CLXVI. METHODS FOR ASSESSING THE LEVEL OF NUTRITION OF THE HUMAN SUBJECT. ESTIMATION OF VITAMIN B₁ IN URINE BY THE THIOCHROME TEST¹

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IN earlier work [Harris & Leong, 1936; Harris *et al.* 1938] it was shown that the extent of the body's "reserves" of vitamin B₁ might be measured by means of a test on the urine, similar in principle to that previously introduced for vitamin C [Harris *et al.* 1933; Harris & Ray, 1935]. The urinary output was found to depend on the past intake, being negligible in beri-beri or in "conditioned" deficiencies of vitamin B₁ and being lowered in certain other abnormal states.

To simplify the procedure, several investigators [e.g. Westenbrink & Goudsmit, 1937, 1, 2; 1938; Karrer, 1937; Ritsert, 1938; Marrack & Höllering, 1939] have proposed to substitute the thiochrome reaction of Jansen [1936] for the biological (bradycardia) method of assay originally employed. Various alternative techniques for the thiochrome test have been recommended by these different workers and these may give rather widely divergent results. Since also the accuracy of the thiochrome method as applied to urine has been questioned [e.g. Pyke, 1939; cf. also Henry *et al.* 1938], it seemed advisable to undertake a thorough study of the details of the procedure, and at the same time (and perhaps even more important) to standardize the results obtained against direct biological assays.

The principal difficulty which we have encountered has been the presence in urine of various non-specific substances which may seriously interfere in the measurement of fluorescence, upon which the thiochrome reaction depends. To overcome this and other sources of error we are recommending in the present paper a number of modifications in the technique, which have the advantage also of improving the simplicity and sensitivity of the method. As will be shown, the results given by this procedure have been proved to be accurate when checked against biological tests, and vitamin B₁ added to urine has been quantitatively recovered. By the method described tests may be completed on a batch of, say, three to six urines within about an hour; the error is ordinarily less than about 10%, and a specimen of not more than about 5 ml. suffices for the examination.

Principle of the thiochrome method

The principle of the thiochrome reaction is to transform the vitamin B₁ into its fluorescent derivative, thiochrome, by means of a mild alkaline oxidation with ferricyanide, and to measure the degree of the fluorescence against some suitable standard.

According to the procedure of Westenbrink & Goudsmit [1937, 1, 2] the vitamin B₁ is first adsorbed on franconite and the fluorescence is subsequently measured by a photoelectric fluorimeter. The procedure of Hennessy & Cerecedo [1939] is similar except that the vitamin B₁ is

¹ Communicated to the Biochemical Society, 6 May 1939 [Wang & Harris, 1939].

adsorbed on synthetic zeolite, while Marrack & Höllering [1939] adsorb on franconite but measure the fluorescence by a comparison with the naked eye. Ritsert [1938] omits altogether the preliminary adsorption, but provides for the large blank-correction either by (a) his "compensation method", or (b) by the so-called "automatic equalization". Like Ritsert we consider it of great importance to pay attention to the presence of interfering substances, but our procedure differs essentially from his in that our aim has been to concentrate as far as possible on their removal from the sphere of the reaction rather than to attempt to allow for them by a very large and sometimes uncertain blank correction (see below, p. 1359).

Examination for possible sources of error

In a series of preliminary tests the various possible sources of error were examined. The most important of these were found to include the following:

- (1) Incomplete removal of the vitamin on adsorption.
- (2) Interference from non-specific fluorescing substances present in urine.
- (3) Interference from reducing substances other than the vitamin.
- (4) Interference from pigments.
- (5) Fluorescence caused by use of impure reagents.

These difficulties may be briefly discussed in turn.

(1) *Incomplete adsorption.* While the vitamin may generally be removed nearly quantitatively from such specimens of urine as are rich in it (provided that enough of the adsorbent is used, and the right conditions of pH etc. are maintained), it is more difficult to effect anything approaching complete separation when working with dilute samples (see Table VI). Another objection to the use of adsorption, so far as the thiochrome test is concerned, is that it brings about a concentration from the urine not only of the vitamin itself, but also of other reducing substances which increase the interference at a later stage by requiring the addition of an undue amount of the ferricyanide reagent (see no. 3 below).

