The first I would attribute mainly to pyridoxine deficiency; the second to deficiency of some other member of the vitamin-B group, pantothenic acid or riboflavin being the most likely. No doubt mixed types occur, since nearly always when one member of the vitamin-B group is deficient, others are also. It is not surprising, therefore, that pyruvate-tolerance tests are abnormal in most cases. Solely from the point of view of therapy, it follows that the ideal treatment is daily intravenous injections of aneurine, with other members of the vitamin-B group, including pantothenic acid and pyridoxine, in large doses. For the purpose of elucidating these syndromes, however, it would be of great value to treat some cases with pyridoxine or pantothenic acid alone, and to observe the results, being prepared to add the other vitamins if necessary.

With regard to animal experiments, a different approach is indicated. The feeding trials of Follis and Wintrobe and of Swank and Adams need to be repeated, but with the emphasis on function, not histology, and with careful assessment of motor and sensory changes by clinical examination of the living animal and by electromyography. This is really only a special case of the generalization that in medicine, except to the anatomist, details of structure are significant only in their relation to function. Swank and Adams report, for example, that the motor roots of certain pyridoxine-deficient animals were normal, but this gives no information about the presence or absence of muscle weakness, since the motor nerves might well be functionless at some point distal to the motor root, and retrograde degeneration of nerves does not occur to any great extent (though one might have expected chromatolysis of the anterior horn cells to be visible).

Summary

The importance of recognizing neuropathy in cancer patients is mentioned, and two cases of proximal motor neuropathy in association with carcinoma are described. In the first, there was also cardiac weakness similar to the mixed type of beriberi. Both cases subsequently recovered neurologically, and there is definite evidence that vitamin B was responsible, particularly pyridoxine. In both the carcinoma is still present, although it has responded to irradiation. These cases should be differentiated from cases of true peripheral neuropathy. A hypothesis based on competition for the vitamin by the tumour cells is suggested. It is also suggested that pyridoxine should be included when treating these cases in future.

My thanks are due to Mr. Maxwell Ellis, Mr. W. A. Mill, Mr. A. H. Hunt, and Dr. Worster Drought for permission to publish case reports of patients under their care. I am also indebted to Dr. M. Lederman and many of my colleagues for their co-operation, and also to Dr. Frank Sargent, who first suggested to me the theory of substrate competition.

[Since this paper was written an account of eight cases of proximal motor neuropathy in association with carcinoma of the bronchus has been published by R. A. Henson (*Proc. roy. Soc. Med.*, 1953, 46, 859).]

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METHOD OF ESTIMATING ISONIAZID IN BIOLOGICAL FLUIDS

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Several colorimetric methods have been proposed for estimating isoniazid (isonicotinic acid hydrazide) in biological fluids (Kelly and Poet, 1952; Rubin *et al.*, 1952; Ginoulhiac, 1952; Smolarek and Stahl, 1953; Wojahn and Wempe, 1953; Jacobs, 1953).

The following method was developed in an attempt to improve the specificity of isoniazid estimations. It depends on the reaction between isoniazid and picryl chloride (2-chloro, 1,3,5-trinitrobenzene) to form a coloured compound, which may be measured photoelectrically.

While our work was in progress a method for the estimation of isoniazid with picryl chloride was described (Kirschbaum, 1952), but not its application to biological fluids.

Method for Plasma

Reagents.—Whenever available, all reagents used were of analytical reagent grade.

10% (W/V) sodium tungstate.2H₂O

2/3N sulphuric acid

N sodium hydroxide

N/10 hydrochloric acid

Ammonium sulphate

n-butanol 1 part, diethyl ether 3 parts

Phosphate buffer pH 7: 60 ml. of M/5 disodium hydrogen phosphate added to 40 ml. of M/5 potassium dihydrogen phosphate.

Phosphate buffer pH 6.5: 30 ml. of M/5 disodium hydrogen phosphate added to 70 ml. of M/5 potassium dihydrogen phosphate

5N acetic acid

Picryl chloride (Hopkin and Williams). This reagent should be washed twice in distilled water, recrystallized twice from chloroform, and air-dried. It is stored in a dark bottle in the refrigerator.

A freshly prepared solution—35 mg. of picryl chloride in 10 ml. of ethanol—is required for the estimation.

Procedure

Plasma (heparinized) from 10 ml. of blood is required for the estimation.

Protein Precipitation.—8 ml. of distilled water, 4 ml. of plasma, and 2 ml. of sodium tungstate solution are measured into a 1-oz. (30-ml.) screw-capped bottle. After mixing well, 2 ml. of 2/3N sulphuric acid is slowly added, with constant shaking. The lid is screwed lightly in position, and the bottle placed in a boiling-water bath for five minutes, cooled, and centrifuged for 10 to 15 minutes—that is, enough to make possible the withdrawal of 12 ml. of supernatant protein-free fluid.

