CCXLIV. THE THIOCHROME TEST FOR ANEURIN (VITAMIN B₁) IN URINE AS AN INDEX OF NUTRITIONAL LEVEL

By GEORGE MASON HILLS

From the Courtauld Institute of Biochemistry, Middlesex Hospital, London

(Received 20 October 1939)

THE method of Jansen [1936] for estimating aneurin by measuring the blue fluorescence of the thiochrome produced on oxidation was applied by Westenbrink & Goudsmit [1937, 1] to the estimation of the vitamin in urine for clinical purposes. The specificity of the method seemed doubtful, as the figures they published in a later paper [Westenbrink & Goudsmit, 1938, 1] for the normal 24 hr. excretion were somewhat higher than those obtained by Harris *et al.* [1938] using the bradycardia method. Karrer [1937] and Ritsert [1938], using modified thiochrome methods, as well as many other workers more recently, reported values rather higher than those of Harris *et al.* [1938]. In an attempt to simplify the original method of Westenbrink & Goudsmit [1937, 1] for routine use, it was considered desirable to use the response to a standard test-dose, rather than the 24 hr. excretion, as an index of nutritional level. These authors have themselves examined the effect of test-doses [Westenbrink & Goudsmit, 1937, 2; 1938, 1, 2], mainly on the 24 hr. excretion, but suggest that the morning excretion, normally low after a light Dutch breakfast, may be used as a rapid test of nutritional level if 1 mg. of aneurin is given with the breakfast. Such a test has been made the basis of the present work, and comparison with the values obtained for the 24 hr. excretion show that the test can not only be completed more rapidly, but is also much more sensitive owing to the increased concentration in the urine. Wang & Harris [1939] have lately reported that the results they obtained by a modified thiochrome test agreed with those obtained on the same specimens of urine by biological assay.

Methods

(1) *Collection of specimens.* The urine was preserved at an acid pH, 0.5 ml. of conc. HCl or glacial acetic acid being added to the amount collected during each period of 3 hr. Toluene was added as well to prevent the growth of moulds. After collecting a 24 hr. specimen, a breakfast low in aneurin was given (e.g. white bread and butter, honey, tea), the urine being collected for a further 3 hr. A second 3 hr. specimen was then obtained after a similar meal supplemented with 1 mg. aneurin by mouth.

(2) *Analytical technique.* Normal urines and those from cases of suspected deficiency were filtered from deposits if necessary and diluted so that the amount excreted per hr. was made up to 500 ml. Specimens suspected of containing more than about 20 μ g. per hr. were diluted to contain less than this amount in 500 ml. The diluted specimens were brought to pH 3–5 if necessary. 75 ml. of the diluted urine were stirred mechanically in a centrifuge tube for 1–2 min. with 25 $\pm$ 2 mg. of "Clarit" acid clay (kindly supplied by the courtesy of Messrs Lever Bros.). A second aliquot was measured out and a similar amount of acid clay added during the stirring of the first. Two further tubes were set up to

prepare adsorbates from the same amount of urine with different amounts of added aneurin (less than 4 $\mu\text{g.}$). The adsorbates were collected by centrifuging for 2–5 min. and the supernatant fluid was discarded. The wet adsorbates were treated in the same tubes with 2 ml. pure methyl alcohol. 1 ml. water was added to one of the tubes, which was to serve as a blank, prepared from urine without added aneurin. The contents of the four tubes were stirred with a stream of N_2 and 1 ml. 7.5 N NaOH was added to each, followed by 1 ml. 1.25 % $\text{K}_3\text{Fe}(\text{CN})_6$ to each of the tubes except the blank. 12.5 ml. isobutyl alcohol saturated with water were added to each tube in turn and stirring was continued for 1–2 min. After thorough mixing the tubes were allowed to stand for a few minutes and the upper layer was decanted through a dry 8.5 cm. filter paper (previously purified by exhaustive extraction with wet isobutyl alcohol) into a test tube specially selected for fluorescence measurement and graduated for the collection of 10 ml. After the collection of 10 ml. of filtrate the tube was corked to prevent clouding through evaporation of some of the methyl alcohol during the fluorescence measurements. The fluorometer of Cohen [1935], constructed with improvements to be described later, was used. The intensity of the fluorometer lamp was standardized between readings by means of a solution of quinine sulphate in 0.1 N H_2SO_4 containing about 0.2 mg. quinine sulphate per 100 ml.

The aneurin content of the sample is most easily determined by a graphical method, illustrated in Fig. 1. Provided that the aneurin content is not too great, it is linearly related to the fluorescence (curve II) of the oxidized extracts. The intercept (ab) of this line on an abscissa at a level corresponding to the fluorescence of the unoxidized blank (abc) gives the aneurin content of the sample.

