160. THE ESTIMATION OF VITAMIN B₁ IN URINE

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VITAMIN B₁ can be estimated by measuring the intense blue-violet fluorescence of its oxidation product thiochrome [Jansen, 1936]. When applied to urine the thiochrome method gives results of the same order as the bradycardia method [Karrer, 1937; Wang & Harris, 1939] and the colorimetric method of Melnick & Field [1939, 1, 2], but interfering substances in urine have made quantitative estimations difficult. Urine gives a "blank" fluorescence which is reduced to an unknown extent by the ferricyanide employed to oxidize the vitamin to thiochrome. The recovery of thiochrome from vitamin B₁ added to urine is incomplete and variable.

In the method described here the synthetic zeolite used as an adsorbent for the vitamin has more specific properties than the activated earths formerly employed. The "blank" fluorescence is much less than in other methods, and the recovery of thiochrome, although incomplete, is constant under suitable conditions. The method, which is simple and rapid, has been applied only to human urine.

A study of the rate of urinary excretion of ingested vitamin B₁ has given information about controlling factors, and the minimum period of urine collection required in tests of the nutritional level has been estimated.

Methods

The technique has already been outlined [Jowett, 1939]. It is simpler than that of Hills [1939], which was published later. The adsorbent used is Decalso, a product of the Permutit Co., Ltd. Fluorescence is measured with the Pulfrich photometer.

Treatment of urine. 24 hr. specimens are collected in bottles containing 5 ml. glacial acetic acid. Urine so acidified (of pH 3.4-4.8) may be kept at room temperature for several days, and in the refrigerator for several weeks, without loss of vitamin B₁. For shorter periods of collection proportionately less acetic acid is added, but the resulting pH is then sometimes higher and requires adjustment to about 4.5. If a heavy precipitate is present the urine is warmed before it is sampled. Urine need not be filtered before analysis unless blood is present, in which case cell-pigments must be removed.

Reagents. Vitamin B₁ solution, prepared by diluting the contents of a "Benerva" (Roche) ampoule to 10 µg./ml. with a buffer solution of pH 4 (0.03 M acetic acid + 0.0075 M acetate), maintains its strength in a refrigerator for at least 3 months. *iso*Butanol (B.D.H. Laboratory Reagent) may be used as received, but gives a lower "blank" when redistilled, as stated by Wang & Harris [1939]. The pure solvent may be recovered after the analysis by fractionation of the neutralized wet alcohol mixture. Powdered Decalso is prepared from the dry crystalline material by grinding it to a fine powder, which is suspended in water and decanted several times to leave coarse particles behind. Fine

particles are removed by rejecting the supernatant after very brief centrifuging. The precipitate is washed on the centrifuge with alcohol and acetone, dried roughly at 60–80°, then completely in a desiccator evacuated with a water-pump, and finally powdered gently and kept in a desiccator.

Analytical procedure. It is convenient to analyse several urines at the same time. In each analysis 3 equal amounts of urine are taken, one for the "blank", a second for the "test" and a third for estimating vitamin recovery. The third specimen may be omitted for some of the urines if the conditions give uniform recovery. A "reagent blank", omitting urine, may also be required.

1–20 ml. of acidified urine, containing preferably 1–3 μ g. vitamin B₁, are measured into a centrifuge bottle containing 1.5 ml. 0.2 *N* acetic acid, the purpose of which is to buffer the Decalso. To the third bottle in each analysis are added 6 μ g. vitamin B₁. Each bottle is filled to the shoulder with water, the total volume being about 23 ml., after which 150 (± 7) mg. powdered Decalso are introduced and shaken into suspension. Centrifuging for a suitable short period packs the Decalso lightly. The supernatant (the pH of which may require determination with B.D.H. "4.5" Indicator) is poured off carefully and the bottles are inverted to drain for a few minutes. The neck of each bottle is wiped, 1.5 ml. methanol are added and the Decalso is shaken loose from the bottom.

The following procedure is then applied to each bottle in turn, "blanks" being treated first, then "tests" and finally "vitamin recovery" bottles. The Decalso is shaken into suspension and a stream of N₂ is passed through the liquid. 1 ml. 5.6 *N* NaOH is added, followed by 2 ml. 0.75% K₃Fe(CN)₆ (or by 2 ml. water for a "blank"). 10 ml. isobutanol are added and the mixture is stirred with N₂ for 15–30 sec. longer. The procedure takes about 1½ min. Finally 9.5–10 ml. of the supernatant alcoholic layer are pipetted into a test tube containing 0.5 ml. ethanol and shaken. Ethanol clears the slightly turbid fluid.

