121. VITAMIN METHODS

1. AN IMPROVED PROCEDURE FOR ESTIMATING VITAMIN B_1 IN FOODSTUFFS AND BIOLOGICAL MATERIALS BY THE THIOCHROME TEST INCLUDING COMPARISONS WITH BIOLOGICAL ASSAYS

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DURING the past two years a systematic study has been made in this laboratory of the various possible sources of error and the technical difficulties involved in the thiochrome test, based on the now well-known reaction first described by Jansen [1936]. These errors and difficulties are more serious than is generally appreciated. In an earlier paper [Wang & Harris, 1939] a procedure was described for urine, by which interference from non-specific substances and various other problems of technique were successfully overcome. Our results were controlled by biological tests, and this proof of the reliability of the method applied to urine has been further confirmed since [see accompanying paper, Harris & Wang, 1941]. The present communication describes how the technique used by Wang & Harris may be adapted for estimating vitamin B_1 in foodstuffs and in animal and plant tissues. As we have again repeatedly checked our results by biological assays, we feel considerable confidence in the method which we are now recommending.

Need for investigating potential errors in the thiochrome test

It is curious that although thiochrome determinations have been carried out by various workers, e.g. by Westenbrink & Goudsmit [1938] on animal tissues, by Wiegland [1938] and by Houston *et al.* [1940] on milk, and by Pyke [1939] on a long list of 190 food materials—little effort has been made hitherto (except for the few estimations on milk by Houston *et al.* [1940]) to test the findings by direct comparison against biological results, and even more surprising is the fact, which seems to have escaped general notice, that many of the results published are systematically low when compared with the usually accepted values as based on biological tests. This liability to error, in the absence of precautions such as those worked out in the paper, has again been forced on our notice in the results of a recent series of collaborative trials in which we were able to participate; values based on the present method agreed well with the biological tests, whereas those based on other thiochrome methods were frequently low. (For a full tabulation of these comparative results, see Wang [1941].)

That there was a need to work out a procedure at once reliable and reproducible has also been brought home to us repeatedly during the past year or two by investigators in other laboratories, who have told us of the difficulties

which they have experienced when attempting to estimate the vitamin by other methods based on the thiochrome reaction. We believe we can claim that the process described in this paper is satisfactory, since, apart from the agreement with the biological findings referred to above, it has been used for continuous routine tests for many months by three independent workers in this laboratory, and their results have tallied closely; also workers from four other institutes, who at their request were provided with details of the method during 1939 or 1940, after having experienced difficulties with other techniques, report that it has worked without trouble and given reproducible results. It has also stood the test of use by students of the advanced biochemical class at Cambridge during the past two sessions.

Comparison with azo method. The improved thiochrome test seems preferable to the alternative chemical method, based on the azo reaction [Prebluda & McCollum, 1939; Melnick & Field, 1939; cf. Kinnersley & Peters, 1934] in being more sensitive and in not requiring an elaborate process of preliminary purification in order to prepare a concentrate suitable for the reaction. We are able to determine as little as 0·03 μg. of aneurin per ml. in extracts from foodstuffs, with an error of $\pm 10\%$. With extracts containing more than 0·1 μg. of aneurin per ml., the error is usually about $\pm 5\%$.

Preliminary control tests

The thiochrome test depends on the conversion of vitamin B_1 into its oxidation product, thiochrome, under the action of alkaline ferricyanide, and on the measurement of the fluorescence produced by the latter, generally after extraction in *iso*butanol.

As the procedure for foodstuffs and tissues now to be discussed is based on the method of Wang & Harris [1939] for urine, it will be convenient first to consider the special measures taken in the latter method to overcome the various technical difficulties which arise. These have been adopted and, where necessary, modified in the present process; and in addition certain new problems have had to be faced. The three principal features of Wang & Harris's method comprised:

(1) Extraction, prior to oxidation, with *iso*butanol, to remove interfering substances.

(2) Subsequent washing of the *iso*butanol layer (containing the thiochrome) for further purification from both pigments and non-specific fluorescing substances.

(3) Visual comparison, to distinguish between specific blue fluorescence due to the thiochrome and any non-specific yellowish fluorescence.