(3) *The fluorometer.* Only those details in which the instrument differs from that of Cohen [1935] are given. The source was a 500 watt low pressure Hg arc in a straight quartz tube and the beam was filtered through Wood's glass of 3 mm. thickness. The test tube was about 14 cm. from the source and as near as possible to the photoelectric cell. Heat from the source was absorbed by means of a fused quartz window in the lamp house instead of window glass. Scattered ultra-violet light was prevented from affecting the cell by means of a Wratten 2A filter. A suitable blue colour-filter was used to bring the maximum response of the cell to wave-length 460–470 $m\mu$, corresponding to the maximum fluorescence of thiochrome [Kuhn & Vetter, 1935]. Using Weston "Photronic" cells, the makers' spectral sensitivity curves and the published transmissions of Wratten filters show that the type I cell requires a Wratten 49A filter while the type II cell requires a Wratten 48. The latter cell is more sensitive and has greater relative sensitivity in the blue, but is less permanent.

The test tubes used in the fluorometer were of thin non-fluorescent glass of nominal size $5 \times \frac{3}{4}$ in. All tubes were rejected which did not conform to the following specifications:

- (a) External diameter less than 19 mm.
- (b) Internal diameter greater than 15 mm.

Fig. 1. Fluorescence and added aneurin. Case, H.F. iii. Specimen, 2. 3 hr. after 1 mg. aneurin. Curve I, 75 ml. dilution 0.50 l. per hr. Curve II, 75 ml. dilution 1.00 l. per hr.

(c) When graduated to contain 10 ml. the mark was at a height of 47.5 ± 1.0 mm.

(d) Fluorescence of 10 ml. quinine sulphate within $\pm 2\%$ of the mean. (More rigorous selection was impossible owing to fluctuation of the lamp intensity.)

(e) Constancy of the small fluorescence when they contained 10 ml. wet *iso*-butanol.

The galvanometer had an internal resistance of 189 ohms and a sensitivity of 3045 mm. per microamp. for 1 m. scale distance, but as this gave a deflexion of about 500 mm. per $\mu\text{g.}$ aneurin it was reduced to about 50 mm. per $\mu\text{g.}$ by means of a 1000 ohm shunt. These deflexions were obtained when using the more sensitive type II "Photronic" cell.

Discussion of the method

The technique adopted is closely similar to the simplification by Jowett [1939] of the original method of Westenbrink & Goudsmit. The methods agree in eliminating washing and drying of adsorbates, in the use of only one concentration of oxidizing agent and in the use of a blue-violet filter in the fluorescence measurement. Jowett, however, used a Zeiss-Pulfrich photometer for visual estimation of the fluorescence. Although this is an improvement on the simpler visual method first adopted by Karrer & Kubli [1937], we have rejected visual methods for routine use on account of errors due to the personal factor, in spite of the elimination of error due to fluctuations in the intensity of the source in the simple fluorometer of Cohen.

In order to give workers who have not used the thiochrome method some idea of the possible size of errors which may be involved, fairly detailed protocols are given in some of the tables illustrating various aspects of the method. The three types of specimen are denoted, for the sake of brevity as 24, 3₀ and 3_{B1} respectively. The fluorescence is expressed as the percentage of that produced under similar conditions from 1 $\mu\text{g.}$ aneurin oxidized in pure solution with 0.3 ml. 1.25% $\text{K}_3\text{Fe}(\text{CN})_6$.

(1) *Collection of specimens.* Oral administration of the test-dose was preferred, since after injection the very high concentration in the blood allows overflow through the kidney even in cases of deficiency [Westenbrink & Goudsmit, 1938, 1]. A 3 hr. test-period was chosen since it has been shown that the maximum excretion after a test-dose by mouth occurs during the 2nd hour. Collection for a 3rd hour was made to minimize errors due to the retention of urine formed previously but not voided, and the subject was encouraged to take enough fluid to ensure an output preferably not less than 100 ml. per hr. for the same reason. Although the test will not distinguish between inadequate reserves and faulty absorption, this is not of great clinical importance, since under the latter circumstances a conditioned deficiency will be present.

(2) *Adsorption.* Westenbrink & Goudsmit [1937, 1] found that the extra fluorescence from aneurin added to urine was often considerably less than that from aneurin oxidized in pure solution, although there was no significant loss due to incomplete adsorption. The fluorescence from added aneurin was increased by dilution, but under their conditions the apparent recoveries were only 57% for night urine diluted 10 times and 71% for day urine diluted 3 times. Using "Clarit" as adsorbent, with 24 hr. specimens diluted to 7.5 l. corresponding to the degrees of dilution used by Westenbrink & Goudsmit, the apparent recovery has varied from 33 to 74%. Increase of the dilution to 12 l. per 24 hr. gave the increased "recoveries" shown in Table I. Although the means for different

Table I. *Fluorescence from added aneurin oxidized in the presence of urine*

Nature of urine	No. of samples	Fluorescence	
		Mean per $\mu\text{g.}$	Standard deviation
N 24	6	87	14
N 3 _O	6	80	13
N 3 _{B₁}	6	81	25
H 24	8	69	18
H 3 _O	8	73	16
H 3 _{B₁}	8	74	10

N = normal.

