in acetylating capacity among the monkeys from different districts also in the homogenate experiments or not. Any close correlation was not established between the rate of acetylation in the liver and the blood level 6 hours after the dose of 4 mg/kg of body weight of isoniazid as shown in Fig. 7.

COMMENT & SUMMARY

We have not been successful to establish a trimodal distribution curve of the biologically active isoniazid levels in monkeys up to now. The curve was bimodal as far as the test dose and the time of blood collection in the present study were concerned.

Also in the case of monkeys, the liver was the most active site of acetylation but the kidney and spleen had the capacity to a certain extent. There was little correlation between acetylating activity of the liver and 6 hour blood level after 4 mg/kg of INH.

Further study on relationship between the rate of acetylation and blood levels other than 6 hours after the dose of 6 mg/kg of isoniazid is now in progress.

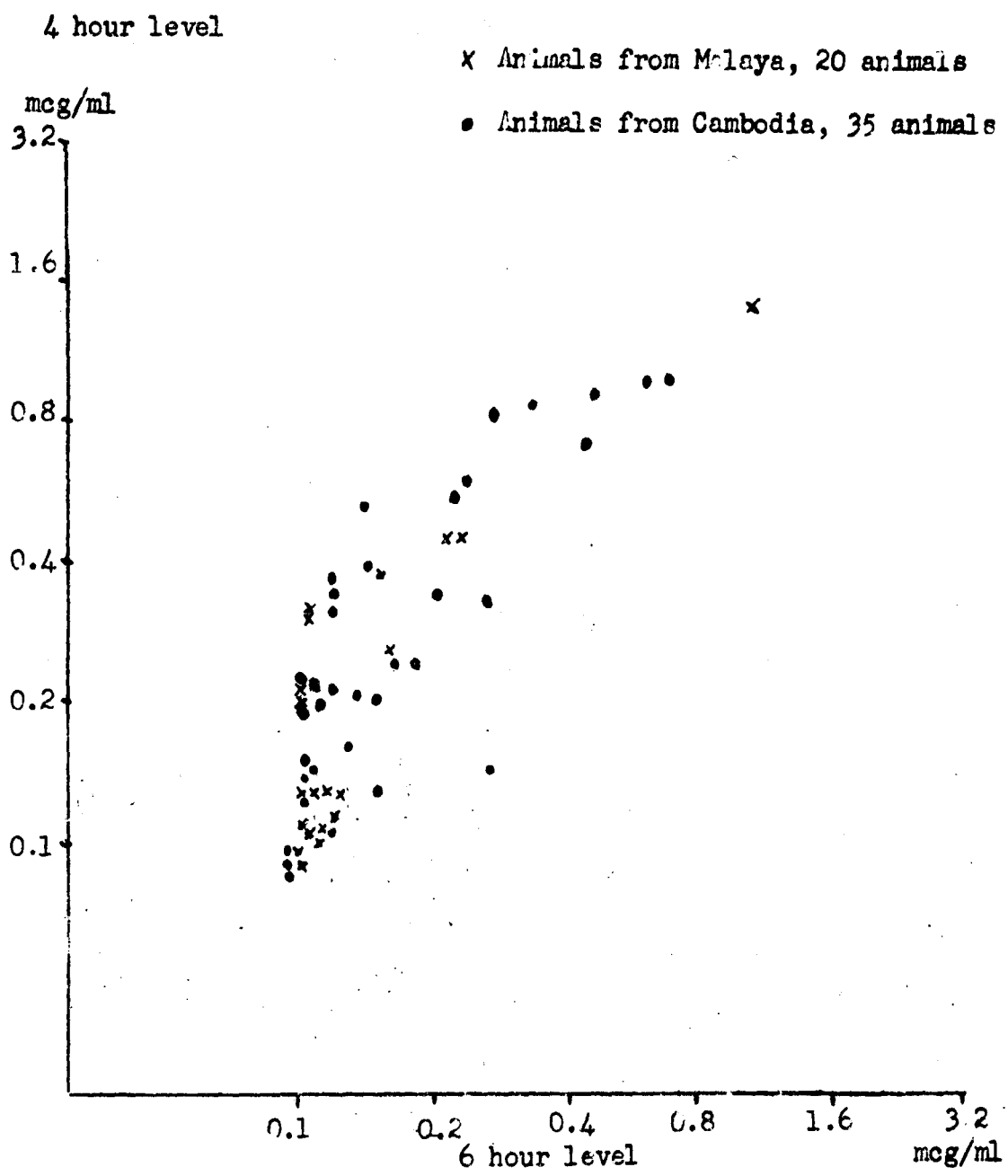


Fig. 1 Relationship between Blood Levels 4 and 6 hours after Administration of 4 mg/kg of INH to Monkeys