(2) *Non-specific fluorescing substances.* We have found that urine itself may contain variable amounts of preformed thiochrome, and this, together with other non-specific fluorescing substances, will be estimated in the final comparison unless steps are taken to obviate this source of error. The non-specific substances produce a yellowish-white fluorescence, in contrast with the bluish or violet fluorescence given by the thiochrome. The presence of the non-specific fluorescing substances seems to affect also the actual colour of the fluorescence given by the thiochrome itself.

(3) *Interference from reducing substances.* Reducing substances other than vitamin B₁ which are present in urine, and which as mentioned under no. 1 above may become concentrated by preliminary adsorption, interfere by making it necessary to add a large excess of ferricyanide to the urine beyond the small amount theoretically needed to oxidize the vitamin B₁ alone. The objection to this large addition of ferricyanide is twofold: (1) it destroys some of the thiochrome produced by the reaction; (2) it has a variable effect on the non-specific fluorescing substances, making it impossible to allow for them accurately in a blank control.

(4) *Interference from pigments.* The presence of natural pigments in the urine may mask the fluorescence of the thiochrome in the final solution to such an extent as to make matching difficult.

(5) *Fluorescence caused by use of impure reagents.* Virtually all the filter papers examined were found to yield fluorescing substances which would interfere with the test. Sodium sulphate, used for drying the solution of thiochrome in isobutanol, may also sometimes be contaminated with fluorescing materials.

Table I outlines the procedure used by past workers, together with the corresponding possible sources of error just referred to which may be expected to interfere with the accuracy of the test under the various conditions.

Table I. *Methods for estimating vitamin B₁ in urine by thiochrome reaction*

Worker	Procedure	Possible sources of error
(1) Westenbrink & Goudsmit [1937, 1, 2]	Adsorb on franconite. Compare by photo- electric fluorimeter	(a) Vitamin B ₁ incompletely ad- sorbed (b) Reducing and colouring sub- stances adsorbed along with vitamin B ₁ , necessitating unduly large addition of K ₃ Fe(CN) ₆ (c) Non-specific fluorescing sub- stances interfere with fluori- meter reading
(2) Karrer [1937]	Adsorb on franconite. Compare by naked eye	Similar to no. 1
(3) Hennessy & Cerecedo [1939]	Adsorb on zeolite. Compare by fluori- meter	Same as no. 1
(4) Marrack & Höllering [1939]	Adsorb on franconite. Compare by naked eye	Probably similar to no. 1
(5) Ritsert [1938]	No preliminary ad- sorption. Compare by naked eye. Blank allowed for (a) by "compensation me- thod", (b) by "auto- matic equalization"	Procedure tedious for method (b), and "blank correction" relatively inaccurate (see below, p. 1359)

Special features of the technique recommended

After numerous trials and control tests a procedure was eventually evolved which seems to overcome these several sources of error, which gives accurate results when tested against biological assays, and which is rapid and simple. The principal points of this procedure include the following:

- (1) No preliminary adsorption.
- (2) Removal of preformed thiochrome and other interfering substances by preliminary extraction of urine with *isobutanol*.
- (3) Regulation of amount of K₃Fe(CN)₆ added.
- (4) Treatment of filter paper and reagents.
- (5) Further removal of interfering substances by washing the final *isobutanol* solution of thiochrome with water.
- (6) Direct visual comparison of fluorescence.
- (7) Allowance in blank for residue of interfering substances not previously removed.

The reasons for these steps may be briefly noted as follows:

(1) *Absence of preliminary adsorption.* As will be clear from the foregoing discussion the advantages of omitting the adsorption, apart from the convenience and great saving of time, are that the danger of loss of vitamin is obviated and there is no undesirable over-concentration of interfering substances.

(2) *Preliminary washing with isobutanol.* With urines containing appreciable amounts of preformed thiochrome or with specimens which are highly pigmented or relatively poor in the vitamin, this procedure significantly improves the accuracy of the method.

(3) *Regulation of $K_3Fe(CN)_6$* . As already explained the addition of excess of ferricyanide interferes with the reliability of the method, and to overcome the error we ascertain in a separate control test what amount is necessary.