Measurement of fluorescence. Fluorescence is measured with the Zeiss-Pulfrich photometer by the substitution method through an L III filter, which transmits blue and violet light. The fluorescent solution, in an uncovered rectangular cell 3 cm. long, is matched against a piece of Ilford Fluorazure Screen mounted at 45° from the vertical, the fluorescence of which is reduced by filter paper pressed tightly against it with glass. Cell and screen are illuminated from above by ultraviolet light, filtered through Wood's glass, derived from a Hanovia Universal Fluorescence Lamp, the position of which is roughly reproducible. The use of vertical illumination eliminates special focussing and filters.

Readings can be made within 5 min. of entering the dark room. If eye-fatigue is minimized by rapid matching of not more than 20 solutions on each occasion, an accuracy of 1–5% is obtained after some practice. Matching is best at moderate intensities. Fluorescence is expressed on an arbitrary scale as the ratio of the drum-readings multiplied by 100. Each analysis or set of analyses is calibrated by the inclusion of specimens with added vitamin. After subtraction of the appropriate "blank", fluorescence is proportional to thiochrome concentration over a range extending at least to that derived from 25 μ g. of adsorbed vitamin.

Discussion of the method

Decalso in the convenient powder form has hitherto not been used for purifying vitamin B₁, though American workers have used it in a column. Other cations in urine compete with vitamin B₁ for adsorption, and only under restricted conditions is adsorption of the vitamin complete. Melnick & Field [1939, 2], whose less sensitive method requires about 200–400 ml. urine, had to

resort to a troublesome extraction before the adsorption. In the present method direct treatment of urine is satisfactory if the pH is correct and the ratio of urine volume to weight of Decalso is not too high. The optimum pH under the analytical conditions is about 4.5 and the yield is lowered by about 10 % at pH values of 3.9 and 5.1. The combined buffering action of acetic acid and Decalso, which takes up hydrogen ion, usually leads to a pH very close to 4.5 without special adjustment of the pH of the acidified urine. Since the salt content of urine is very variable no definite upper limit can be set to the amount of urine from which the vitamin is completely adsorbed. Adsorption is 95–100 % complete when the urine volume is equivalent to 1/300 of a 24 hr. specimen (about 3–8 ml.), but doubling this amount of urine may sometimes lead to less complete adsorption. It is not essential that adsorption should be complete, since the recovery can be estimated in each analysis, but if the recovery is much lowered the accuracy is reduced.

Recovery. The thiochrome derived from adsorbed vitamin by the combined processes of elution, oxidation and extraction described is only about 68 % of that derived from the vitamin in solution treated in the same manner in the absence of Decalso. The incomplete recovery is apparently due to incomplete elution of the vitamin or to its destruction during elution. In founding a quantitative method on an incomplete recovery, the recovery should be consistent, even though it can be estimated in each analysis. Investigation has shown that the recovery varies little either when different batches of reagents are used or when the analytical conditions are varied far beyond the limit of accidental variation of technique. The recovery from pure solutions of vitamin is about 68 % over a wide range of concentration. The recovery from a large number of urines under the conditions described (about 95 % adsorption) averages 65 %, with a standard deviation about ± 3 . Definite alteration of recovery has been observed only when too much urine is taken or in analysing unfiltered specimens of menstrual urine containing blood. The very low recovery in presence of blood, which is due to destruction of vitamin B_1 by haematin in an alkaline medium [cf. Schroeder, 1939], was found to rise to normal when the clotted red cells were filtered off before analysis.

Other thiochrome methods give much less consistent recoveries as far as details are available. Westenbrink & Goudsmit [1937] obtained varying yields up to 70 %, and according to Hills [1939] the method of Wang & Harris [1939] gives similar recoveries. Hills [1939] obtained an average yield of 76 % with a standard deviation of ± 17 , the great variability being due to interfering substances in urine, for increasing the amount of adsorbent lowered the yield. In the present method excess of Decalso does not lower the yield. It is possible that the use of Decalso in a column, without previous extraction of urine but with separate procedures for elution and oxidation of the vitamin, may give a high and consistent yield of thiochrome at the expense of more labour. In the only work on such lines [Hennessy & Cerecedo, 1939] urine is analysed only incidentally and the method cannot be regarded as established for urine.