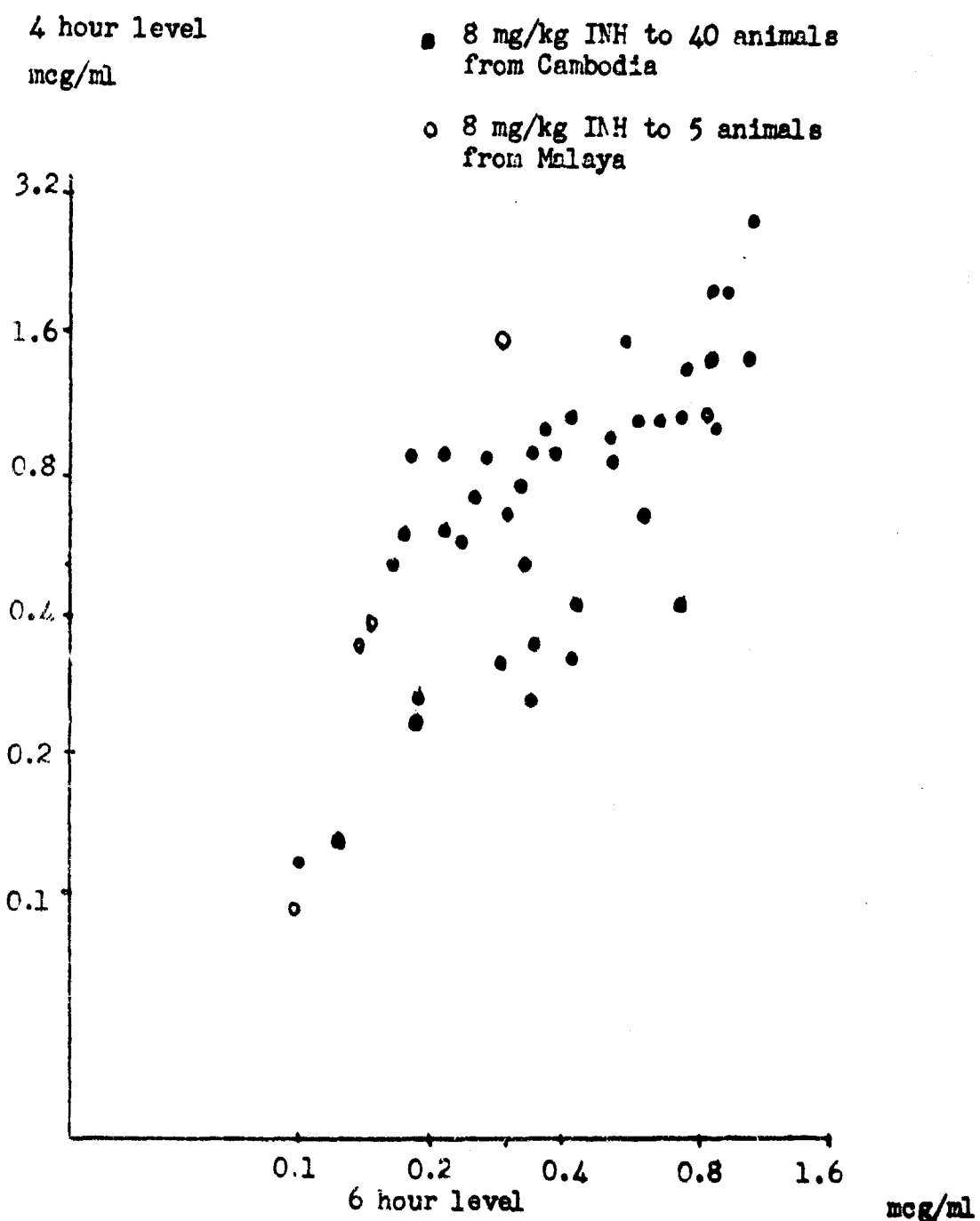


Fig. 2 Relationship between Blood Levels 4 and 6 hours after Administration of 8 mg/kg of INH to Monkeys

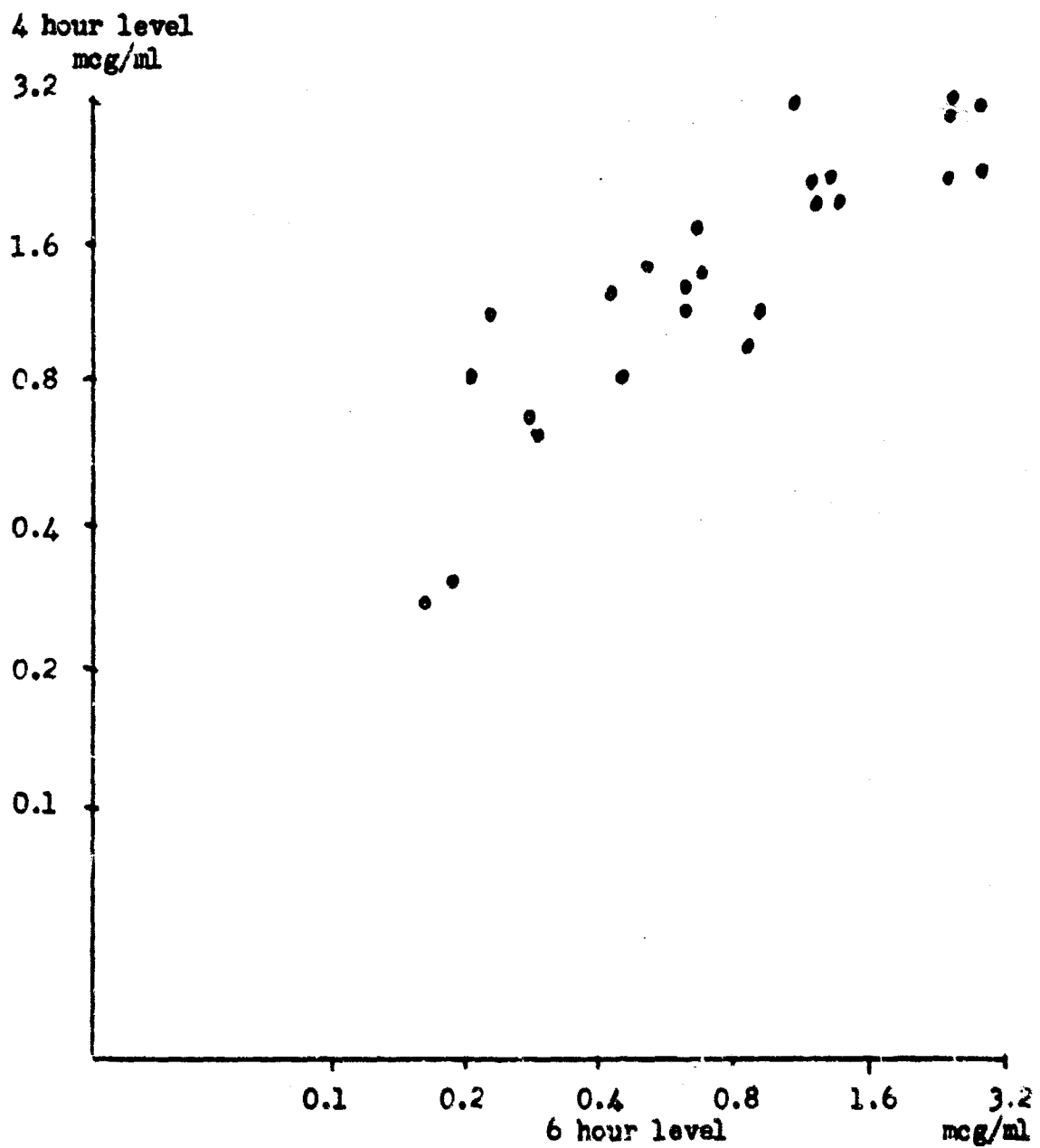


Fig. 3 Relationship between Blood Levels 4 and 6 hours after Administration of 16 mg/kg of INH to Monkeys (25 animals from Malaya)