(4) *Treatment of filter paper and reagents*. The presence of fluorescing substances in the filter paper may alone give rise to a considerable error if not allowed for.

(5) *Washing of isobutanol layer*. This we regard as an important step, as it removes a large portion of the non-specific fluorescing substances and helps to clear the solution from further traces of pigments. The water also extracts anions, which in certain fluorimetric tests are known to have a variable influence on the degree of fluorescence.

(6) *Visual comparison of fluorescence*. For the sake of simplicity and saving of expense and time, a matching of the end-point by the eye seems to be desirable. The accuracy is not less than with a photoelectric apparatus, and the prior removal of the greater part of the blank enables the matching to be done with accuracy.

(7) *Blank control*. To allow for any residuum of interference a blank correction is introduced. However, the blank is of a small order of magnitude in comparison with the "unknown", instead of being of a large order as when interfering substances are not first-removed.

Ritsert (see Table I) proposed to allow for a blank correction either (a) by his "compensation" method or (b) by the so-called "automatic equalization". The former alternative involves the addition of an isobutanol extract of the untreated urine to the control for the final matching; the disadvantage of this procedure seems to be that the blank is excessively large in proportion to the small amount of the unknown and there is also interference from any preformed thiochrome in the urine. The second alternative ("automatic equalization") involves the use of a blank consisting of urine from which the vitamin has been removed by adsorption on franconite. Unfortunately such a procedure may entail a partial and variable removal of the blank fluorescence (so that the extent of the blank cannot be adequately controlled) and also an incomplete removal of the vitamin B₁ itself.

Outline of procedure

The process recommended involves five successive steps. (1) A small volume of urine is washed with an equal bulk of isobutanol. (2) The washed urine is treated with methanol, ferricyanide and NaOH, to convert the vitamin B₁ into thiochrome. (3) The "thiochrome" so formed is extracted with isobutanol. (4) The isobutanol layer is washed with water, cleared with Na₂SO₄ (or ethanol), and a specimen taken for measurement of fluorescence. (5) In the measurement of fluorescence a standard solution of thiochrome is added drop by drop to a blank used as a control, while at the same time an equal volume of isobutanol solvent is added to the unknown, until the fluorescence of the two is matched.

Working details

It is important that strict attention should be paid to procedure if reliable results are to be obtained, and accordingly the working details are given below in full.

Reagents.

$K_3Fe(CN)_6$, 2 % aqueous solution.

NaOH, 30 % aqueous solution.

Methanol.

isoButanol (redistilled).

Na₂SO₄, anhydrous.

All reagents, particularly the Na_2SO_4 and the redistilled *isobutanol*, should be tested for freedom from fluorescence in blank determinations.

Apparatus.

Ultraviolet mercury-vapour lamp fitted with filter of Wood's glass.

Three glass-stoppered graduated cylinders, 25 ml. capacity.

Centrifuge.

Filter papers, extracted with *isobutanol* for not less than 12 hr. in a Soxhlet apparatus until found to be free from fluorescent substances in blank determinations.

Procedure.

(1) *Preliminary washing.*¹ Measure 5–8 ml. of urine into a 15 ml. graduated centrifuge tube, add an equal volume of *isobutanol* and 2 drops of 10 % HCl. Shake vigorously for 2 min. and centrifuge. Note the final volume of the aqueous layer. Discard the *isobutanol* layer.

(2) *Oxidation.* Measure three 1–2 ml. portions of the extracted urine, depending on the concentration of vitamin B_1 in it, into 3 graduated glass-stoppered cylinders of 25 ml. capacity. Add to each 2 ml. of MeOH. To the first two of these cylinders add 1 ml. of 30 % NaOH and mix. To the first cylinder add 2 % $\text{K}_3\text{Fe}(\text{CN})_6$ from a graduated pipette cautiously drop by drop, shaking between each addition until the colour is just slightly more yellow than in the second cylinder which has no $\text{K}_3\text{Fe}(\text{CN})_6$ and which is to be used subsequently as a control. Avoid an excess of ferricyanide; this is very important, otherwise it will be impossible to get a perfect match of fluorescence. The slight colour should stay for at least half a minute. Note the volume of $\text{K}_3\text{Fe}(\text{CN})_6$ so required. Add exactly the same amount of $\text{K}_3\text{Fe}(\text{CN})_6$ to the third cylinder (i.e. the one without NaOH), mix, and then add 1 ml. of 30 % NaOH. Mix and allow to stand for 1 min.