H = hospital case.

types of specimen show some uniformity, the variations between individual specimens, indicated by the standard deviation, emphasize the advisability of calibrating the fluorescence of added aneurin in the presence of each urine examined. Such calibration serves also to check the possibility of abnormal interference with the fluorescence which occurs in some specimens, and which might vitiate the value of the results for other reasons. Filtration of the specimens had been avoided, owing to the possibility of loss of aneurin by adsorption on the deposits or on the filter paper, until it was found that in the presence of deposits the fluorescence of the *isobutanol* extract, even in the case of the blank, was considerably reduced, presumably by non-fluorescent substances which reduced the intensity of ultra-violet light in the bulk of the solution by absorption. This effect is shown in Table II.

Table II. *Effect of filtration of urine on fluorescence*Subject H.F. iii. Sample 3_{B₁} (cf. Fig. 3, column 1)

	Fluorescence	
	Unfiltered urine	Filtered urine
Blank	58	108
Oxidized	69	169
Added aneurin (per $\mu\text{g.}$)	23	65
Apparent assay ($\mu\text{g.}$)	0.40	0.95

Table III. *Effect of amount of "Clarit" on fluorescence from added aneurin and on blank*

Subject	Nature of urine	Fluorescence of adsorbate			
		Blank with		Added vitamin B ₁ with (per $\mu\text{g.}$)	
		25 mg.	50 mg.	25 mg.	50 mg.
N.F. i	24	102	146	97	76
	3 _O	117	167	92	67
	3 _{B₁}	115	144	92	73
N.M. v	24	96	153	108	79
	3 _O	165	165	73	69
	3 _{B₁}	162	152	78	66

In order to reduce the adsorption of interfering substances as much as possible, not only is the maximum convenient dilution recommended, but the amount of adsorbent has also been reduced, as in the method of Marrack & Höllering [1939]. Table III shows that reduction of the amount of "Clarit" from 50 to 25 mg. per 75 ml. diluted urine not only increases the fluorescence per $\mu\text{g.}$ added aneurin but usually reduces the fluorescence of the blank. With

the smaller amount of adsorbent, the adsorption of aneurin is incomplete, since up to 25 % greater fluorescence is often obtained when aneurin is added to the adsorbate than when it is added to the corresponding amount of urine before adsorption (Table IV). Since the fluorescence increases linearly with the amount

Table IV. *Loss of aneurin on adsorption*

Subject	Nature of urine	No. of specimen	Fluorescence per $\mu\text{g.}$ vitamin B ₁ added to		% adsorbed
			Urine	Adsorbate	
N.M. i	24	2	80	83	96
H.M. iii	24	4	75	98	76
			72	96	
H.M. v	24	3	83	94	87
			82		
Mean =					86

of added aneurin, there must be a corresponding loss of the aneurin of the specimen for which compensation is made in the calibration, provided that this is carried out by adding aneurin to the urine before adsorption. If, for convenience in transmitting samples from a distance, adsorbates without added aneurin have been made, it has seemed advisable to increase the results by some 15–20 % to allow for the loss; this empirical correction must be regarded as an approximation only, since the loss seems to vary in different samples.

Wang & Harris [1939] have criticized the use of adsorption as a means of removing aneurin from interfering substances in urine and prefer to oxidize the urine directly. The method cannot be conveniently applied to aliquots of more than 2 ml. (i.e. 0.15 % of a 24 hr. specimen of average volume) and is therefore best adapted for use with concentrated urines of high aneurin content. For greater accuracy with urines of low aneurin content the present method which uses 0.625 % of a 24 hr. specimen as the normal aliquot is preferable. The method has been compared with that of Wang & Harris [1939] in the case of specimens of high aneurin content, and it has been found that with the same sample (2 ml.) the present method usually gives lower blanks and greater fluorescence per $\mu\text{g.}$ added aneurin, in spite of the fact that Wang & Harris [1939] claim quantitative recovery (Table V). This discrepancy is accounted for

Table V. *Comparison of the present method with that of Wang & Harris [1939]*

All determinations made on 24 hr. specimens. 2 ml. aliquot except in the case of H.M. v. 3 when 8.1 ml. were used for the determination by the present method

Specimen	Method	Fluorescence			Assay $\mu\text{g.}$	% of W. & H.
		Blank	Oxidized	1 $\mu\text{g.}$ vitamin B ₁		
*	Present	57	145	81	2.15	100
	W. & H.	68	145	71	2.15	100
*	Present	50	95	86	1.10	67
	W. & H.	51	110	71	1.65	100
H.M. ii. 3	Present	39	130	79	1.15	68
	W. & H.	68	178	64	1.70	100
H.M. v. 3	Present	34	224	83	2.30	57
		38	208	82	2.10	52
	W. & H.	36	100	63	1.00	100

* No reference no. given since the response to a test-dose was not determined and the case is not further discussed in this paper.

by the fact that these workers used a visual method by which it is impossible to assess the reduction of the fluorescence of thiochrome when added to the blank *isobutanol* extract of urine. Wang & Harris obtained the same results with their specimens as by the bradycardia method, while the present technique gives values on the average 30% lower than those obtained by their thiochrome technique.