Other precautions taken over from the method for urine include:

(4) Control of the amount of ferricyanide used, to prevent (*a*) a partial destruction of the thiochrome formed and (*b*) variable effects on non-specific fluorescent substances. The destructive effect of excess of ferricyanide is seen from Table 1.

Table 1. *Influence of variations in amount of potassium ferricyanide added*

	Test 1	Test 2	Test 3	Test 4
Vitamin B_1 present, μg.	0·40	0·40	0·40	0·40
H_2O, ml.	1·48	1·4	1·0	0·5
MeOH, ml.	2·0	2·0	2·0	2·0
NaOH, 30% solution, ml.	1·0	1·0	1·0	1·0
$K_3Fe(CN)_6$ 2% solution, ml.	0·025	0·10	0·50	1·0
Vitamin B_1 found, μg.	0·41	0·40	0·35	0·32
Recovery of vitamin B_1, %	102	100	87·5	80

(5) Omission of adsorption which generally caused a partial loss or inactivation of vitamin B_1, judged from recovery experiments on the eluate [cf. also Jowett, 1940].

(6) Treatment of the reagents used, to remove fluorescing or other interfering substances.

(7) Inclusion of a blank control, to allow for a small residuum of interference.

In work on foodstuffs two major additional problems have to be faced which do not arise with urine: (8) the preliminary digestion or extraction of the material to get the vitamin into a form suitable for the estimation and (9) the conversion of cocarboxylase into aneurin and its differentiation from the latter. These and other related questions may next be discussed.

(8, 9) *Preliminary digestion. Conversion of cocarboxylase into aneurin*

For converting cocarboxylase into aneurin, phosphatases derived from various sources can be used. Thus, Hennessy & Cerecedo [1939] used a preparation of beef kidney. This however has been criticized by Melnick & Field [1939] as giving an incomplete conversion. We have tested takadiastase [Kinnersley & Peters, 1938] and dried yeast [Melnick & Field, 1939], and prefer the former. The disadvantage of yeast is that an additional blank has to be run side by side with the test on the 'unknown' to allow for the aneurin present in the yeast itself; with takadiastase (Parke Davis) on the contrary no blank is needed as the enzyme preparation is free from vitamin B_1.

We have found that the most convenient procedure for removing any protein from the unknown, and otherwise obtaining an extract suitable for the test, is by digestion with papain. Other workers [e.g. Pyke, 1939] have used pepsin followed by takadiastase. The advantage of papain is that it can be combined with takadiastase in a single digestion at one *p*H value, whereas if pepsin is used two consecutive digestions at different *p*H values are necessary.

(10) *Partial solubility of thiochrome pyrophosphate in* isobutanol

Some error is caused in the estimation of the aneurin-cocarboxylase ratio in a mixture of the two, if the method adopted by most past workers is followed. This has involved a determination of aneurin both before and after hydrolysis of cocarboxylase. The increased aneurin found after hydrolysis has been taken as the cocarboxylase. This procedure is based on the assumption that thiochrome pyrophosphate, formed by the action of the K_3FeCy_6 on unchanged cocarboxylase, is insoluble in the *iso*butanol layer. But this is not strictly true. We have found that thiochrome pyrophosphate is soluble to a considerable extent in the so-called '*iso*butanol layer' which is really a mixture of *iso*butanol, methanol and water. Since thiochrome pyrophosphate shows the same blue fluorescence as thiochrome, its presence in the *iso*butanol layer causes an apparent increase in the thiochrome reading. The error thus introduced may be quite large when the amount of free aneurin is low in comparison with the cocarboxylase. This is shown in Table 2. The error is almost completely eliminated in our process by the washing of the *iso*butanol layer with water. This operation has the additional advantage that it removes excess of alkali from the *iso*butanol layer, and thereby considerably improves the stability of the thiochrome (Table 2).