Cylinder no. 1, used to determine the amount of $\text{K}_3\text{Fe}(\text{CN})_6$ needed, is not further required.

(3) *Extraction of thiochrome.* Add 10 ml. of *isobutanol* both to cylinder no. 2 and to no. 3, shake vigorously for 2 min. and allow the layers to separate.

(4) *Washing and drying.* Cylinders nos. 3 ("unknown", with ferricyanide) and 2 (control, without ferricyanide) are now treated identically as follows. Remove the bottom (aqueous) layer with a long pipette fitted with a rubber teat.² Add 4 ml. of water to each cylinder and shake vigorously for 1 min. Control tests indicate that under these conditions no appreciable quantity of the thiochrome enters the watery phase. Again allow the layers to separate. Make up the *isobutanol* layer to 15 ml. with fresh *isobutanol* and remove it to a dry conical flask. Add gradually approximately 4 g. of anhydrous Na_2SO_4 to each flask, shaking during the addition; filter through the *isobutanol*-extracted filter

¹ For routine determinations this step is not usually necessary, but for accurate work and with certain urines containing large amounts of interfering fluorescing substances it is desirable (and may be essential) to give the urine this preliminary washing. With an occasional specimen a second and even third washing may be necessary. Control tests show that no significant amount of vitamin B_1 is lost in the washing.

² When working with large batches of urines time may be saved by performing the operations in a specially graduated separating funnel, so that the bottom layer can be quickly run off on each occasion and there is no need to transfer the *isobutanol* layer. A battery of such funnels can be used together and shaken simultaneously in a mechanical shaker.

papers into clean dry test tubes¹ and take 10 ml. of the clear filtrates for comparison of fluorescence. (Alternatively, in place of drying with Na_2SO_4 the solution may be clarified by adding 1 ml. of ethanol and the filtration may then be omitted.)

(5) *Comparison of fluorescence.* Place the two test tubes side by side in front of the Wood's glass window of the ultraviolet lamp, inclining them towards the body at an angle of approximately 60° to the horizontal so that the eye can look into the tubes. Add the standard (see below) little by little from a graduated pipette to the control until the fluorescence exactly matches the unknown. Add to the "unknown" an amount of *isobutanol* equal in volume to the amount of the standard added to the control, so as to make the final volumes in the two tubes the same. The contents of the tubes may be mixed after each addition by pouring backwards and forwards into another dry test tube. From the volume of the standard used, the content of aneurin can be calculated; e.g. if

x = the number of ml. used of standard, having a concentration of y μg . per ml.,

v = the volume of urine used,

V = the total output of urine per 24 hr.

the aneurin content of the urine = $\frac{xy}{10} \times \frac{V}{v}$ μg . in the 24-hr. specimen.

Preparation of standard.

"Benerva" ampoules (manufactured by Roche Products, Ltd.) can be conveniently used as the standard for aneurin. Prepare a solution containing 1–2 μg . per ml. by diluting a measured volume of the original solution with H_2O and adjust it to a *pH* value of 3. This solution may be kept for 2–3 days in an ice chest. Measure 1 ml. of the standard into a graduated glass-stoppered cylinder and treat it in exactly the same way as for the urine, using one drop only of the 2% $\text{K}_3\text{Fe}(\text{CN})_6$.

Results: normal subjects

As will be seen from Table II, eleven normal subjects have been examined, consisting of laboratory assistants and research workers at Cambridge. The lowest average excretion for any one subject was about 30 I.U. per day and the highest 130 I.U. The usual range was between 50 and 80 I.U.