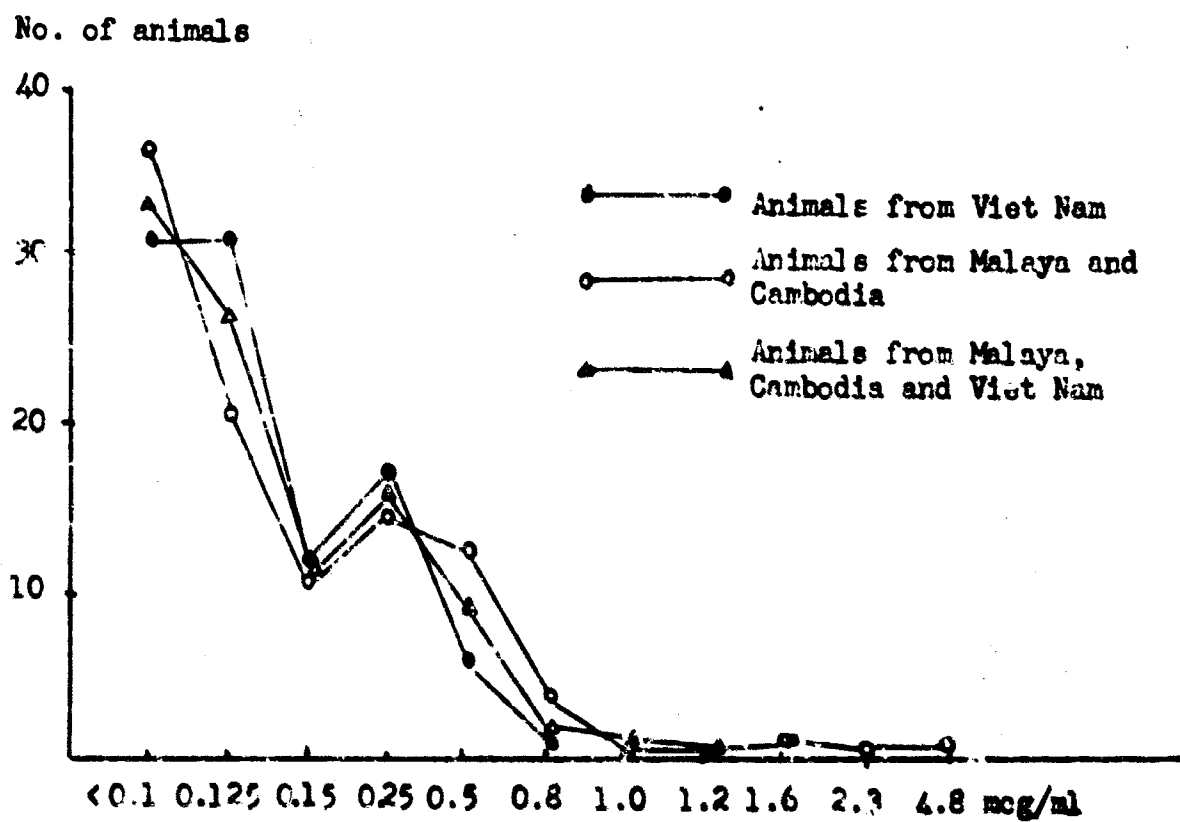


Fig. 4 Distribution Curve of Biologically Active Isoniasid Levels 6 hours after the Dose of 1mg/kg of INH

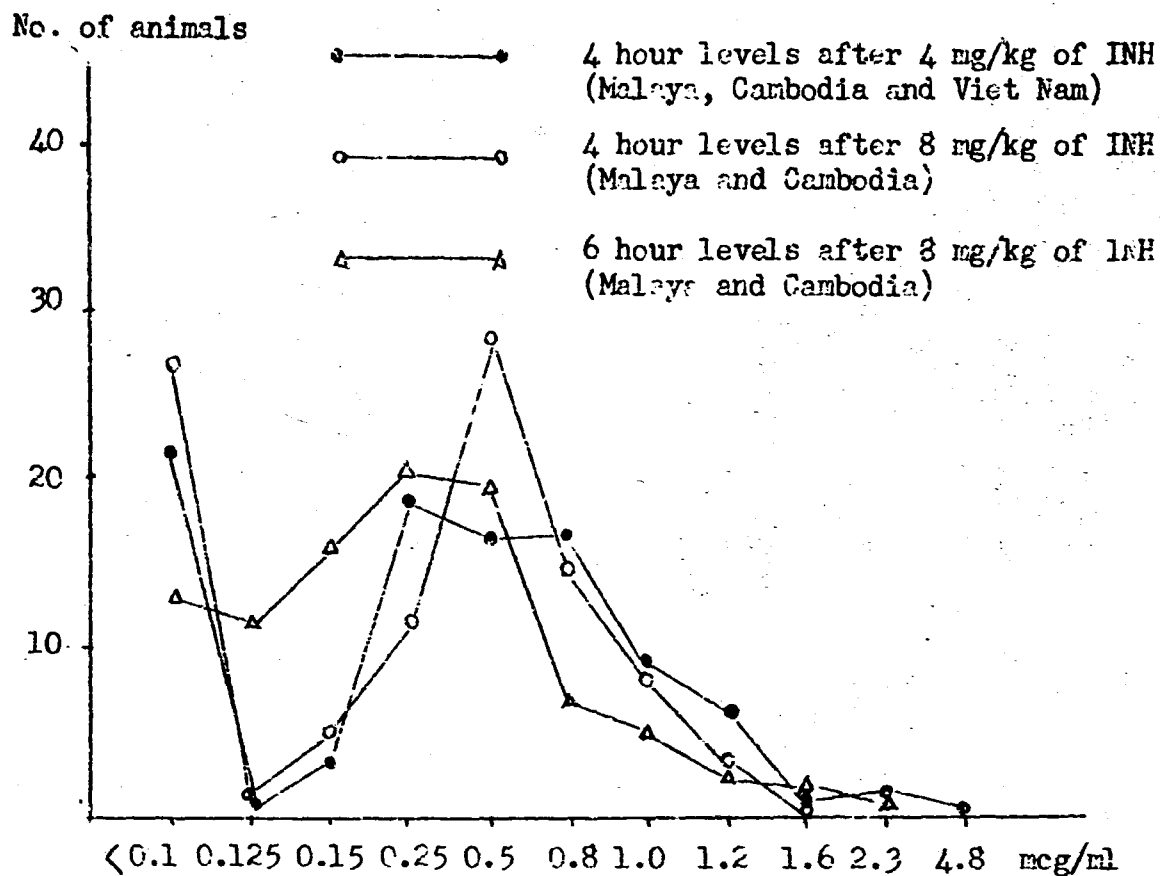


Fig. 5 Frequency Distribution Curve of Biologically Active Isoniazid Levels of Monkeys