(3) *The blank test.* The use of an unoxidized sample as a blank is not entirely satisfactory, since the unknown substances responsible for this fluorescence might be either increased or reduced in amount during the oxidation of aneurin to thiochrome. The latter seems more likely, since it is frequently found that samples low in aneurin show diminished fluorescence on oxidation, unless a blue filter is used to eliminate, as far as possible, fluorescent light not due to thiochrome (Table VI). Ritsert [1938] avoided this difficulty by using as a blank

Table VI. *Effect of blue filter on assay*

Subject, H.M. iii (Fig. 3, col. 1, 24 hr. sample). Weston "Photronic" cell, type I

Wratten filters	2A	2A + 49A
Wave-length for maximum sensitivity	575 m μ	460 m μ
	Fluorescence	
Blank	159	77
Oxidized	149	98
Added aneurin (per μ g.)	73	65
Assay of aliquot (μ g.)	- 0.15	+ 0.30
Content of total (μ g.)	- 25	+ 50

that amount of an *isobutanol* extract prepared from urine freed from aneurin by adsorption which would give the same amount of white or greenish fluorescence as was judged by the eye to be present in the oxidized sample, which showed in addition the blue-violet fluorescence of thiochrome. Thiochrome was added to the blank till a match was obtained with the fluorescence of the *isobutanol* extract from an oxidized sample of the urine being tested. The amount of added thiochrome gave the aneurin content of the sample. The method is not theoretically sound since different urines differ qualitatively as well as quantitatively in the blank fluorescence. Moreover, the technique requires considerable visual acuity on the part of the observer and is likely to be profoundly susceptible to errors due to the personal factor. The average human eye, with sensitivity at 465 m μ less than 10% of that at 555 m μ (the wave-length of maximum sensitivity), is less ideally adapted for the estimation of the fluorescence of thiochrome in the presence of a green blank than a selenium cell, even without a filter, for which the corresponding relative sensitivity may be 65–90%, depending on the type of cell. Although the eye, unlike the photoelectric cell, distinguishes qualitatively between the green blank and the blue-violet of thiochrome, the presence of the two renders matching extremely difficult in any visual method (except that of Jowett [1939] using a violet filter), especially as the amount of green fluorescence may be different in the oxidized and unoxidized samples. Even with a filter the blank has a greater relative effect on the eye than on the selenium cell, owing to the smaller relative sensitivity of the former in the blue-violet region.

A further difficulty arising from the use of an unoxidized sample as a blank is due to the absorption of ultra-violet light by interfering substances, with a consequent reduction of the intensity of fluorescence in the bulk of the solution. If the concentration of such substances is altered during the process of oxidation,

the blank will differ from the oxidized samples in other respects than thiochrome content. Occasionally samples occur, especially with highly pigmented urines, in which visual observation detects practically no fluorescence in the layers of isobutanol extract remote from the source of light. As this interference is invariably reduced after oxidation, the oxidized sample shows an increase in fluorescence not due to the formation of thiochrome and the assay is consequently too high. A further type of interference which occurs in pigmented urines is due to traces of a yellow pigment which is present in the blank and absorbs some of the blue fluorescent light, but which is absent from or diminished in the extracts of the oxidized samples. An example is shown in Table VII in

Table VII. *Effect of pigment in blank extract*

Specimens, H.F. iii. i

Specimen	Fluorescence		
	24	*3 _O	3 _{B₁}
Blank	120	58	108
Oxidized	153	91	169
1 μ g. added vitamin B ₁	83	60	65
Assay of aliquot (μ g.)	0.40	0.55	0.95
Content of total (μ g.)	65	11	19

* Blank extract orange, others pale yellow.

which the low blank for the 3 hr. specimen on a low aneurin intake (3_O) was due to pigment which was largely absent from the oxidized samples. As a result the apparent excretion is greater than the average for 3 hr. during the previous 24 hr. on a good hospital diet. It appears that the blank in this extreme case might be too low by 30%.

Since it is impossible to estimate the effect of oxidation on the blank it is necessary to keep it as low as possible. In general this can be achieved by reducing the aliquot or by increasing the dilution when purification is carried out by adsorption. Accordingly, where the results obtained by the present method differ from those obtained by the method of Wang & Harris [1939], as shown in Table V, we believe them to represent more nearly the true aneurin content of the sample. Our lower blanks cannot be due to absorption of ultra-violet or fluorescent light, since such interference would give high results, while actually the results are lower than those obtained by the technique of Wang & Harris.

Reduction of the blank by reduction of the aliquot is limited by the minimum amount of aneurin which it is desired to detect. It has not yet been found possible to reduce the blank significantly by other means. After observing slightly lower blanks with samples freshly diluted at room temperature than with cold samples of the same dilution after a few hours in the ice-chest, the effect on the blank of adsorption at 37° was found to be only a very slight decrease.