Westenbrink & Jansen [1938] originally proposed to determine cocarboxylase by a process in which the preliminary hydrolysis into aneurin is omitted. The fluorescence of the *iso*butanol layer is taken as representing the free aneurin, and the fluorescence of the aqueous layer after the extraction with *iso*butanol as

Table 2. *Estimation of free aneurin in the presence of cocarboxylase. Interference from cocarboxylase removed by washing the* isobutanol *layer with* H_2O

Total amount present, as μg. of aneurin	Proportion of cocarboxylase and aneurin present		Free aneurin found, %	
	Cocarboxylase %	Aneurin %	Without washing	With washing
0·38	100	0	16	1
0·28	95	5	26	5·7
0·26	77	23	31	21
0·4	50	50	57	48

representing the cocarboxylase. The method has the objection just indicated—namely partial loss of the cocarboxylase derivative (thiochrome pyrophosphate) into the *iso*butanol; and in addition there is frequently serious interference from other fluorescent substances present in the aqueous phase. Such interfering substances are eliminated by the washing in our process.

(11) *Protective action of methanol. Optimum addition of NaOH*

Westenbrink & Goudsmit [1938], Ritsert [1938] and Karrer & Kubli [1937] all omit the use of methanol in the oxidative process. This modification has the advantage that less thiochrome pyrophosphate dissolves in the *iso*butanol layer, because of the decreased solubility of water in *iso*butanol in the absence of methanol. On the other hand it has the serious objection that in the absence of methanol accurate quantitative estimation of the vitamin is not possible owing to the destruction (presumably by oxidation) of the vitamin or of the thiochrome formed from it. Only 50–70% of the true value is found (Table 3). In our

Table 3. *Effect of MeOH on the recovery of thiochrome*

Vit. B_1 present, μg.	0·20	0·20	0·20	0·20	0·20*	0·20*	0·20*	0·20*	0·20*	0·40
H_2O, ml.	0·5	0·5	1·0	1·0	1·5	1·5	2·0	2·0	3·5	0·5
MeOH, ml.	2·0	2·0	2·0	2·0	1·5	1·5	1·0	1·0	0	2·0
NaOH, 30% solution, ml.	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0
$K_3Fe(CN)_6$, 2% solution, ml.	0·04	0·2	0·04	0·2	0·04	0·2	0·04	0·2	0·08	0·08
Vit. B_1 found, μg.	0·19	0·19	0·19	0·19	0·19	0·17	0·16	0·16	0·14	0·41
Recovery, %	95	95	95	95	95	85	80	80	70	102
Vit. B_1 present, μg.	0·40	0·40	0·40	0·40	0·40*	0·40	0·40*	0·40*	0·40*	0·40*
H_2O, ml.	1·0	1·0	1·0	1·0	1·0	1·5	2·0	2·0	3·0	3·0
MeOH, ml.	2·0	2·0	2·0	2·0	2·0	1·5	1·0	0	0	0
NaOH, 30% solution, ml.	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0
$K_3Fe(CN)_6$, 2% solution, ml.	0·08	0·04	0·04	0·04	0·04	0·08	0·08	0·04	0·08	0·08
Vit. B_1 found, μg.	0·41	0·40	0·40	0·40	0·38	0·40	0·37	0·20	0·24	0·20
Recovery, %	102	100	100	100	95	100	93	50	60	50

* Methyl alcohol was added afterwards to the *iso*butanol extract, to compensate for the difference in volume.

experience it is essential that methanol be added to the extent of at least 50% by volume before the addition of the NaOH, in order to ensure that there is no destruction of the vitamin (Table 3)[1]. Provided that this proportion of MeOH is

[1] In presence of MeOH higher fluorescences were obtained when the $K_3Fe(CN)_6$ was added before the NaOH, while in absence of MeOH the reverse held good. The latter procedure however gave slightly lower values and involved risk of loss of vitamin if there were delay between the additions of the reagents.

Table 4. *Effect of varying the amount of NaOH added*

	Test 1	Test 2	Test 3	Test 4
Vitamin B_1 present, μg.	0·40	0·40	0·40	0·40
H_2O, ml.	2·3	2·0	1·5	0·5
MeOH, ml.	2·0	2·0	2·0	2·0
NaOH, 30% solution, ml.	0·2	0·5	1·0	2·0
$K_3Fe(CN)_6$, 2% solution, ml.	0·06	0·06	0·06	0·06
Vitamin B_1 found, μg.	0·34	0·37	0·40	0·41
Recovery, %	85	92·5	100	102

Table 5. *Influence of time allowed for the reaction on the percentage recovery*

1 ml. aneurin solution containing 0·20 μg. aneurin treated with 2·0 ml. MeOH, 0·04 ml. of 2% $K_3Fe(CN)_6$ and 1 ml. of 30% NaOH. The mixture was allowed to stand for varying lengths of time before extraction with *iso*butanol.