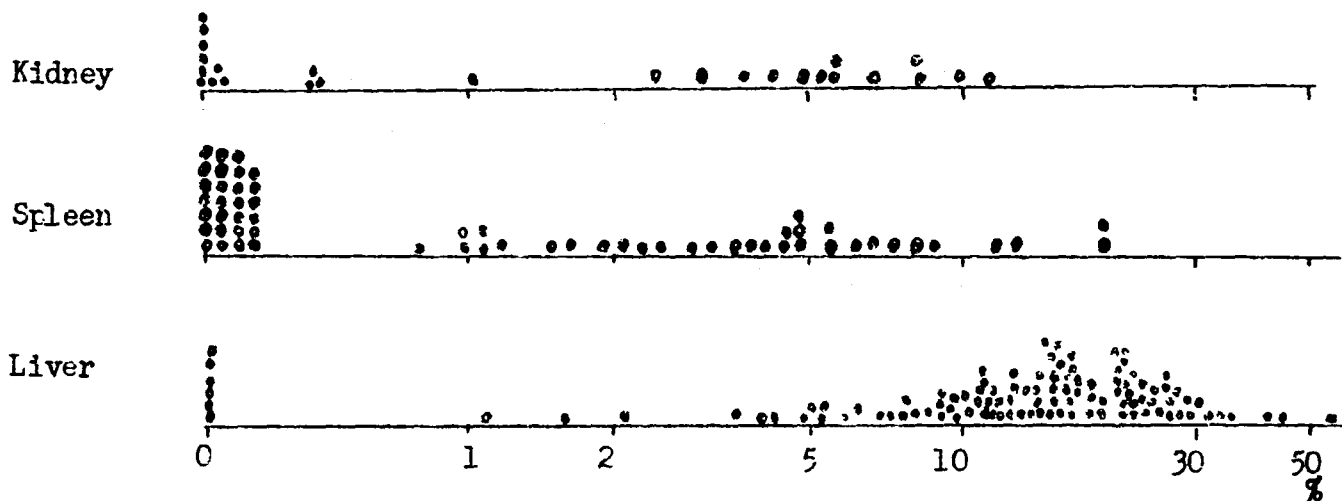


Fig. 6 Frequency Distribution of Rate of Acetylation of Isoniazid in the Liver, Spleen and Kidney

Rate of acetylation
of INH in the liver

x Animals from Malaya

● Animals from Cambodia

○ Animals from Viet Nam

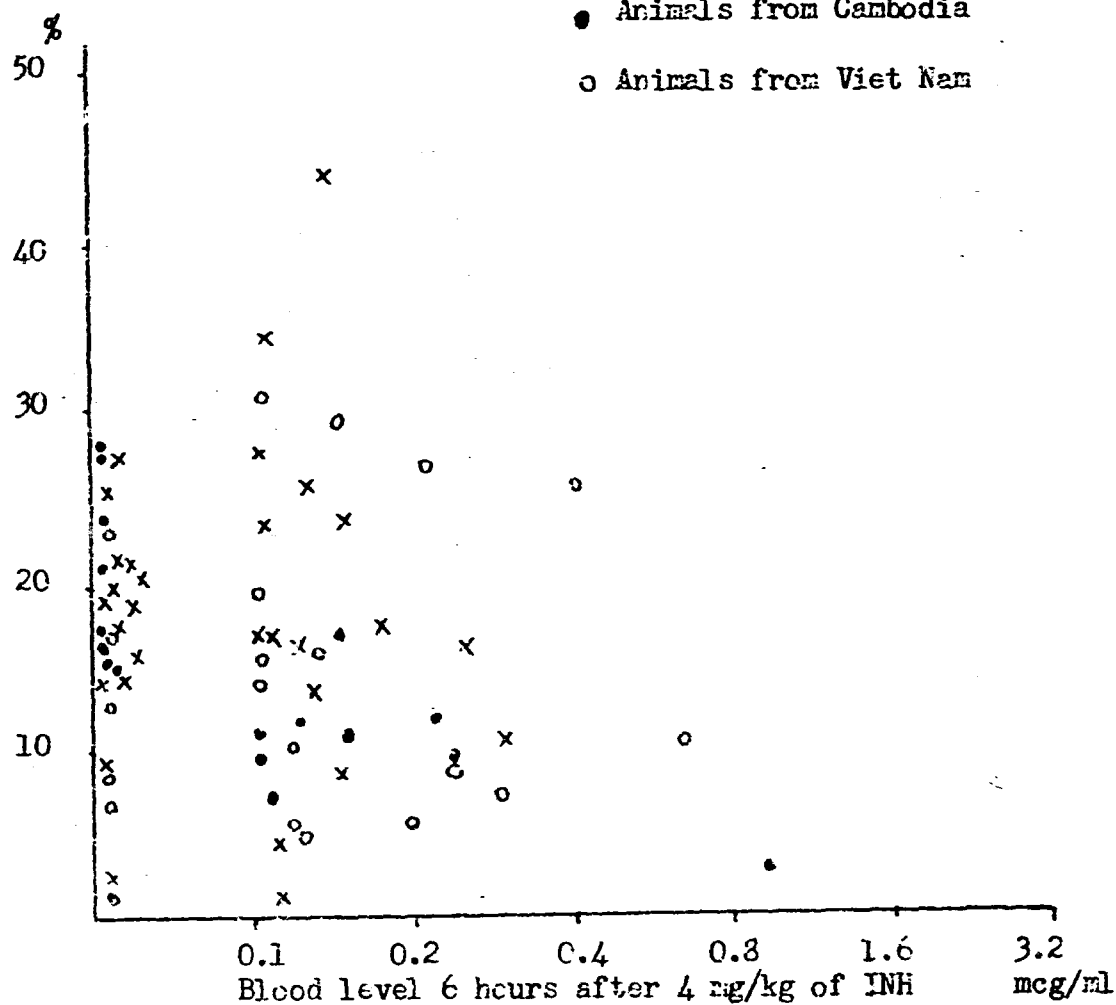


Fig. 7 Relationship between Isoniazid Plasma Level and Rate of Acetylation in Monkey Liver

PART II

Preliminary Report on A New Biochemical Method for Determining Total and Free Isoniazid in Biological Fluid

INTRODUCTION

A number of biochemical methods have been published for determining free isoniazid concentration in serum or urine (Short (10), Hughes (3), Hunter (4), Guthberton et al. (2), Scott (9), Scarid (8), Foole and Meyer (7), Maher et al. (5), Belles and Littlemar (1), Peters (6), etc.). Being quite difficult to have an accurate value for the concentration below 1 mcg/ml by employing the techniques above mentioned except the Peter's method, we have used the vertical diffusion culture method—a kind of bioassay method—for determining the blood level in our previous reports.

Recently a new biochemical method which enables us to measure a relatively low concentration of free isoniazid has been developed in our laboratory.