Results with special cases

Through the kind co-operation of a number of medical practitioners, to whom we wish to express our indebtedness, we have been able to examine the urinary output of a number of patients (Table III). An interesting feature is the lowered excretion noted in several women during and after pregnancy. This result accords with the view that additional vitamin B_1 is needed by women during gestation and lactation, and that their reserves are liable to be depleted to a dangerously low level, either because of the increased demands of the foetus or the sucking child or sometimes as a result of hyperemesis. It is now well recognized that a conditioned deficiency of vitamin B_1 may be the cause of polyneuritis in pregnancy. A significant finding was that the low excretion of vitamin B_1 in the urine of the pregnant women was found, in tests by Mr G. G. Banerji, to be sometimes associated with increased excretion of bisulphite-binding substances [cf. Banerji & Harris, 1938].

¹ Care must be taken to procure test tubes the glass of which is free from fluorescence in the light of the mercury-vapour lamp.

Table II. *Excretion of vitamin B₁ by normal subjects*

Initials	Date	Remarks	Daily excretion of vitamin B ₁ in I.U.		
			Single days	Range	Average
G. G. B.	3. i. 39	Normal diet	65	65- 83	74
	17. i. 39	"	83		
Y. L. W.	14. xii. 38	After test dose	(200)	90-110	105
	12. iv. 39	Normal diet	110		
	2. vi. 39	"	110		
	3. vi. 39	"	110		
	6. vi. 39	"	90		
A. D.	9. ii. 39	Normal diet	47	—	47
	17. ii. 39	"	47		
G. G.	20. xii. 38	Normal diet	90	70- 90	77
	16. ii. 39	"	70		
	17. ii. 39	"	70		
K. R. R.	9. ii. 39	On brown bread	140	71-140	106
	30. iii. 39	On white bread	71		
J. Y.	17. i. 39	Normal diet	68	33- 68	51
	2. vi. 39	"	53		
	3. vi. 39	"	33		
W. D. R.	18. iv. 39	Marmite daily	110	110-160	132
	25. iv. 39	"	160		
	26. iv. 39	"	140		
	27. iv. 39	"	120		
L. J. H.	25. iv. 39	Normal diet	54	53- 65	57
	26. iv. 39	"	53		
	27. iv. 39	"	55		
	28. iv. 39	"	65		
S. A. C.	20. xii. 38	Normal diet	93	35- 93	55
	3. vi. 39	"	36		
	4. vi. 39	"	35		
A. W.	28. iii. 39	Normal diet	33	—	33
S. I.	9. ii. 39	Normal diet	54	—	54

Table III. *Excretion of vitamin B₁ by patients*

Patient's initials (with name of doctor)	Date	Diagnosis or notes	Vitamin B ₁ excreted I.U. in 24 hr.
Mrs P. (Dr J. Yudkin)	9. i. 39	Subacute combined degeneration, pernicious anaemia	15
	10. i. 39		26
— C. (Dr J. Yudkin)	13. iii. 39	Disseminated sclerosis	16
Mrs W.	27. iii. 39	Anorexia, neurasthenia	29
	29. iii. 39		16
Mrs B. (Dr Silberstein)	20. iv. 39	8 months pregnant	39
— S. (Dr Taylor)	21. iv. 39	Peripheral neuritis	26
	24. iv. 39		0
	25. iv. 39		0
Mrs N. (Dr Lawrie)	21. iii. 39	Late pregnancy; toxæmia of preg- nancy. Same after delivery	32
	19. iv. 39		12
	20. iv. 39		16
Mrs W. (Dr Nicholson)	26. v. 39	Anorexia nervosa	19
	27. v. 39		20
W. T. (Dr Nicholson)	8. v. 39	Lassitude	97

The subnormal values found in certain other patients may presumably be attributed to "conditioned" deficiency (or partial deficiency) arising from faulty absorption or utilization, or restricted diet [cf. Harris *et al.* 1938]. The low "reserves" in the patient (Mrs W.) suffering from anorexia nervosa are of interest. Her medical attendant reported that she was suffering from persistent vomiting and taking little nourishment beyond some milk and two slices of bread and butter per day.

Agreement with biological standardization

To test the accuracy of the results obtained in the thiochrome tests, a comprehensive series of biological controls was carried out as follows. A group of 5 specimens of urine was chosen, varying in activity from the very subnormal figure of 16 I.U./24 hr. to the very high one of 304 I.U. The latter represents an excretion after test-dosing (p. 1367).