The "blank" fluorescence. In the absence of ferricyanide, fluorescent substances in urine give rise to the appearance in the isobutanol solution of a fluorescence which has been regarded as a "blank" to be subtracted from the "test" fluorescence. Hills [1939] has however found evidence that ferricyanide reduces to an unknown extent the green fluorescence arising from urine constituents. The uncertainty introduced into the method of Hills is considerable, for the amount of vitamin estimated is less than $4 \mu g.$ and the "blank" fluorescence expressed in terms of vitamin is 1–2 $\mu g.$ when the usual amount of about

9 ml. urine is analysed, or about 0.6 μ g. with 2 ml. urine. The methods of Westenbrink & Goudsmit [1937] and of Wang & Harris [1939] give "blanks" of the same order.

The present method gives much lower "blanks", and it becomes worth while to distinguish between a "reagent blank" (determined in absence of urine) and the observed "total blank", the difference being called the "urine blank". In terms of vitamin recovered from urine, the "reagent blank" with redistilled isobutanol is about 0.09 μ g. and is unaffected by ferricyanide. The "urine blank" with amounts of urine of the order of 1/300 of the 24 hr. output averages 0.1–0.15 μ g. In 800 analyses only 2 or 3 "urine blanks" as high as 0.4 μ g. have been found. The "total blank" fluorescence is usually blue in colour.

The "urine blank" is still high enough to introduce uncertainty into the determination of low vitamin concentrations. If from the "test" reading the "reagent blank" is subtracted instead of the "total blank", the calculated 24 hr. output of vitamin averages about 35 μ g. higher (the corresponding figure in Hills's method being about 7 times as great).

An attempt has been made in former methods to eliminate the "blank" difficulty by the use of a supposedly optimum amount of ferricyanide. It has been found by Hills with Clarit, and confirmed in this laboratory for Decalso adsorbates from urines of very low vitamin content, that although a certain amount of ferricyanide may give a maximum "test" reading, this amount is insufficient to give full thiochrome production from added vitamin. The supposedly optimum amount has to be determined or guessed. There are also the objections (1) that destruction of non-thiochrome fluorescence, though lessened, has not been shown to be eliminated, (2) that the amount of ferricyanide required to give maximum "test" readings will depend on the ratio of vitamin to fluorescent substances in the sample of urine and (3) that the conversion of added vitamin into thiochrome will be more variable and more liable to technical error.

In the present method a considerable excess of ferricyanide is used. About 0.1–0.2 ml. 0.75% $K_3Fe(CN)_6$ gives the maximum fluorescence with urines of low vitamin content, while about 0.2–0.4 ml. lowers this fluorescence and raises the yield of thiochrome from added vitamin to levels which are not altered by amounts up to 2 ml.

Since the "urine blank" is usually small it is difficult to investigate, but several rough tests agree in suggesting that the excess of ferricyanide approximately halves the "urine blank".

(1) Sulphite destroys vitamin B₁ more rapidly than the substances responsible for the "urine blank", e.g. in one experiment 0.02 *M* sulphite at 100° and pH 4.9 destroyed half the vitamin every 1½ min., while the "urine blank" decreased only 20% in 14 min. With urine freed from vitamin by sulphite the effect of ferricyanide on the residual "urine blank" can be determined.

(2) The "urine blank" does not usually rise in proportion to the amount of urine analysed. By analysing several different amounts of urine of high "blank" and low vitamin content, a rough estimate can be made of what proportion of the "urine blank" applied as a correction gives the most consistent estimate of vitamin content.

(3) If the "total blank" is subtracted, urines of low vitamin content very occasionally give a negative vitamin value which becomes positive if the correction for the "urine blank" is halved.