PROCEDURE

1. Reagent and Vessel
 - a. Fig. 1 Extraction vessel
2. Quantitative Determination of Total INH
 - a. Quantitative determination of total INH in urine
3. Quantitative Determination of Free INH
 - a. Quantitative determination of free INH in aqueous solution
 - b. Quantitative determination of free INH in serum
 - c. Quantitative determination of free INH in urine

RESULTS

1. Calibration Curve

- a. Fig. 2 Calibration curve of INH in aqueous solution
- b. Fig. 3 Calibration curve of INH in serum
- c. Fig. 4 Calibration curve of INH in urine

2. Recovery

- a. Table 1 Recovery tests of INH in aqueous solution
- b. Table 2 Recovery tests of INH in aqueous solution, serum & urine
- c. Table 3 Duplication test

3. Comparison between Bioassay and Chemical Assay

- a. Table 4 Comparison of chemical assay and biological assay of INH in aqueous solution
- b. Table 5 Comparison of chemical assay and biological assay of INH in serum
- c. Table 6 Free INH concentration in serum after oral administration of INH (4 mg/kg body weight)

4. Determination of INH Derivatives

- a. Fig. 5 Determination of free INH in aqueous solution of INH and related substances by means of our method
- b. Fig. 6 Recovery of free INH in aqueous solution of different kinds of INH derivatives

COMMENT & SUMMARY

Although it is needless to say that more detailed critical examination is necessary to put it in practical use, our new method for determining total and free INH in biological fluid (tangstate method) seems to be more reliable and convenient than most of biochemical techniques published hitherto. As the color at the end reaction is indigo blue, it can be quite easily distinguished. We are going to investigate in our future study whether this method would be able to replace the bioassay method, especially the vertical diffusion culture method.

PROCEDURE

1. Reagent and Vessel

An extraction vessel with ground stopper as indicated in Fig. 1 was used.

Reagents used were as follows:

- 1) $(\text{NH}_4)_2\text{SO}_4$ (anhydrous crystal)
- 2) 1N-NaOH aq. solution
- 3) N/10-Hcl aq. solution
- 4) Dichlorethan
- 5) Isoamylalcohol
- 6) 5% NaCN aq. solution
- 7) F-tangstate reagent
(Brown's uric acid reagent,
Brown, H.J.: J. Biol. Chem.
158: 601, 1945)
- 8) 66% urea aq. solution

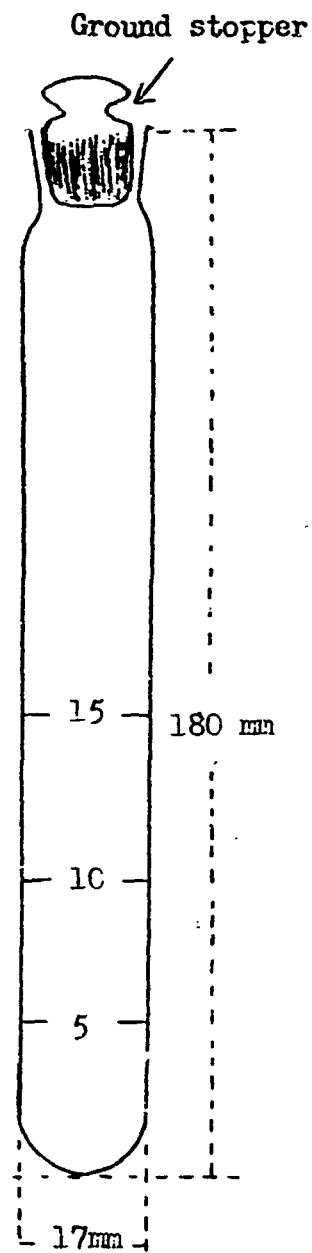


Fig. 1

2. Quantitative Determination of Total INH
 Quantitative Determination of Total INH in Urine

Sample	Blank
Urine	Water
Material	1.0 ml
(NH ₄) ₂ SO ₄	1.5 gm
IN-NaOH	1.5 ml
Dichlorethan	7.0 ml
Isoamylalcohol	3.0 ml

Shake for 15 minutes
 Centrifugation (1500 r.p.m.) 5 minutes

Upper layer (solvent layer)	5 ml
N/10-HCl	5 ml

Shake for 15 minutes
 Centrifugation (1500 r.p.m.) 5 minutes

Upper layer (HCl layer)	3 ml
-------------------------	------

Place in a boiling water bath for one hour

↓
Cool

↓
Make up to 15 ml with N/10-HCl (X)

(X)	1.5 ml
5% NaCN	0.5 ml
66% Urea	0.5 ml
Tangstate reagent	0.4 ml

Place for one hour at room temperature

Optical density of the Sample at 660 mμ was read against the Blank.

3. Quantitative Determination of Free INH

Quantitative Determination of Free INH in Aqueous Solution

Sample	Blank
aq. solution of INH	Water
Material	1.0 ml
(NH ₄) ₂ SO ₄	1 gm
Dichlorethan	7 ml
Isoarylalcohol	3 ml

Shake for 15 minutes
Centrifugation (1500 r.p.m.) 5 minutes

Upper layer (solvent layer)	8 ml
N/10-HCl	2 ml

Shake for 15 minutes
Centrifugation (1500 r.p.m.) 5 minutes

Upper layer (HCl layer)	1.5 ml
5% NaCN	0.5 ml
66% urea	0.5 ml
F-tangstate reagent	0.4 ml

Place for one hour at room temperature

↓
Optical density of the Sample at 660 mμ was read against the Blank.

Quantitative Determination of Free INH in Serum



Material	1 ml
(NH ₄) ₂ SO ₄	1.2 gm
1N-NaOH	0.25 ml
Dichlorethan	7 ml
Isoamylalcohol	3 ml

Shake for 15 minutes
Centrifugation (1500 r.p.m.) 5 minutes

Upper layer (Solvent layer)	8 ml
N/10-HCl	2 ml

Shake for 15 minutes
Centrifugation(1500 r.p.m.) 5 minutes

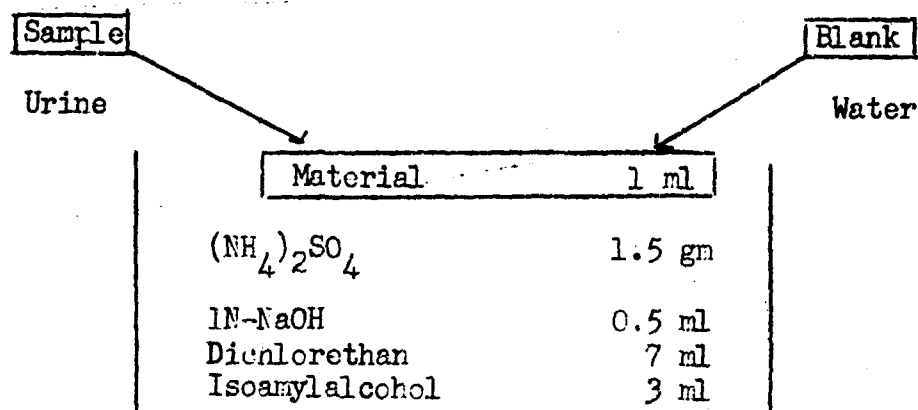
Upper layer (HCl layer)	1.5 ml
5%-NaCN	0.5 ml
66%-urea	0.5 ml
Tangstate reagent	0.4 ml

Place for one hour at room temperature

↓

Optical density of the Sample at 660 mμ was read against the Blank.