As will be seen from the results summarized in Table VII and charted in Fig. 1, there is a most satisfactory parallelism between the chemical and biological results, although the specimens of urine cover no less than a twenty-fold range in their activities.

Details of biological test. For protocols of the full experimental details see Tables IV and V. In each instance the vitamin B₁ was adsorbed from a large bulk of the urine by treatment with "Clarit" acid-clay, and the biological tests were carried out by the bradycardia method as

Table IV. *Details of biological standardization. Series I*

Sample	Vitamin B ₁ content of urine, I.U. test	No. of I.U. administered per day by thiochrome test	Days, cured	
			Individual animals	Average
Urine 1. Patient (after test dose)	304	1	0, 2, 1-25, 1-25, 2, 0-5, 0, 3-25	1-3
		2	3, 1-5, 3, 3, 2, 2	2-4
		3	2, 2, 3-5, 2-5, 4, 3-5, 4-5	3-1
Urine 2. Normal con- trol G. G. B.	83	1	2-5, 1-5, 1, 1	1-5
		2	2-5, 4, 3-5, 2-5	3-1
		3	3-5, 2, 4, 4	3-5
International standard	—	1	1, 2, 2, 2-5	1-4
		2	1-5, 4, 1-5, 2, 1	2-0
		3	3, 2-5, 3-5, 5, 4	3-6

Table V. *Details of biological standardization. Series II*

Sample	Vitamin B ₁ content of urine, I.U. test	No. of I.U. administered per day by thiochrome test	Days, cured	
			Individual animals	Average
Urine 3. Normal control Y. L. W.	108	1	1·5, 1, 2, 1·5, 2	1·6
		2	4, 2, 2·5, 2, 3	2·7
		3	4, 3, 3·5, 3, 3·5	3·4
Urine 4. Normal control K. R. R.	71	1	0, 2, 2, 2	1·5
		2	3·5, 2·5, 2, 2, 2	2·4
		3	3, 2, 4·5, 3·5	3·3
Urine 5. Patient Mrs W.	16	1	2, 2	2
		2	3·5, 2	2·8
Urine 6. Patient Mrs N.	16	1	0, 1·5, 0·5, 1, 1·5	0·9
International standard	—	1	2, 2, 2, 2, 0	1·6
		2	2, 2, 5, 2, 2·5	2·7
		3	3, 3·5, 3, 4, 3	3·3
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Table VI. *Calculation of biological activities*

Specimen	Vol. urine per day ml.	Vitamin B ₁ excreted per day, by thiochrome test I.U.	Amount of acid-clay used per 100 ml. urine g.	% vitamin B ₁ adsorbed (thiochrome analysis)	Dose of adsorbed B ₁ given (by thiochrome) I.U.	No. of doses given for level	Days cured av.	Activity as read from reference curve, I.U.		Vitamin B ₁ found in adsorbate per ml. urine, I.U.		Difference %
								Per dose given	Per unit given	By brady-cardia	By thiochrome	
Series 1. Patient Mrs P. (after test dose)	1380	304	2.5	96	1	8	1.3	1.1	1.1	0.23		
					2	6	2.4	2.1	1.05	0.22		
					3	7	3.1	2.7	0.9	0.19		
								Av. 1.02	Av. 0.21	0.21		0
Normal control G. G. B.	1380	83	1	72	1	4	1.5	1.3	1.3	0.056		
					2	4	3.1	2.7	1.35	0.058		
					3	4	3.5	3.0	1.0	0.043		
								Av. 1.22	Av. 0.052	0.043		- 17
Series 2. Normal control Y. L. W.	850	108	2	76	1	5	1.6	0.9	0.9	0.091		
					2	5	2.7	2.2	1.1	0.106		
					3	5	3.4	3.0	1.0	0.099		
								Av. 1.00	Av. 0.099	0.099		0
Normal control K. R. R.	2520	71	0.7	71	1	4	1.5	0.8	0.8	0.016		
					2	5	2.4	1.8	0.9	0.018		
					3	4	3.3	2.9	0.97	0.019		
								Av. 0.89	Av. 0.0178	0.020		+ 11
Patient Mrs W.	1200	16	1	50	1	2	2	1.4	1.4	0.009		
					2	2	2.8	2.3	1.15	0.0075		
								Av. 1.28	Av. 0.0083	0.0065		- 22