Time allowed before extraction, min.	1	5	5*
Vitamin B_1 present, μg.	0·20	0·20	0·20
Vitamin B_1 found, μg.	0·19	0·19	0·19
Recovery, %	95	95	95

* 0·25 ml. of 2% $K_3Fe(CN)_6$ added in this instance.

present, the NaOH can then be safely added in the quantities necessary to effect complete conversion of the vitamin B_1 into thiochrome (Table 4). Under these conditions the thiochrome formed is found to remain undestroyed, even when the reaction mixture is allowed to stand for as long as at least 5 min. before the extraction with the *iso*butanol (Table 5).

(12) *Factors inhibiting the hydrolysis of cocarboxylase*

We have found that some animal tissues, e.g. liver, contain a factor or factors which appear to inhibit the hydrolysis of the naturally present cocarboxylase (or other precursor, if such there be). As is shown in Table 6, the hydrolysis of

Table 6. *Recovery tests after digestion for different periods of time and under varying conditions*

		Amount recovered after digestion for 1 hr.		Amount recovered after digestion for 3 hr.		Amount recovered after digestion for 24 hr.	
		μg.	%	μg.	%	μg.	%
Cocarboxylase, specimen 1	Takadiastase	80	—	—	—	82	—
Cocarboxylase, specimen 2	Takadiastase	86	—	—	—	84	—
Cocarboxylase, specimen 3	Takadiastase	70	—	—	—	72	—
Liver, specimen 1	Takadiastase	1·1	—	2·2	—	7·9	—
Liver, specimen 1 + cocarboxylase ≡ 7·1 μg. vit. B_1	Takadiastase	2·1	14	4·8	37	14·3	90
Liver, specimen 2	Takadiastase	0·44	—	—	—	—	—
Liver, specimen 2 + 10 μg. vit. B_1	Takadiastase	10·2	98	—	—	—	—
Liver, specimen 3	Takadiastase + papain	8·8	—	10·6	—	11·9	—
Liver, specimen 3 + cocarboxylase ≡ 17·5 μg. vit. B_1	Takadiastase + papain	26·7	102	29·7	109	29·7	98
Liver, specimen 3 + 17·5 μg. cocarboxylase	Takadiastase	13·9	—	26·7	—	29·7	—
Liver, specimen 4, heated for 10 min. at 100° before digestion	Takadiastase	1·8	—	—	—	8·3	—
Liver, specimen 4 + cocarboxylase ≡ 7·1 μg. vit. B_1	Takadiastase	4·2	34	—	—	15·4	100

the cocarboxylase in liver by means of takadiastase was slow and incomplete, whereas pure synthetic cocarboxylase under the same conditions was completely hydrolysed after incubation for 1 hr. The inhibition is largely removed by the incubation with papain. This is an additional reason for the method of digestion favoured by ourselves.

It does not seem that this phenomenon of 'inhibition' is due to enzymic action, e.g. to phosphorylation of the aneurin set free, for we have found that added free aneurin can be recovered quantitatively unchanged, and further that preliminary heating to destroy enzymes did not abolish the inhibition.

(13) *Masking effect of pigments on the fluorescence*

We have observed that with blood, liver and certain cereals, if the digest did not receive the preliminary extraction with *iso*butanol before oxidation, coloured substances were present which masked the fluorescence of the thiochrome in the final comparison. With some materials the interference was so great that it completely masked the fluorescence of the thiochrome; both in the blank and the unknown only the colour of the natural pigment could be seen. Table 7 illustrates the beneficial effect of the preliminary extraction with *iso*butanol.