Quantitative Determination of Free INH in Urine



Shake for 15 minutes
Centrifugation (1500 r.p.m.) 5 minutes

Upper layer (solvent layer)	2 ml
N/10-HCl	2 ml

Shake for 15 minutes
Centrifugation (1500 r.p.m.) 5 minutes

Upper layer (HCl layer)	0.2 ml
5% NaCN	0.5 ml
66% urea	0.5 ml
Tangstate reagent	0.4 ml
Water	0.8 ml

Place for one hour at room temperature

↓

Optical density of the Sample at 660 m μ was read against the Blank.

RESULTS

1. Calibration Curve

Optical density (-log T)

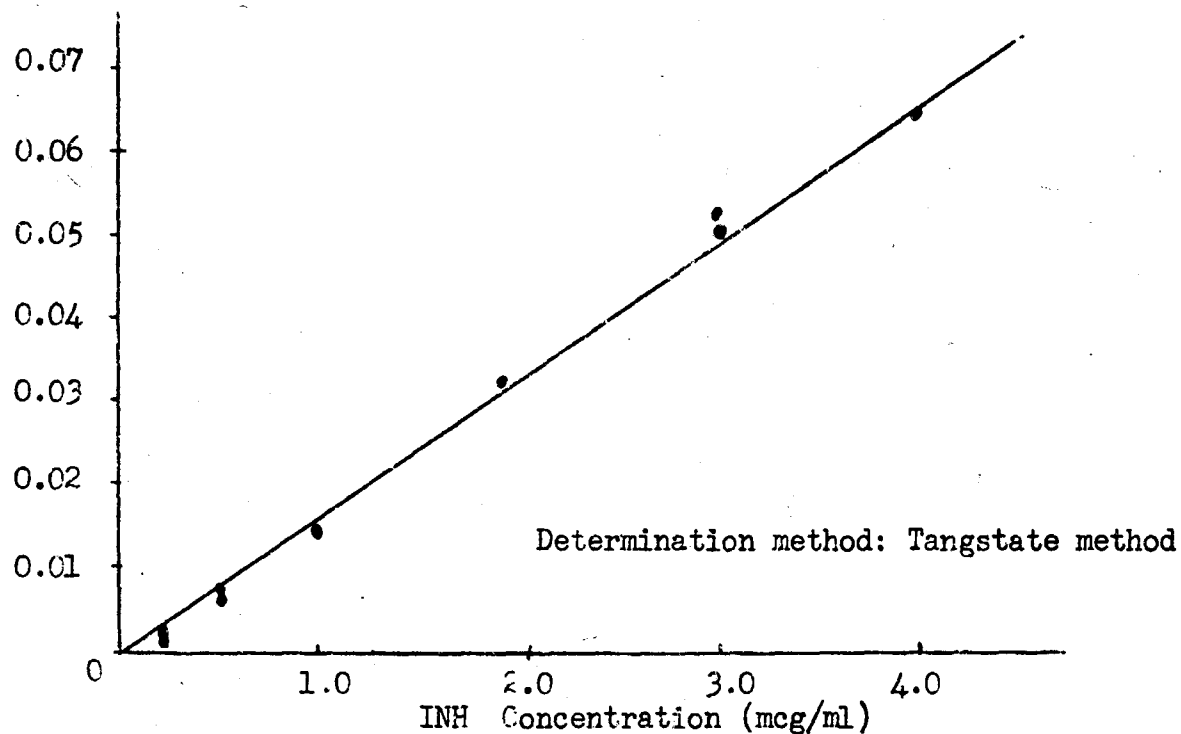


Fig. 2 Calibration Curve of INH in Aqueous Solution

Optical density (-log T)