Table VII. *Results of biological standardization*

	Vitamin B ₁ excreted, i.u. per day	
	By thiochrome	By bradycardia
Series 1:		
Patient (after test dose)	304	304
Normal control G. G. B.	83	99
Series 2:		
Normal control Y. L. W.	108	108
Normal control K. R. R.	71	63
Patient Mrs W.	16	20

Fig. 1. Agreement between chemical and biological determinations on specimens of urine of varying activity (excretion ranging from 304 to 20 i.u. daily). Columns = biological tests. Crosses = thiochrome estimations.

Fig. 2.

Fig. 3.

Fig. 2. Dose-response curve, series I. $\times$ = international standard. $\circ$ = urine no. 1. $\square$ = urine no. 2. The reference curve is based on the responses with the international standard. The scale of doses for the two specimens of urines is drawn in proportion to the final calculations of their biological activities.

Fig. 3. Dose-response curve, series II. $\times$ = international standard. $\circ$ = urine no. 3. $\square$ = urine no. 4. $\bullet$ = urine no. 5. Reference curve drawn from responses with international standard; scale for urines in proportion to the biological activities as determined.

previously described [Harris & Leong, 1936; Harris *et al.* 1938]. Care was taken to effect as complete an adsorption as possible of the vitamin (e.g. by the use of a sufficiently large amount of the acid-clay and attention to various other details of procedure), the effectiveness of the removal being checked by cross tests by the thiochrome reaction. With each specimen of urine, 3 levels of dosage were given corresponding with a calculated intake of 1, 2 and 3 I.U. according to the results of the chemical estimations.¹ Simultaneously, doses of 1, 2 and 3 I.U. of a standard preparation of vitamin B₁ were given, and the responses compared. For the evaluation of results, dose-response curves were constructed (Figs. 2 and 3), and the activity of the urine was calculated in terms of international units (Tables VI and VII).

Since the various specimens of urine were tested biologically in two separate main experiments, carried out at an interval of several weeks apart, separate tests on the standard material were done on each occasion, so as to allow for any variation in the experimental conditions. In series I (Table IV) the bradycardia tests were done by one of us (Y. L. W.) without any previous experience of the method. In the second series (Table V), to reduce any possibility of subjective factors unconsciously influencing the findings, the results of the chemical tests were known only to Y. L. W. and the bradycardia tests were carried out by a laboratory assistant under the direction of L. J. H., neither of the latter having any knowledge of the nature of the different materials under examination. Incidentally, the very close agreement between the series of values "found" and those "calculated", in the last two columns of Table VI, furnishes, we think, further independent evidence of the reliability of the bradycardia method [see also Baker & Wright, 1938; Lunde *et al.* 1938; Pedersen, 1938].

Sensitivity of method

Duplicate tests have been carried out comparing results on the same specimen of urine as well as on other specimens having similar activities. The results indicate that with urines having a fairly high content of vitamin B₁ there is no difficulty in detecting differences in activity of somewhat less than 10%. With urines low in vitamin B₁ differences of between 10 and 20% can be detected. These findings have been confirmed by matchings carried out in this laboratory by workers from three other institutes.

Recovery tests with added vitamin B₁

As a further test of the accuracy of the method, various known amounts of vitamin B₁ were added to different specimens of urine, and the amounts were estimated by the thiochrome test carried out according to the directions given above. Typical examples are given in Table VIII. It will be noted that the percentage recovery varied from 94 to 108, the error being always less than 10% under the conditions studied.

Table VIII. *Recovery of added vitamin B₁*

Urine	Original concentration of vitamin B ₁ μg./ml.	Vitamin B ₁ added μg.	Additional vitamin B ₁ found μg.	Recovery %
(a)	0.04	0.06	0.065	104
	0.04	0.11	0.103	94
	0.04	0.13	0.14	108
(b)	0.085	0.09	0.085	94

¹ Except that with one specimen of urine insufficient material was available for testing more than two levels and with another one level. In these instances the percentage accuracy is somewhat less.