Table 7. *Removal of interfering pigments by preliminary extraction with isobutanol*

	Aneurin found, μg.		
	Without extraction	After extraction	Recovery %
Whole rat blood + cocarboxylase $\equiv$ 7·1 μg. vit. B_1 (digested with takadiastase for 5 hr.)	0	3·8	50*
Whole rat blood + free aneurin, 7 μg. (digested with takadiastase for 5 hr.)	0	5·8	80
Maize, specimen no. 1	0	0·11	—
Maize, specimen no. 2	0	0·11	—

* The hydrolysis of cocarboxylase was probably incomplete under these conditions.

(14) *Differences in tints of fluorescence*

Sometimes it is observed on matching that the tint of the fluorescence in the unknown or in a control solution of pure thiochrome is distinctly different from that in the blank, e.g. the latter may be tinted white or yellow, while the unknown is more usually a clear bluish-violet. This effect arises from the destruction by the ferricyanide of certain non-specific fluorescing substances which are present along with the vitamin B_1 in the sample, but which differ from vitamin B_1 in the shade of the fluorescent tint. This oxidation by the ferricyanide does not occur in the blank, which contains no ferricyanide. The difference between tint of unknown and blank is more pronounced when the amount of ferricyanide added is in great excess. In our method the difficulty is largely overcome: first, by careful regulation of the amount of ferricyanide added so that only a slight excess of it is present; secondly, by the preliminary extraction before oxidation, with *iso*butanol, which removes the greater part of the non-specific fluorescent substances; and thirdly, by the use of suitable filters, e.g. Wratten 18 A and 49 A.

This difficulty of a difference in tint is a possible objection to all modifications of the thiochrome method. It is the chief reason why objective measurements, as with a photoelectric cell, are not necessarily better, and may in fact be far less accurate, than the direct visual comparison. With the visual comparison differences in tint may be looked for and allowed for, whereas with a mechanical recording of fluorescence they may be more easily overlooked.

Working directions

The procedure described below has been found suitable for use with a very wide range of cereal products, yeast and yeast extracts, and for meat or animal tissues generally. It has also worked well with the somewhat more limited number of fruits and vegetables so far tested (including cauliflower, spinach, potatoes, oranges.[1]

To obtain accurate results it is essential that the following process be adhered to closely. Determinations should be carried out in duplicate to check any possibility of random errors.

A. *Extraction and digestion*

The process of preliminary extraction and digestion can be varied somewhat according to the nature of the product to be examined.

(1) *For cereals.* With dry materials such as whole grain, flours, biscuits etc., grind up the specimen to as fine a state as possible and take a representative sample. With moister preparations, e.g. breads, it is often convenient to break up into small crumbs and spread out to dry in a thin layer in air at room temperature before sampling; alternatively, a representative specimen of the fresh moist crumb may be extracted and digested direct without drying. It is important not to leave the moist material standing too long before examination, as the vitamin is liable to become oxidized at an alkaline or almost neutral reaction.[2] Take 1–2 g. (or more for a relatively inert material, e.g. up to 5 g.) and heat in a conical flask with about 15 ml. of acidulated water (2 drops of conc. HCl per 50 ml. of water) at 100° on a water bath for 10 min. with continual stirring. Then add 5 ml. of 0·1 *M* acetate buffer, *p*H 4; mix and cool to 40°. Readjust the reaction accurately to *p*H 4 if necessary by addition of a drop or two of 10% HCl, using bromocresol green as external indicator. A pasty suspension results, and two alternative methods may be used for the subsequent digestion of it: (*a*) with takadiastase plus papain or (*b*) with takadiastase alone. The former is preferred as being more accurate, particularly with materials of low activity, and is also more convenient if a large number of materials has to

[1] A more detailed study of plant products is in progress.

[2] Even at the neutral or mildly alkaline reaction of commercial yeast extract, serious and progressive deterioration occurs (see Table 8).

Table 8. *Deterioration of yeast extracts on standing*

Material	Length of time kept standing	Vitamin B_1 I.U. per g.
Commercial yeast extract, 'M'	Examined on purchase	5·8
	After 3 months	3·7
	After 2 years	1·0
	After 4 years	0·8
Commercial yeast extract, 'Y'	Examined on purchase	8·2
	After 2 months	6·6

be examined simultaneously. Probably its only disadvantage compared with method (*b*) is that it takes longer to carry out, since it entails leaving the digestion mixture overnight.