Prolonged storage of samples may lead to a considerable increase in the fluorescence of the blank, its colour differing from that due to thiochrome (Table VIII, specimen N.M. ii. 24). The substances responsible are destroyed on oxidation since the fluorescence of oxidized samples is not increased and is the typical blue-violet of thiochrome, not the green of the blank. It is inadvisable to delay the preparation of adsorbates even though the aneurin itself may not be greatly reduced (specimen N.M. i. 3_{B₁}).

Table VIII. *Effect of storage*

The stored samples were diluted and kept at room temperature for 80–90 days

Specimen	Sample	Fluorescence	
		Fresh	Stored
N.M. ii. 24	Blank	89	131
	Oxidized	151	117
	1 μ g. vitamin B ₁	73	64
	Assay (μ g.)	0.85	–0.20
N.M. i. 3B ₁	Blank	86	71
	Oxidized	309	250
	1 μ g. vitamin B ₁	55	63
	Assay (μ g.)	4.05	2.85

(4) *Calibration.* For accurate calibration the amount of added aneurin must be at least equal to that present in the sample. On the other hand, if the total amount exceeds 7–10 μ g. (the actual value varying to some extent with different specimens) the linear relation between fluorescence and aneurin content fails as in curve I, Fig. 1, in which the sample was subsequently shown to contain 4.8 μ g. by the good linear curve with determinations at twice the normal dilution (Fig. 1, curve II). At the normal dilution the addition of an equivalent amount of aneurin shows an intensity of fluorescence already departing markedly from the linear relation.

The discrepancy between the fluorescence due to aneurin added to urine and that obtained when aneurin is oxidized in pure solution is an indication of the presence of interfering substances in the urine. Since, as shown above, the amount of such interference is not likely to have remained constant during the oxidation, the blank determination is subject to an uncertain degree of interference. Accordingly, where the apparent recovery of added aneurin is less than 60% the result should be checked by determination with adsorbates made at a higher dilution, in which the ratio of interfering substance to aneurin is likely to have altered. Unfortunately, accurate comparison is only possible with values

Table IX. *Effect of dilution*

Subject, H.M. ii (Fig. 3, column 2). Aliquot, 75 ml. diluted urine

Specimen		Dilution, l. per hr.	
		0.50	1.00
		Fluorescence	
24	Blank	55	46
	Oxidized	163	108
	1 μ g. vitamin B ₁	42	74
	Assay of aliquot (μ g.)	2.60	0.85
	Content of total (μ g.)	415	270
3 _O	Blank	44	47
	Oxidized	115	90
	1 μ g. vitamin B ₁	60	71
	Assay of aliquot (μ g.)	1.20	0.60
	Content of total (μ g.)	25	25
3 _{B₁}	Blank	45	39
	Oxidized	405	257
	1 μ g. vitamin B ₁	64	70
	Assay of aliquot (μ g.)	5.65	3.10
	Content of total	115	125

near the upper limit of the normal range, since increase of dilution reduces the maximum size of the aliquot which can be handled conveniently. An example is given in Table IX in which the two 3 hr. samples show good agreement at our standard and at twice our standard dilution. Since the low fluorescence of added aneurin in the 24 hr. sample was much improved by further dilution, the assay obtained in the latter case is much to be preferred.

(4) *The use of one concentration of oxidizing agent.* Since the amount of ferricyanide required for optimal increase of fluorescence depends on the total amount of oxidizable substances rather than on the amount of aneurin, attempts were made to avoid the need for determining the optimal conditions in each case by the use of excess, the redox potential being poised by the addition of ferrocyanide. No success was attained with ratios of oxidant to reductant between 0.7 and 40. It appeared that an initially high proportion of oxidant was required to oxidize the aneurin, but the fluorescence was reduced if such conditions were maintained by excess of a poised reagent.

In a series of determinations on 50 subjects requiring 0.1–0.4 ml. 5% $K_3Fe(CN)_6$, it was found that 31 cases required 0.2–0.3 ml., while for all cases, with and without added aneurin, the values with the latter amount were never lower than the optimal value by more than 10%. With significant amounts of aneurin in the sample, sub-optimal oxidation was compensated by similar sub-optimal oxidation of added aneurin in calibration. It was therefore decided to use 1 ml. 1.25% $K_3Fe(CN)_6$ which provides a slight excess in all cases.

Before increasing the sensitivity of the fluorometer to allow the use of a blue-violet colour filter, it was found that samples low in aneurin frequently gave an apparently negative assay under these conditions (e.g. Table VI). Such samples would have been reported as of zero content in any method based on maximum obtainable fluorescence. Since using the colour filter no such negative values have occurred, except after prolonged storage (Table VIII). Jowett [1939] also reported the harmless effect of excess of oxidizing agent when using a filter. It is evident that thiochrome is not significantly susceptible to excess of oxidizing agent under these conditions, as would be expected from the vast excess used in its preparation by Barger *et al.* [1935]. The error introduced by the oxidative destruction of substances responsible for the blank is considerably reduced by the use of a colour filter.