Experiments with test doses

It was previously recommended [Harris *et al.* 1938] that for assessing the level of nutrition of a human subject measurements should be made first of the "resting level" of excretion and then of the response to standard test doses (350 I.U. for an adult of 10-stone body-weight), the latter giving information as to the degree of saturation of the tissues.

The same procedure has been followed in this work with the thiochrome method. Fig. 4 gives examples from two normal subjects and one subnormal

Fig. 4. Resting levels of excretions and response to test doses, in two normal subjects and one deficient subject. Open columns = without test dose. Dark columns = after test dose. Test dose, 350 I.U. per 10 stone of body-weight given at arrow.

case. In the normal individuals an immediate response was seen after the first test dose, the increased excretion remaining approximately constant on each day that a test dose was continued and amounting to about 25% of the extra vitamin given.

With the partially deficient subject the plateau of excretion was not reached until after the second day of test dosing.

On ceasing the dosing, the output of the vitamin almost immediately fell to the original level (Fig. 4).

Standards

In the earlier work by the biological method [Harris *et al.* 1938] the two lowest normal excretions found were 12 I.U. in one series of cases and 15 I.U. in another. In the present more extended series of tests the lowest normal value found was about 30 I.U. We think that the tentative minimum standards previously suggested probably erred in the direction of being unnecessarily conservative. Judging from the present observations, which are based on large numbers of concordant chemical and biological tests, we believe that a value of less than 30 I.U. represents certainly a "sub-average" state of nutrition, and probably it would be fair to describe it as "subnormal". Much more work will be necessary to determine the level of deficiency which may be correlated with the appearance of definite clinical symptoms. A still more difficult task will be to establish a

true "optimum" value, since it is clear that the amount of vitamin B₁ needed for optimal well-being is many times that needed to prevent actual symptoms of disease. However, provision of a simple and reliable chemical test should go far to accelerate progress in this direction. It is just necessary to add that before pronouncing a judgment on any given case it seems advisable to examine the effect of daily test doses given *per os*. A deficient case differs from the normal in showing one or more day's delay before a plateau of excretion is reached [see Harris *et al.* 1938].

As mentioned above, the normal values previously found ranged from about 12 to 40 I.U., as compared with 33 to 130 I.U. in the present tests. It is possible that there was some systematic loss in these earlier preliminary experiments (e.g. during adsorption), but there is no reason to doubt that the original categories were justified in so far as they showed that there was a negligible excretion in beri-beri and nutritional or conditioned polyneuritis, a diminished excretion in the subjects from the poor industrial area as compared with the well-fed controls, and varying degrees of deficiency in medical cases associated with anorexia, restricted diets, faulty absorption or gastro-intestinal obstruction.

SUMMARY

A study has been made of the thiochrome method as applied to urine and the results obtained have been standardized against direct biological tests.

The principal sources of error to be overcome were found to include: (1) incomplete adsorption; (2) interference from (a) non-specific fluorescing substances, (b) other reducing substances, (c) pigments.

With the modifications in the technique now recommended, the accuracy as well as the sensitiveness and simplicity of the method have been improved (a set of 6 or more tests can be carried out within an hour), interference from non-specific fluorescing substances or inhibiting substances reduced and the results obtained have been proved to be accurate when checked against biological assays. Added vitamin B₁ has been recovered with an error of less than 10%. With average urines, differences of less than 10% in activity can be detected, and 5 ml. are sufficient for a determination.

Some special features of the technique recommended are: (1) the omission of any adsorption process, (2) regulation of the amount of potassium ferricyanide added, (3) special treatment of the filter paper and reagents used, (4) removal of interfering substances, both by preliminary washing of the urine with *isobutanol* and subsequent extraction of the *isobutanol*-thiochrome phase, (5) direct visual comparison of fluorescence.

The usual range of excretion for normal subjects was found to be from 50 to 80 I.U. daily, with a maximum of 160 and a minimum of 30 (in place of the more conservative estimate of a minimum of 12-15 I.U. previously tentatively suggested). Test doses should be given to confirm a diagnosis of deficiency; examples are shown.

Lowered reserves of the vitamin were seen in pregnancy and in "conditioned deficiency" attributed to anorexia or faulty utilization.

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