(*a*) *Digestion with takadiastase and papain.* To the pasty suspension at *p*H 4 and temperature 40°, now measuring a little over 20 ml., add 0·1 g. of takadiastase plus 0·1 g. of papain. Incubate overnight at 40–45° with the addition of a few drops of toluene as preservative. Make up to 25 ml., centrifuge and discard the insoluble residue.[1]

(*b*) *Digestion with takadiastase.* To the pasty suspension at *p*H 4 and temperature 40°, add about 0·1 g. of takadiastase and incubate for some 15 min. at about 40° with occasional shaking. (After this 15 min. incubation, it may be occasionally advisable, in instances in which the original sample was not sufficiently fine and homogeneous, to centrifuge and re-extract the residue with 7–8 ml. of acidulated water at 70–80° for 15 min. with stirring, and combine the extracts and residue.) If necessary, incubate again a second time, for 1–2 hr. at 40–45°; this second incubation may be omitted for most cereals, which contain negligible amounts of cocarboxylase. Now make up the volume of extract plus residue to 25 ml. or other convenient volume. Centrifuge and discard the residue.[1]

(2) *For meat and meat products.* First remove any obvious fat from the lean, then grind 1–2 g. of the latter (more for inert materials) to a fine pulp in a mortar with one drop of conc. HCl plus a few ml. of 0·1 *M* acetate buffer (*p*H 4), using sand if necessary. Decant off the suspension into a measuring flask, washing the sand remaining in the mortar 2 or 3 times with the buffer solution. Make up the total volume of extract including residue to about 10 ml. (or other convenient volume), check the *p*H (adjusting to *p*H 4 with a drop or two of acid if necessary), and incubate with 0·1 g. of takadiastase plus 0·1 g. papain at 40–45° overnight in presence of a few drops of toluene. After cooling readjust the fluid to a convenient volume (10–25 ml.). Centrifuge the digest, and discard the insoluble residue.[2]

(3) *For yeast and related products.* Take 1 g. or more of the finely ground sample, add about 15 ml. of acidified water and adjust the reaction to *p*H 4 with buffer. Digest with papain and takadiastase as described under paragraph 1 (*a*) but with the addition of 1–2 ml. of toluene to inactivate the yeast; make up to a suitable volume (e.g. 50 ml. for specimens of dried brewer's yeast of average potency, *ca.* 20 I.U. per g., or in proportion) and centrifuge.

In the case of *yeast extracts*, first dilute the extract until the concentration of vitamin B_1 is approximately 0·5–1·0 μg. per ml. Adjust the reaction to *p*H 4 with acetate buffer or HCl and incubate overnight, as in paragraph 1 (*a*) above, with takadiastase and papain in presence of a few drops of toluene; make up to volume and centrifuge.

(4) *For plant tissues*, e.g. potatoes, spinach, cauliflower etc. Grind about 10 g. of the sample with 2–4 drops of conc. HCl to a fine pulp in a mortar. Transfer the suspension to a conical flask, make up the volume with water to about 30–40 ml. and immerse in boiling water for 10 min. Cool, adjust reaction to *p*H 4, then digest with takadiastase and papain, make up to volume and centrifuge, as in paragraph 1 (*a*).

[1] *Calculations of volume.* In determining the total volume of the digest it is best to include the small undigested residue in the measuring flask. This is more accurate than attempting to allow for it, since imbibition of water by the protein residue may greatly increase its apparent bulk, so that any supposed correction for the insoluble residue would in fact be fictitiously high.

[2] See above footnote.

(5) *For milk, milk powder, egg products.* With milk powder, first dilute by mixing about 5 g. with about 30 ml. of water; milk can be used without preliminary dilution, using about 20–30 ml. Adjust the reaction to *p*H 4, and proceed as in paragraph 1 (*a*).

B. *Preliminary washing with* iso*butanol*

With a pipette, transfer 5 ml. of the clear digest to a centrifuge tube. Extract by shaking for 1 min. with an equal volume of *iso*butanol[1] which has been previously saturated with water. Centrifuge, and draw off the upper *iso*butanol layer, using a pipette or glass tube provided with a teat. Discard the *iso*butanol layer. Note the final volume of the aqueous layer.