(5) *The use of wet isobutanol.* The solvent may be recovered in the wet state simply by steam-distillation after washing with water to prevent the accumulation of methyl alcohol which would alter the limits of miscibility with the alkaline aqueous phase. The wet solvent is advantageous in the removal of fluorescent substances from the filter-papers to be used in clearing the *isobutanol* extracts. Since its boiling-point is as low as 88° a continuous extractor heated on the water bath may be used. A form of extractor in which a column of papers is surrounded by the hot vapour is preferable to the Soxhlet extractor, recommended by Wang & Harris [1939], since extraction is more rapid and more complete.

Discussion of results

Fig. 2 summarizes the results obtained with a series of neurological cases of which the clinical diagnoses are given in Table X, and with a few normal controls who were research workers, except N.M. iv (technical assistant) and N.F. i (wife of N.M. v). The two series are arranged in ascending order of excretion after the test-dose. For ease of comparison with the excretion after the test-dose the 24 hr. excretion has been plotted as the average for 3 hr. Fig. 3 gives similar

data showing the increase of excretion for a number of patients undergoing treatment with aneurin, together with an example of the spontaneous variations which may occur in a normal case N.M. i.

Fig. 2. Aneurin excretion and response to test-dose. Shaded rectangle = average 3 hr. excretion. Continuous rectangle = excretion after 1 mg. aneurin by mouth. A broken line indicates the level of excretion on a low aneurin intake.

Fig. 3. Response of cases to treatment with aneurin. Details as in Fig. 2. ---- Normal limits for 3 hr. excretion after 1 mg. test-dose. --- Normal limits for average 3 hr. excretion during 24 hr. with dietary aneurin only.

Table X. *Clinical diagnoses of hospital cases*

Subject	Diagnosis
Males	
H.M. i	Polyneuritis
H.M. ii	Alcoholic periphe. neuritis
H.M. iii	Sub-acute combined degeneration with pernicious anaemia
H.M. iv	Polyneuritis
H.M. v	Peripheral neuritis
Females	
H.F. i	Nutritional polyneuritis
H.F. ii	Peripheral neuritis
H.F. iii	Polyneuritis
H.F. iv	Polyneuritis
H.F. v	Thyrotoxicosis and nutritional polyneuritis

The results for the 24 hr. excretion are roughly parallel with those obtained after the test-dose, but considerable variations occur, especially with urines of low aneurin content. It is impossible to say to what extent these variations are due to real differences in the aneurin content of the samples and how far they are affected by variations in the amounts of interfering substances. In an attempt to allow for this uncertainty determinations were made on the control samples excreted after a breakfast low in aneurin, in order to determine the actual response to the test-dose. This determination is, however, highly susceptible to errors due to incomplete emptying from the bladder of the con-

centrated urine of the previous night, which may contain much of the aneurin excretion of the previous 24 hr. [Westenbrink & Goudsmit, 1937, 2]. This is clearly the case with the specimens shown in Fig. 3, case H.M. ii, column 4, where the excretion on low aneurin intake is apparently more than twice as much as the average excretion during the previous 24 hr. Errors due to interfering substances cannot have affected these results significantly, since they were carried out at twice the normal dilution, the blank was low, namely, 5–12 % of the fluorescence of the oxidized samples and the fluorescence from added aneurin was high, 80–90 %. If retention of 70 $\mu\text{g.}$ out of a possible 150–200 $\mu\text{g.}$ in the night urine had occurred this would suffice to bring the “3₀” specimen to the level of the previous “24”. Anomalous values for other samples for the control period may also be due to this cause (Fig. 2, hospital cases, F. i, F. ii, F. iii). In all these cases, however, visual observation of the blank showed traces of yellow pigment and little fluorescence in the layers remote from the fluorometer lamp, though this interference was diminished or not detectable in the oxidized samples. The assays are evidently too high, as discussed already with special reference to case H.F. iii (Table VII). With case H.F. i, moreover, the value for the 24 hr. may be too low owing to the small volume (245 ml.). Similarly with H.M. i, the total absence of aneurin after the test-dose, in spite of its presence in the previous samples, may be due to the low volume of urine voided (60 ml.). Other anomalous figures, e.g. H.F. v and N.M. v, are not outside the limits of experimental error for the fluorometer measurement, which, for a specimen of low aneurin content determined under the standard conditions, might amount to $\pm 1 \mu\text{g.}$ calculated on a 3 hr. basis.

On account of these anomalies, the 3 hr. excretion after the test-dose, without correction for the excretion during the previous control period, is considered to be the best index of nutritional level when the thiochrome test is used. It is preferable that the test-period should be preceded by a few hours of low aneurin intake to avoid errors due to retention of the concentrated urine of the previous night.