The most suitable "blank" to subtract is therefore taken to be midway between the "total blank" and "reagent blank". These two values represent

the limits of choice of "blank". The error involved in the mid-choice cannot on the average exceed $\pm 18 \mu\text{g.}$ for a 24 hr. urine, and it is probably less. The calculation from the fluorescence readings is illustrated by the following example: "reagent blank" 0.7; "total blank" 1.9, "test" 12.3, "test + 6 $\mu\text{g.}$ vitamin" 73.5. The amount of vitamin is $6 (12.3 - \frac{1}{2} (0.7 + 1.9)) / (73.5 - 12.3)$, or 1.08 $\mu\text{g.}$

Application of the method. According to Robinson *et al.* [1940], men on a diet low in vitamin B_1 excrete 0–90 $\mu\text{g.}$ daily in their urine. The present method has a "blank" error that may possibly average as high as 18 $\mu\text{g.}$ per 24 hr. and a photometric error (for low values) of about 5 $\mu\text{g.}$ per 24 hr., and it therefore does not measure such excretions accurately. The dietary excretion is however only of limited value in assessing the nutritional level. According to Robinson *et al.* [1940] urine must be collected for 24 hr. while the subject is on a diet of adequate vitamin content. The method does not apply in achlorhydria or during alkaline medication, it entails delay before therapy, and it cannot be used to follow the progress of therapy.

The method of determining urinary excretion after an oral dose of vitamin has fewer limitations. With a small dose, such as the 1 mg. recommended by Hills [1939], the excretion depends appreciably on the current vitamin intake. A 5 mg. dose, however, leads to a vitamin excretion which is much less influenced by current dietary or recent therapeutic intake and is also large enough to possess therapeutic value. The present analytical method is well suited for determining the excretion following a 5 mg. dose, which for a man adequately supplied with the vitamin amounts to 400–1200 $\mu\text{g.}$ in 24 hr. The uncertainty due to the "blank" becomes unimportant and the standard error of a single estimation is about 7%, due mainly to photometric error. Since the range of normal response and the variability of abnormal response are great, this accuracy is quite satisfactory. With care and duplication more accurate values may be obtained for special purposes.

The rate of urinary excretion of vitamin B_1

The urinary response to an oral dose of 5 mg. of vitamin B_1 can be estimated by measuring the excretion during the following 24 hr. Experiments reported here indicate that shorter periods of urine collection will serve. They also cast light on the part played by the stomach and kidney in absorption and excretion of the vitamin.

Four adult male laboratory workers ingested 5 mg. of the vitamin immediately after breakfast. While the excretion in the first 2½ hr. was variable, the proportion of the 24 hr. excretion of vitamin which took place in the first 5 hr. was fairly constant at about 68% (Table 1). Correction can be made for the excretion of vitamin of dietary origin, assuming this to be the same as on the previous day and to be spread evenly over the 24 hr. The 5 hr. excretion of vitamin derived from the test-dose is then found to be about 75% of the 24 hr. figure.

Table 1. *Excretion of vitamin B_1 , 5 and 24 hr. after ingestion*

Subject	$\mu\text{g.}/24 \text{ hr. on}$ normal diet	$\mu\text{g.}/24 \text{ hr. after}$ test dose	% of 24 hr. excretion after test dose which is excreted in	
			2½ hr.	5 hr.
C.	39	652	35	65.5
D.	80	768	22	69
J.	144	1002	41	69
R.	121	704	—	70

In an otherwise similar experiment with 8 mental patients the 5 hr. excretion was determined on a different day from the 24 hr. excretion; the former averaged 71% of the latter, but there was greater variation among the individuals. In one case the percentage was only 27, but the 5 hr. urine volume was only 90 ml., which suggests that retention of urine in the bladder can lead to low results in a 5 hr. test.

Table 2. *Excretion at short intervals after a dose*

Exp. 1 (day 1)		Exp. 2 (day 3)		Exp. 3 (day 13)	
Time hr.	μg. vitamin B ₁	Time hr.	μg. vitamin B ₁	Time hr.	μg. vitamin B ₁
0-1	28	0-0.8	7	0-0.5	6
1-2	151	0.8-1.5	47	0.5-1.6	99
2-3	137	1.5-2.5	129	1.6-2.4	50
3-4	43	2.5-3.1	134	2.4-3.1	37
4-5	38	3.1-3.6	57	3.1-4.0	45
5-6	31	3.6-5.0	98	4.0-5.0	18
6-7	19	5.0-6.6	61	5.0-8.0	74
7-8	21	6.6-8.0	43	8.0-23.0	182
8-23	187	8.0-24.0	226		