Extraction of Isoniazid into Butanol-Ether Mixture.—11 g. of ammonium sulphate, 0.5 ml. of N sodium hydroxide

solution, 1 ml. of phosphate buffer pH 7, 40 ml. of butanol-ether mixture, and 12 ml. of supernatant protein-free fluid are measured into a glass-stoppered 2-oz. (60-ml.) bottle. The bottle is shaken vigorously for five minutes (mechanically) and then allowed to stand until the butanol-ether and aqueous phases separate. The butanol-ether phase is transferred as completely as possible to a fresh 2-oz. glass-stoppered bottle.

Extraction of Isoniazid into Hydrochloric Acid.—The butanol-ether extract is shaken for two to three minutes with two separate volumes of 5 ml. of N/10 hydrochloric acid. The combined acid extracts are then placed in a 6 by 1 in. (15 by 2.5 cm.) test-tube.

Colour Reaction.—1 ml. of N sodium hydroxide and then 2 ml. of phosphate buffer pH 6.5 are added to the acid extract. After mixing well, 0.5 ml. of freshly prepared picryl chloride solution is added and the tube heated for 20 minutes in a boiling-water bath. The tube is cooled and 0.4 ml. of 5N acetic acid is added, followed by 6 ml. of *n*-butanol. After being shaken for approximately one minute the tube is centrifuged and the butanol phase transferred to a test-tube, two drops of ethanol being added to prevent any cloudiness due to water droplets.

Colour Measurement.—Readings are made against water in a Hilger absorptiometer, using a 4-cm. (8-ml.) light-path cell and an Ilford blue-green filter No. 603 (absorption maximum 485 m μ).

Standard Curve.—Results are calculated by comparison with a standard curve. This is prepared from readings obtained by applying the method to known freshly prepared solutions of 0, 4 μ g., 8 μ g., and 16 μ g. of isoniazid, each level in 4 ml. of pooled, non-haemolysed plasma from blood transfusion material. The reproducibility of the method is satisfactory: the absorptiometer readings are linearly related to the amounts of isoniazid present within the range 0 to 20 μ g. The standard deviation of a single estimation is about $\pm 10\%$. It is necessary to estimate unknown samples and standard samples concurrently.

Method for Urine

The same method is used as for plasma, but protein precipitation is omitted.

Test samples are read against standard aqueous isoniazid solutions treated in the same way.

Specificity of Method

Metabolic Products of Isoniazid Ingestion

The urine of human subjects after isoniazid ingestion is known to contain isoniazid, isonicotinic acid, and isonicotinoyl glycine (Cuthbertson *et al.*, 1953). Isonicotinamide was not detected, but could have been present undetected in amounts of up to about 5% of the dose. The reactions of these substances with picryl chloride have been described (Cuthbertson *et al.*, 1953). Quantities of 150 μ g. of each of the last three substances do not interfere in the recommended method.

Another metabolic product of isoniazid ingestion is also present in human urine, but its nature is unknown. By paper chromatography of concentrated urine solutions using water-saturated *n*-butanol as developing solvent, this substance has approximately the same *R_F* value as isonicotinamide. It reacts with picryl chloride to form a red-coloured compound at alkaline pH and a yellow-coloured compound at acid pH, but does not react with the cyanogen bromide-pyrazolone or cyanogen bromide-benzidine reagents in the same way as isonicotinamide (Cuthbertson *et al.*, 1953).

Since this substance reacts with picryl chloride similarly to isonicotinic acid and isonicotinamide, it is unlikely to interfere in the determination of isoniazid by our method. This assumption is supported by the fact that after isoniazid ingestion some 24-hour human urine samples contain little or no isoniazid, as shown by this method, but do contain appreciable amounts of the unidentified substance, as shown by chromatography.

Wojahn and Wempe (1953) have shown that isoniazid combines through its hydrazide group with an unknown substance or substances in plasma and urine to form micro-biologically inactive or less active compounds. We have no direct evidence to show whether or not these compounds, if different from the unknown metabolite described above, interfere in our method. Circumstantial evidence indicates that they interfere only slightly, if at all. Smolarek and Stahl (1953), using a method that does not measure this type of "bound" isoniazid, found that the average 24-hour urine excretion of free isoniazid in five human subjects receiving a single 500-mg. oral dose of isoniazid was 17.5% of the dose. The individual values varied between 13.6% and 22.5% of the dose. We found that the 24-hour urine contents of isoniazid in two human subjects each receiving 250 mg. of isoniazid orally every 12 hours were 21% and 22% of the dose. Furthermore, approximate assessments of the isoniazid content of urine samples from chromatographic evidence agree with actual estimations.