It is not proposed to consider here the clinical significance of the results in detail as this will be discussed elsewhere (McAlpine, unpublished observations), but certain observations may be of value.

Owing to different metabolic needs and variations in kidney function, it is not to be expected that there will be a definite value above which deficiency can be said to be absent and below which clinical symptoms are apparent. This has already been pointed out by Harris *et al.* [1938]. Out of nine cases with neurological symptoms, Fig. 2 shows that the 24 hr. excretion of four was equal to or greater than the minimum for normal controls, i.e. 50 $\mu\text{g.}$ in 24 hr. or 6 $\mu\text{g.}$ in 3 hr. Only one of these (H.M. iv) showed a value after the test-dose which was higher than the minimum normal value observed here (26 $\mu\text{g.}$), but two other cases did so (H.F. iv and H.F. v). With regard to the 24 hr. values, apart from the possibility of greater relative influences of sources of error in the test, it must be borne in mind that the values are a function, not only of the reserves of the patient but also of the diet during the period of the test. Many of the cases may already be showing a response due to a good hospital diet even before treatment with supplements of aneurin. Thus H.F. ii (Fig. 3) assaying at 15 $\mu\text{g.}$ for the first 24 hr. (col. 1) gave an increased excretion of 50 $\mu\text{g.}$ in 24 hr. on the 5th day, although the excretion after a test-dose was still very small (col. 2). H.F. iv (Fig. 2), giving a dietary history indicating no gross deficiency and a fair response to the test-dose, showed a low 24 hr. excretion (15 $\mu\text{g.}$) due to a diet low in aneurin for the period of the test.

Although it appears that the thiochrome test may give rather uncertain information about the extent of hypovitaminosis, it can be of real value in the control of dosage and in the choice of the most economical route for effective administration. In the absence of such a test it has become customary to give 10 mg. or more per day, often parenterally. Westenbrink & Goudsmit [1938, 1] showed that more aneurin is lost through the kidney when it is given by injection than when given by mouth. The oral route is therefore preferable unless the possibility of faulty absorption is indicated by the absence of aneurin from the urine after a test-dose by mouth. Under such circumstances it is advisable to give aneurin parenterally until the deficiency is remedied sufficiently to give a moderate response to a test-dose orally administered. Further work on the absorption of aneurin is needed since it seems improbable that there should ever be difficulty in the absorption of such a small water-soluble molecule. In conditioned deficiencies due to incomplete utilization of the vitamin of the food, it seems possible that the difficulty may be in freeing it from protein with which it may be bound, as in milk [Houston *et al.* 1939], and possibly in animal tissues generally [Westenbrink & Goudsmit, 1938, 3].

Cases H.M. iii and H.F. iii (Fig. 3), admittedly showing only moderate deficiency when first examined, are examples of the response to oral treatment. H.F. iii received 1 mg. aneurin on the 2nd day as a test-dose and 3 mg. per day on the 5th, 6th and 7th days. 24 hr. specimens were collected on the 1st and 8th days and in each case were followed by examination of the response to a test-dose. The upper limit for the normal cases examined so far was reached by the criterion of either the "24" or the "3_p" specimen. H.M. iii received 1 mg. as test-dose on the 2nd day, 1 mg. per day on the 9th, 10th and 11th days and 3 mg. per day on the 12th, 13th and 14th days. The 24 hr. excretion was examined on the 1st, 8th, 11th and 14th days. By the 11th day (col. 3) it had reached the upper limit for normal cases and had reached the much higher figure of 465 μ g. by the 14th day. This is probably near the saturation level. H.M. ii and H.M. v reached similar levels after prolonged treatment, about 45 and 60 days respectively, receiving as much as 10 mg. per day by injection for much of these periods. The high levels of excretion, 500–600 and 350 μ g. per day respectively, are really due to reserves in the tissues and not directly due to excessively high aneurin intake, since none was given, apart from that in the good hospital diet, for 48 hr. before collecting the urine. Westenbrink & Goudsmit [1938, 1] give 400–500 μ g. as the saturation level for two normal subjects after treatment with 5 mg. per day for several days. The corresponding excretion after the test-dose was of the order of 200 μ g. in 3 hr., the response being less than 20 % of the dose. As this work was mainly concerned with establishing a rapid test for clinical purposes, the excretion subsequently has not been examined, but Westenbrink & Goudsmit showed that quantitative excretion is never attained, as after saturation with ascorbic acid [cf. Abbasy *et al.* 1936]. The fate of the aneurin not excreted intact is at present unknown; the capacity of the tissues for storing it is very limited and saturated subjects are rapidly depleted on a low aneurin diet.

Case H.F. v is an example of refractoriness to treatment, 10 mg. being given daily by intravenous injection for 6 days (4th–9th). The 24 hr. specimens were taken on the 1st, 3rd and 12th days. The failure to respond satisfactorily is probably due to the increased requirement for metabolism in thyrotoxicosis.