Total excretion

(a) By addition of above figures

655

802

511

(b) By analysis of pooled aliquots

735

789

518

The excretion was followed at much shorter intervals with subject J. (Table 2). In Exps. 1 and 2, 5 mg. vitamin B₁ were ingested immediately after breakfast, in Exp. 3, 0.8 hr. before breakfast. Times were reckoned as before from the time of ingestion of the vitamin. The estimated rates of vitamin excretion are plotted in Fig. 1, the times at which the maxima occur being well defined although the heights of the maxima are only approximate. It is clear (Exps. 1 and 2) that the time of the maximum can vary from day to day in the same individual. The rate of excretion falls greatly 2 hr. after the maximum. The maximum occurs earlier when the vitamin is ingested before the meal (Exp. 3), but the amount of vitamin excreted is less. This result agrees with those of Melnick *et al.* [1939], who followed the excretion at 4 hr. intervals when a 5 mg. dose had been ingested either 3 hr. before breakfast or immediately after a heavy meal. These authors suggest that much of the vitamin is destroyed before absorption when taken into a fasting stomach.

Fig. 1.

It is probable that the site of absorption of vitamin B₁ is the small intestine, and injection experiments have shown that once the vitamin reaches the circulation excretion takes place rapidly. If the vitamin is absorbed rapidly from the intestine, then the time-interval between ingestion and maximum excretion of the vitamin will depend mainly on the rate of emptying of the stomach. The rates of excretion shown in Fig. 1 support the view that maximum excretion occurs soon after the passage of most of the vitamin into the intestine, and that the variability of the lag between ingestion and maximum excretion is due to variability in the rate of emptying of the stomach.

The vitamin excretion after ingested doses has already been followed at hourly intervals by Westenbrink & Goudsmit [1938], but the time-relation to meals was not indicated. Their finding that most of the excretion of the dose took place in 3 hr. is therefore not an adequate basis for the method adopted by Hills [1939] of determining the excretion during 3 hr. after ingestion of a test-dose with breakfast.

The present experiments indicate that, when a test-dose is ingested with breakfast, urine should be collected for at least 5 hr. to obtain a fairly uniform proportion of the resulting excretion of vitamin. If there is a likelihood of delayed stomach or bladder function, the period should be extended. The data of Melnick *et al.* [1939] make it probable that a period of 8 hr. is required after ingestion of the dose with a heavy meal.

How the kidney deals with vitamin B₁ does not appear to have been discussed. We are not concerned with the phosphorylated vitamin, which is not found in urine or (apparently) plasma. If the diet is adequate the concentration of the free vitamin in daytime urine is of the order 0.04–0.16 $\mu\text{g./ml.}$ According to analyses by thiochrome methods the concentration in plasma is of the same order, Ritsert [1939] finding 0.03–0.15 $\mu\text{g./ml.}$ in blood and Hennessy & Cerecedo [1939] 0.09–0.12 $\mu\text{g./ml.}$ On the other hand Goodhart & Sinclair [1940], relying on a small difference between total and phosphorylated vitamin determined by different methods, estimate the free vitamin in blood to be 0.01 $\mu\text{g./ml.}$, which is less than in urine. These discordant results do not show whether the kidney concentrates the vitamin from plasma.

A definite decision on this question can be arrived at from the experiments summarized in Table 2. In Exp. 2 during the period 1.5–3.1 hr. after ingestion of 5 mg. of vitamin the average concentration in the urine exceeded 3 $\mu\text{g./ml.}$ If the whole of the 5 mg. dose were present in the blood plasma the plasma concentration would be only 1.5 $\mu\text{g./ml.}$ But vitamin B₁ leaves the circulation rapidly; from the blood analyses of Ritsert [1939] it can be deduced that vitamin injected intravenously into rabbits is distributed as if throughout the body water in about 10 min. Clearly only a small proportion of an ingested dose can be present in the plasma for a period of, say, an hour. The conclusion to be drawn is that the kidney can concentrate vitamin B₁ from plasma to a marked degree, perhaps 20 times or more.

SUMMARY

1. A rapid and simple method of estimating vitamin B₁ as thiochrome is applied to urine. The powdered Decalso used is a more specific adsorbent of the vitamin than are activated earths, and gives lower "blanks" and more consistent yields. Fluorescence is measured with the Pulfrich photometer.

2. The method does not estimate low dietary excretions accurately, but is suitable for measuring the response to test-doses, such response being the best test of nutritional level.

3. Measurements of urinary excretion at short intervals after ingestion of 5 mg. of vitamin show that the time-lag before excretion depends mainly on the time required for the stomach to empty, and that the shortest suitable period of urine collection in saturation tests is 5 hr. The kidney can concentrate vitamin B₁ from plasma.

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