Since isonicotinic acid is a metabolic product of isoniazid ingestion in human subjects, hydrazine could also be liberated. Hydrazine reacts with picryl chloride to form hexanitrohydroazobenzene (Kulberg and Cherkosov, 1952), the light absorption of which would be measured by this technique. However, Kelly and Poet (1952) and Kelly *et al.* (1952) have not detected the presence of free hydrazine in the body fluids of the animals they studied. Using their method, we were unable to detect free hydrazine in the plasma of human subjects receiving orally 200 mg. of isoniazid per subject, or in the plasma of guinea-pigs (body weight 380 to 420 g.) receiving orally 10 mg. of isoniazid per guinea-pig, at $\frac{1}{2}$, 1, 2, 4, or 7 hours after the dose. Also 24-hour urine samples from human subjects receiving 500 mg. of isoniazid orally contained no free hydrazine.

Streptomycin and *p*-Aminosalicylic Acid

Experiments were performed to detect any possible interference with the picryl chloride method by streptomycin or *p*-aminosalicylic acid.

In aqueous solutions of isoniazid the addition of 500 μ g. of streptomycin per ml. caused no increase in the readings obtained.

p-Aminosalicylic acid was found to interfere even in relatively low concentrations. For this reason it was possible to perform isoniazid assays only on patients not at the time receiving any *p*-aminosalicylic acid, but streptomycin therapy was not a factor of importance in this connexion.

Results

Plasma

Isoniazid was estimated in the plasma of 24 patients. Twenty-two received 100 mg. and two 150 mg. of isoniazid orally every twelve hours. Table I gives each patient's dose in mg. of isoniazid per kg. body weight.

Blood samples were taken by venepuncture shortly before the morning dose of the drug, and three further samples were withdrawn at intervals of 30, 60, and 120 minutes afterwards. Preliminary tests had soon shown that the picryl chloride method was not sensitive enough to detect any isoniazid in plasma twelve hours after the last dose, and so it was considered permissible to treat the early morning plasma of each patient as a "blank."

Table I shows the results obtained from these patients. In general, the plasma levels are somewhat lower than the figures quoted in previous publications (Elmendorf *et al.*, 1952; Rubin *et al.*, 1952), and the highest level was almost always found 30 or 60 minutes after the dose.

In 10 patients receiving 1.15 to 1.5 mg. of isoniazid per kg. body weight the average level of isoniazid estimated 30 minutes after the dose was 0.7 μ g./ml., 60 minutes after the dose 0.8 μ g./ml., and 120 minutes after the dose 0.4 μ g./ml. For 14 patients receiving 1.55 to 3.65 mg. of isoniazid per kg. body weight the corresponding 30-minute,

TABLE I.—Concentrations of Isoniazid in the Plasma of Patients After Oral Administration

Case No.	Dose in mg./kg. Body Weight	Plasma Level of Isoniazid in $\mu\text{g./ml.}$ at intervals, after the Dose, of		
		30 Minutes	60 Minutes	120 Minutes
1	1.15	1.6	0.8	0.5
2	1.35	0.8	1.0	0.9
3	1.35	1.6	1.3	0.8
4	1.35	0.0	0.0	0.0
5	1.35	0.0	1.3	0.5
6	1.35	1.1	0.9	0.0
7	1.35	0.0	1.0	1.1
8	1.45	1.1	0.9	1.1
9	1.50	0.5	0.0	1.2
10	1.50	0.0	1.0	0.0
11	1.55	1.0	1.1	0.9
12	1.55	1.0	0.7	0.5
13	1.55	2.0	1.5	0.8
14	1.55	2.8	4.0	3.2
15	1.55	0.6	1.5	1.0
16	1.65	1.3	1.0	0.6
17	1.65	0.5	0.5	0.8
18	1.75	0.9	1.5	2.6
19	1.80	0.2	1.2	0.4
20	1.95	2.0	0.9	0.6
21	1.95	0.0	0.0	0.0
22	2.05	0.8	1.9	2.1
23	2.60	0.2	1.9	0.6
24	3.65	1.0	1.0	0.6
10 R	1.50	0.5	0.2	0.0
21 R	1.95	0.0	0.0	0.5

The plasma isoniazid concentration of Cases 10 and 21 were redetermined three months later as shown under 10 R and 21 R.