Since the normal values shown never reached the saturation level, it seems probable that the intake was sub-optimal although the individuals showed no symptoms. Other workers have reported normal ranges which reach higher

Table XI. *Normal daily excretion of aneurin*

Method	Range μg.	Mean μg.	Reference
Bradycardia	*35-105	*60	Harris <i>et al.</i> [1938]
Thiochrome	—	100	Karrer [1937]
Thiochrome	α 120-330	α 230	Westenbrink & Goudsmit [1938, 1]
	β 60-240	β 120	
Thiochrome	110-520	—	Ritsert [1938]
Bradycardia	*90-480	—	Wang & Harris [1939]
Thiochrome			
Thiochrome	50-170	100	This paper

* Reported as I.U. and converted by the relation, 1 I.U. = 3 μg.

α Male subjects.

β Female subjects.

levels (Table XI). While differences in the lower limits of the values obtained by the thiochrome test may be in part due to different amounts of interfering substances depending on the precise technique adopted, the upper values are less susceptible to such errors. Our values were determined in early spring and it is possible that reserves may have been depleted during the winter, owing to the greater demand for aneurin corresponding to greater calorie requirements, and to a diminished intake, due to possible deterioration in stored foods and to the lower content even of fresh foods, such as milk from stall-fed cattle as compared with pasture-milk (National Institute for Research in Dairying, *Annual Report*, 1938, p. 52).

In conclusion, it may be stated that the excretion of aneurin after a 1 mg. test-dose, determined by the thiochrome test, is a more reliable guide to the extent of hypovitaminosis-B₁ than the 24 hr. excretion, since it is proportionately less susceptible to errors arising from interfering substances in the urine. The patient who is considered to be suffering from a deficiency of aneurin may be given 1 mg. (i.e. 50-100 % of the normal daily requirement [Williams & Spies, 1938]) at the start of the test if a control period is omitted. The result can be available within 4 hr. of the start of the test; on the other hand, if the 24 hr. excretion is to be used as an index of nutritional level, the administration of aneurin must be postponed till the end of the period.

SUMMARY

1. A simplified procedure for the determination of aneurin in urine by the thiochrome test is described.

2. Photoelectric measurement of fluorescence is employed and rendered more specific for thiochrome by the use of a blue filter.

3. The importance of calibrating the fluorescence from aneurin in the presence of each specimen of urine examined is emphasized.

4. Six normal subjects excreted 50-170 μg. in 24 hr., the mean value being 100 μg. The excretion for 3 hr. after 1 mg. aneurin by mouth was 26-110 μg. with a mean of 65 μg.

5. Of 9 neurological cases, 5 gave a lower 24 hr. excretion and 6 gave a lower excretion after the test-dose than the lowest normal case.

6. The excretion of aneurin by individuals who were treated with aneurin, except for a case of thyrotoxicosis, increased considerably. Saturation appeared to be reached at a level of about 500 μg. per day. The corresponding excretion for 3 hr. after the 1 mg. test-dose was about 200 μg.

7. The excretion after a test-dose is suggested as the most reliable index of nutritional level, as its measurement is less susceptible to various sources of error which have been discussed.

I wish to thank Prof. E. C. Dodds, who suggested carrying out this work, and Dr D. McAlpine for the provision of neurological cases. The work was carried out during the tenure of a McKenzie-McKinnon Research Fellowship of the Royal Colleges of Physicians and Surgeons.

REFERENCES

- Abbasy, Gray-Hill & Harris (1936). *Lancet*, **2**, 1413.
Barger, Bergel & Todd (1935). *Ber. dtsch. chem. Ges.* **68**, 2259.
Cohen (1935). *Rec. Trav. chim. Pays-Bas*, **54**, 133.
Harris, Leong & Ungley (1938). *Lancet*, **1**, 539.
Houston, Kon & Thompson (1939). *Chem. Ind. Lond.* **58**, 651.
Jansen (1936). *Rec. Trav. chim. Pays-Bas*, **55**, 1046.
Jowett (1939). *Chem. Ind. Lond.* **58**, 556.
Karrer (1937). *Helv. chim. Acta*, **20**, 1147.
—— & Kubli (1937). *Helv. chim. Acta*, **20**, 369.
Kuhn & Vetter (1935). *Ber. dtsch. chem. Ges.* **68**, 2375.
Marrack & Höllering (1939). *Lancet*, **1**, 325.
Ritsert (1938). *Dtsch. med. Wschr.* **64**, 481.
Wang & Harris (1939). *Biochem. J.* **33**, 1356.
Westenbrink & Goudsmit (1937, 1). *Rec. Trav. chim. Pays-Bas*, **56**, 803.
—— — (1937, 2). *Arch. néerl. Physiol.* **22**, 319.
—— — (1938, 1). *Arch. néerl. Physiol.* **23**, 79.
—— — (1938, 2). *Nederl. Tijdschr. Geneesk.* **82**, 1076.
—— — (1938, 3). *Nature, Lond.*, **142**, 150.
Williams & Spies (1938). *Vitamin B₁ and its use in medicine*. New York.