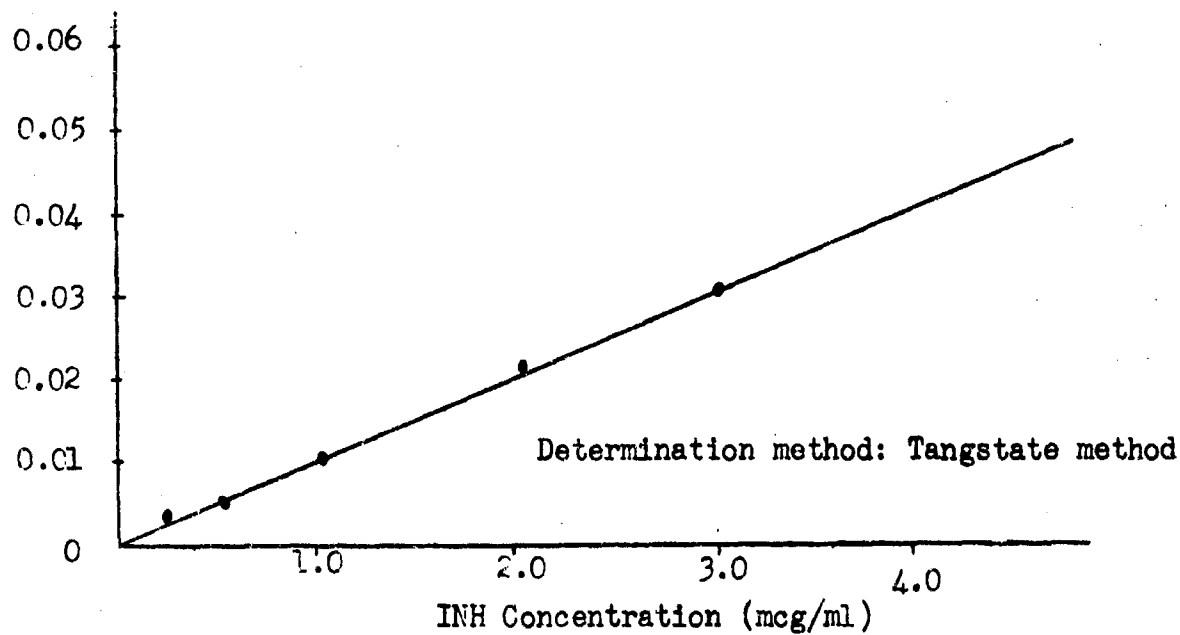


Fig. 3 Calibration Curve of INH in Serum

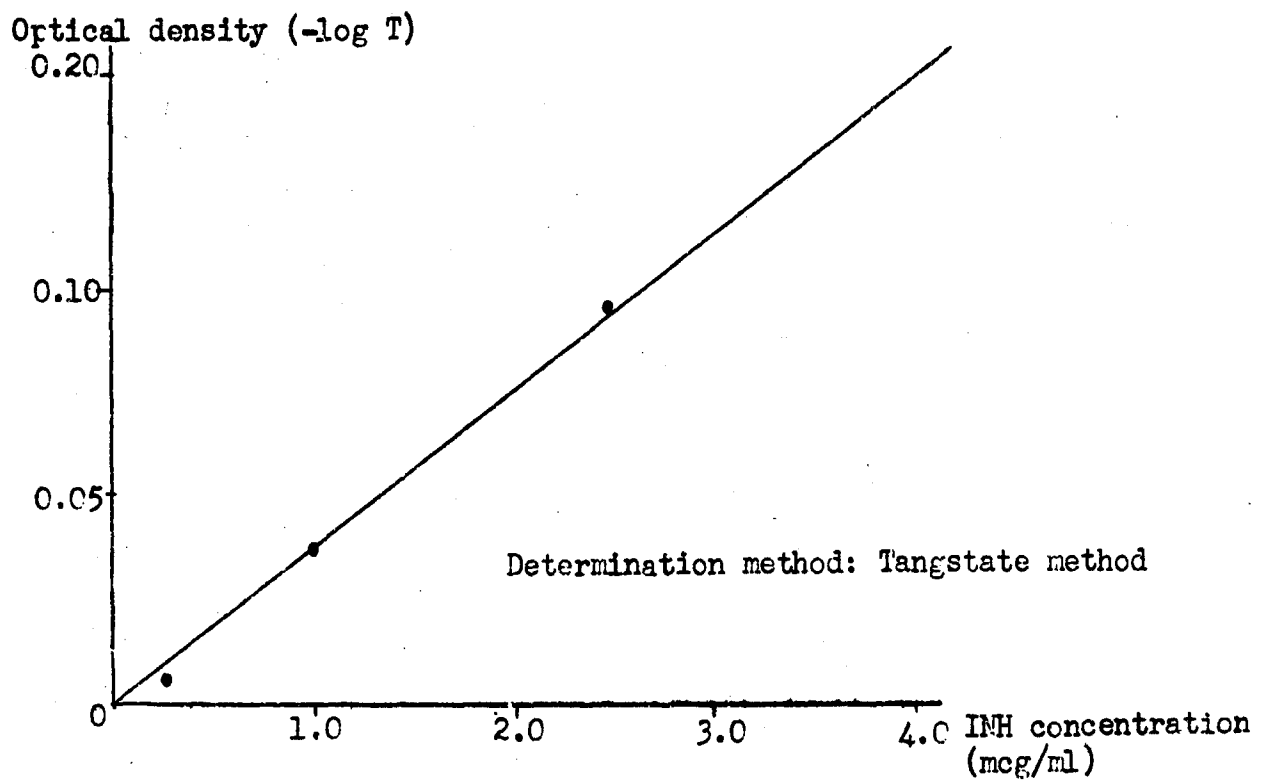


Fig. 4 Calibration Curve of INH in Urine

2. Recovery

Table 1 Recovery Tests of INH in Aqueous Solution

	3 mcg/ml	1 mcg/ml	0.5 mcg/ml
Dupli. No. 1	0.045	0.014	0.010
2	0.050	0.010	0.008
3	0.049	0.019	0.005
4	0.046	0.014	0.008
5	0.054	0.016	0.009
Mean	0.049 \pm 0.0032	0.0146 \pm 0.0029	0.008 \pm 0.0017
Recovered	3.0 mcg/ml	0.9 mcg/ml	0.5 mcg/ml
St. D.	\pm 0.2	\pm 0.18	\pm 0.11

Table 2 Recovery Tests of INH in Aqueous Solution, Serum and Urine

	INH Concentration 3 mcg/ml		
	in aq. solution	in serum	in urine
Dupli.No. 1	0.052	0.030	0.065
2	0.049	0.030	0.066
3	0.051	0.030	0.062
Mean	0.0507	0.030	0.0643
Recovered	3.08 mcg/ml	3.00 mcg/ml	

Table 3 Duplication Test

Dupli. No. 1	0.011	Material: 1 mcg/ml in serum
2	0.009	
3	0.009	
4	0.011	
5	0.010	
6	0.010	
7	0.009	
8	0.010	
	Mean	0.010 \pm 0.001
	Recovered	1 mcg/ml
	St. D.	\pm 0.1

3 . Comparison between Bioassay and Chemical Assay

Table 4 Comparison of Chemical Assay and Biological Assay of INH in Aqueous Solution

INH concent. in aq. sol.	Chemical assay mcg/ml	Biological assay mcg/ml.
3 mcg/ml	2.75	3.20
"	3.06	2.58
"	3.00	2.58
"	2.81	3.20
"	3.30	2.41
Mean , St. d.	2.98 ±0.20	2.79 ±0.34
1 mcg/ml	0.87	1.09
"	0.63	1.09
"	1.17	1.09
"	0.87	1.31
"	0.98	0.94
Mean , St. d.	0.90 ±0.18	1.11 ±0.12
0.5 mcg/ml	0.63	0.62
"	0.50	0.49
"	0.32	0.49
"	0.63	0.44
"	0.56	0.44
Mean , St. d.	0.53 ±0.13	0.50 ±0.07

Table 5 Comparison of Chemical Assay and Biological Assay of INH in Serum

INH concent. in serum	Chemical assay		Biological assay
	(a)	(b)	(a)
3mg/ml	2.9mcg/ml	3.0mcg/ml	2.58mcg/ml
2 "	1.5	2.1	1.68
1 "	1.0	1.0	0.58
0.5 "	0.9	0.5	0.35
0.2 "	0.1	0.3	0.18

Chemical assay (a) and bioassay (a) were simultaneously determined.

Table 6 INH concentration in serum after administration of INH (4 mg/kg of body weight)

Hour	Chemical assay	Biological assay
1	3.2 mcg/ml	>3.2 mcg/ml
2	2.0	>3.2
3	0.9	2.41
4	0.8	1.04
5	0.7	0.65
6	0.5	0.42