60-minute, and 120-minute values were 1.0 $\mu\text{g./ml.}$, 1.3 $\mu\text{g./ml.}$, and 1.0 $\mu\text{g./ml.}$

Three of the 24 patients (Cases 4, 10, and 21) had no detectable isoniazid in any of the blood samples. Nothing in their clinical condition suggested a disturbance of intestinal absorption, and examination of urine for isoniazid and its metabolites was undertaken in Cases 10 U and 21 U (Table II). This subject is considered in the discussion of

TABLE II.—Urinary Excretion of Isoniazid. (a) 100 mg. Isoniazid, Administered Orally 12-hourly; (b) 250 mg. Isoniazid, Administered Orally 12-hourly

Case No.	mg. Isoniazid per kg. Body Weight	$\mu\text{g./ml.}$ Isoniazid in Urine	Total Isoniazid Excreted in 24 Hours (mg.)	% of Dose Excreted in 24 Hours
(a) 25 U	3.3	13	9.7	4.8
26 U	3.5	20	35.0	17.5
27 U	2.7	18.5	32.0	16
28 U	2.3	6.2	11.5	5.7
29 U	3.2	8.0	17.4	8.7
30 U	3.1	8.4	8.0	4.0
31 U	3.9	4.3	5.3	2.6
10 U	3.0	2.0	3.0	1.5
21 U	3.9	2.6	5.7	2.8
			Average	7.1
(b) 28 U	5.7	61	103	21.0
29 U	8.0	42	110	22.0

results of urine assays. Three months later, blood curves were repeated on two of these patients (Nos. 10 R and 21 R), who were still in hospital. These two curves are very similar to those obtained on the first occasion. In both patients the highest level attained was 0.5 $\mu\text{g./ml.}$, and this was present in only one specimen from each patient.

Urine

The results shown in Table II (a) were obtained from nine patients receiving 100 mg. of isoniazid 12-hourly. Whenever possible a 24-hour specimen of urine for use as a "blank" was collected before isoniazid therapy was begun, and the test estimations were performed on aliquots of 24-hour specimens of urine collected during treatment.

The amount of the dose excreted as isoniazid during 24 hours varied widely (1.5% to 17.5%, average 7.1%). These values are appreciably lower than those found by Elmendorf *et al.* (1952), using the assay method of Kelly and Poet (1952). In six patients receiving 3 mg. of isoniazid per

kg. body weight, they found the 24-hour urinary excretion of isoniazid lay between 47.8% and 70.7% of the dose. Our results thus confirm that the method of Kelly and Poet measures something besides isoniazid (Smolarek and Stahl, 1953; Wojahn and Wempe, 1953; Cuthbertson *et al.*, 1953). Samples 10 U and 21 U (Table 11 (a)) are urine samples from the patients whose blood values are shown in Table I as 10 and 21 and 10 R and 21 R. In these two patients the very low excretion values, together with the low blood levels suggested some defect of absorption, but chromatographic analysis (Cuthbertson *et al.*, 1953), of the two urine samples revealed the presence of the metabolites of isoniazid (isonicotinoylglycine, isonicotinic acid, and the unidentified metabolite) in approximately the same amounts as contained in the urine of patients with higher isoniazid levels in blood and urine. Assessment of their clinical response to isoniazid was rendered difficult, for both the patients were also receiving streptomycin.

Patients whose urine samples are given as 28 U and 29 U were subsequently given 250 mg. of isoniazid twelve-hourly. There was an appreciable rise in percentage of the dose excreted as isoniazid during the succeeding 24-hour period (Table II (b)).

Further assessment of the method is desirable and should lead to improved evaluations of results obtained by it. It is hoped that the description given here of both method and results may be of use to those who wish to carry the matter further.

Summary

A colorimetric method is described for estimating isoniazid in biological fluids by means of picryl chloride.

The specificity of the method is assessed with reference to the metabolites of isoniazid.

Results are given for the amounts of isoniazid in plasma and urine from patients receiving the drug.

We wish to thank the physicians and surgeons of the Brompton Hospital for permission to investigate the samples from their patients; Dr. R. N. Johnston, medical registrar, for his great assistance in the collection of samples; and Miss E. Harris and Mr. F. W. Price for technical assistance.

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The professor of medicine at Duke University, North Carolina, Dr. James V. Warren, is to visit Kenya to perform research work on giraffes. The object of this research is to aid those who are designing "G-suits" (gravity suits) worn by the pilots of high-speed jet aeroplanes. In pulling out of power dives or other manoeuvres at high speeds pilots are subjected to pressure eight or nine times the force of gravity. It is generally believed that a pressure of 8 G is the maximum that a pilot can withstand. The G-suits are pressurized and designed to offset the violent pressure changes which cause a reduced flow of blood to the brain and consequent black-outs. Dr. Warren believes that the giraffe, with its head a considerable distance from its heart, has at all times to meet the situation that man faces when he is subjected to centrifugal force in military planes making fast turns and dives. "Maybe," Dr. Warren is reported to have said, "the giraffe has some sort of booster mechanism that helps its heart."—*U.S.A. in Review*, February 24, 1954.