4. Determination of INH Derivatives

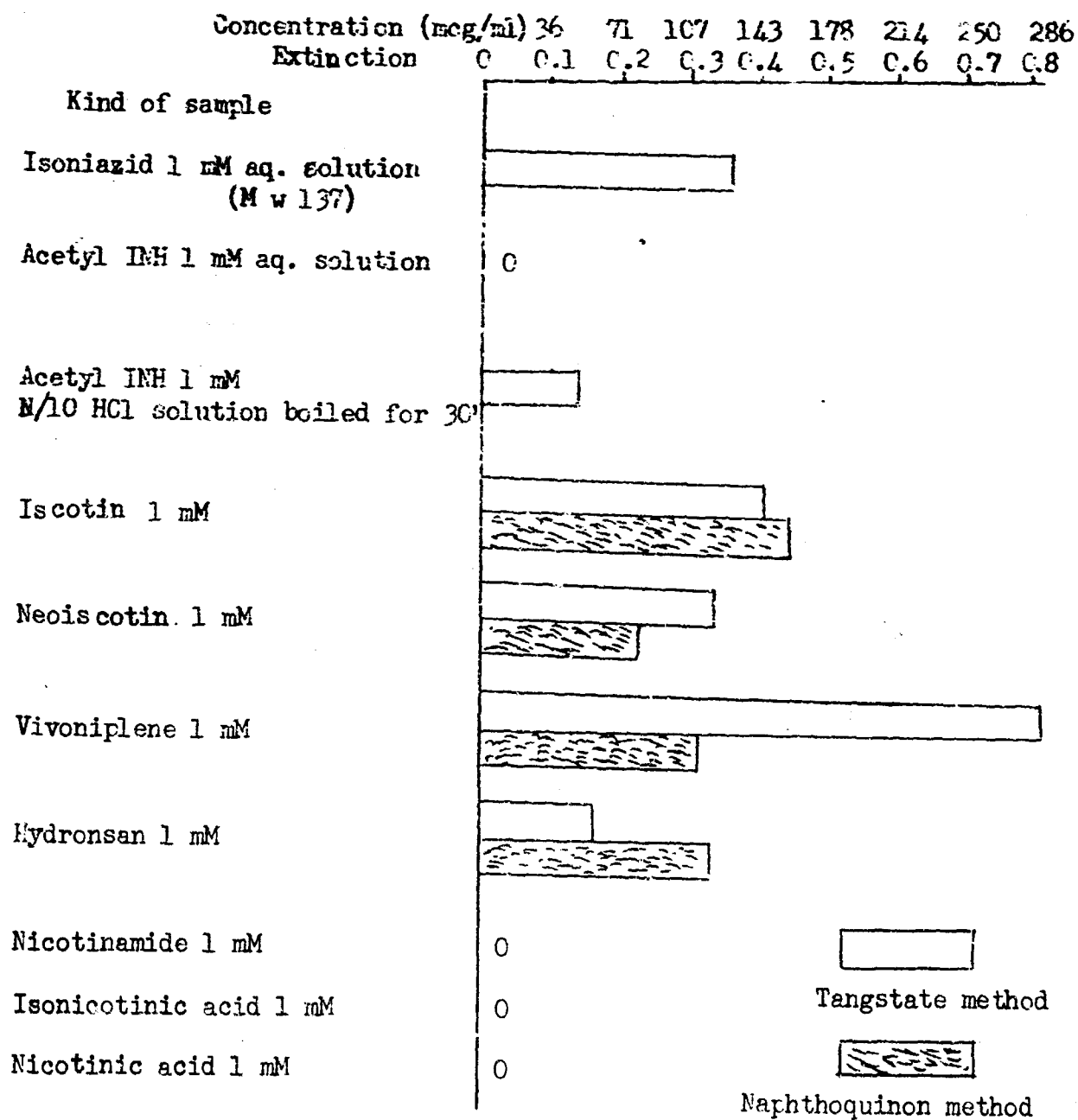


Fig. 5 Determination of Free INH in Aqueous Solution of INH and Related Substances by Means of Our Method

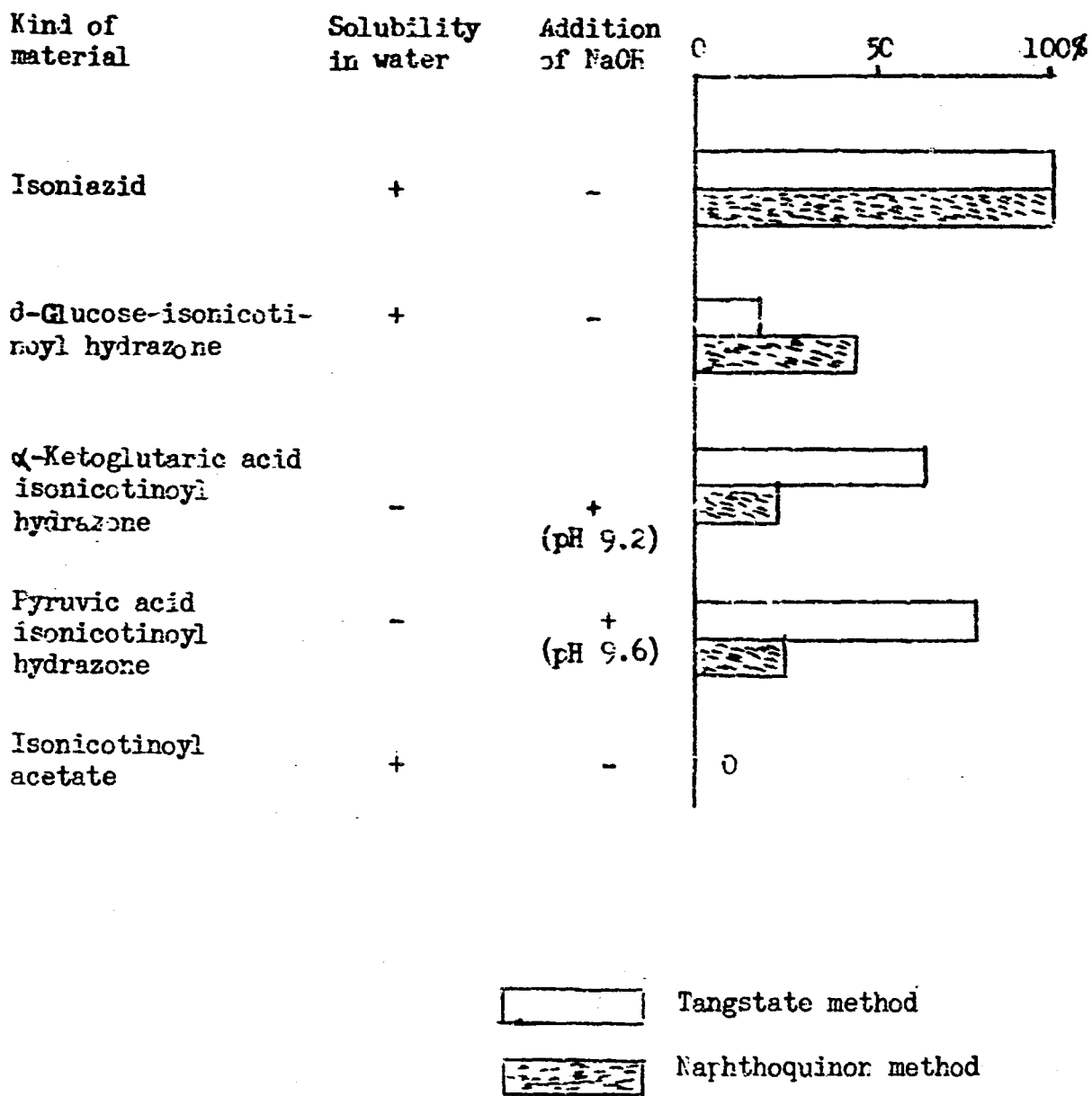


Fig. 6 Recovery of Free INH in Aqueous Solution of Different Kinds of INH Derivatives

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14. KEY WORDS	LINK A		LINK B		LINK C	
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