C. *Oxidation to thiochrome*

Transfer by means of a pipette 1 or 1·5 ml. portions of the aqueous layer into two graduated glass-stoppered cylinders of 25 ml. capacity. If 1 ml. sample is used, add water to make it up to 1·5 ml.: this has the advantage of facilitating the subsequent separation of the layers (1·5 ml. samples can be used with less active products). Add to each cylinder 2 ml. of methanol. Add one drop or more, depending on the amount of reducing substances present, of a 2% solution of potassium ferricyanide to one cylinder only (the 'unknown'). The second cylinder, without the $K_3Fe(CN)_6$, is kept as the 'blank' and, apart from the omission of this reagent, is to be treated in precisely the same manner as the 'unknown'. (It may be worth while, though not absolutely necessary, to run a preliminary trial as described by Wang & Harris [1939] to determine the optimum amount of $K_3Fe(CN)_6$ to add to the 'unknown'.) In a third cylinder (the 'control') place 1 ml. of the diluted standard (see below), make up to 1·5 ml. with water, and add three drops of methanol and one drop only of the ferricyanide solution. Then add 1 ml. of a 30% solution of NaOH to each of the three cylinders. Mix, allow to stand for 1 min. and then add 10 ml. of *iso*butanol[1] to all three to extract the thiochrome produced. Shake the cylinders vigorously for 2 min. and then allow the layers to separate.

D. *Washing with water*

Remove the bottom aqueous layers by means of a long narrow tube provided with a rubber teat. Add 3 ml. of water to each cylinder, now containing the *iso*butanol layers only, and shake for another minute. Allow the layers to separate, then with the tube transfer the three *iso*butanol layers to dry conical flasks and add 2 ml. of 96% ethanol to each, to clarify.

E. *Comparison of fluorescence*

Measure 10 ml. of the clear extract from the 'unknown' into one test tube and 10 ml. from the 'blank' into another. Care must be taken that the test tubes used are made from glass which does not fluoresce in the rays of the mercury-vapour lamp. Match the fluorescence visually without delay. For this purpose place the two test tubes side by side in front of the Wood's glass window of the ultra-violet 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 'control' solution little by little from a graduated pipette to the 'blank' until the fluorescence exactly matches that in the 'unknown'. To check possible uneven illumination of the two tubes, their positions should be reversed frequently

[1] The *iso*butanol is redistilled from the pure reagent. All reagents used, including filter papers, must be tested for freedom from fluorescence [see Wang & Harris, 1939].

ERRATA

Volume 35, Nos. 8 & 9, p. 1058, line 25

for three drops of methanol *read* 2 ml. of methanol

Volume 35, p. 1397, line 13 from bottom

for 80 ft. *read* 8 ft.

during the matching. Add to the 'unknown' an amount of *iso*butanol equal in volume to the amount of the standard added to the blank, 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 closing the tube with the thumb and inverting it twice. It is important that the matching should relate entirely to the bluish fluorescence of the thiochrome in the 'unknown', without respect to any superadded yellow or other colour present in the blank. To obviate this difficulty it is advisable when the tints of fluorescence are different, to use, in addition to the Wood's glass, a Wratten 18 A filter. This filter cuts off the visible light more completely and allows the passage of light waves of 300–400 mμ, which corresponds with the absorption maxima of thiochrome, 368 and 369 mμ. Alternatively, a Wratten 49 A filter may be used in place of the 18 A, when the latter is found inadequate. This filter cuts off any yellow or green colour and allows a better matching. It should, however, be held between the eye and the test tubes and not between the lamp and the test tubes as with the 18 A filter. It is preferable to use filter 18 A rather than 49 A for most purposes.

A little experience is needed to become proficient in making precise matches of fluorescences. The following hints may be found helpful. On entering the dark room it is advisable to allow a few minutes to elapse before attempting a reading, in order to enable the eyes to become adapted.[1] The tubes, which, as already mentioned, are inclined at an angle of 60° to the horizontal, are held side by side with their bottoms resting on a platform covered with mat black cloth to form a suitable dark background for viewing the fluorescence. The observer should look down the tubes and make a *quick decision* which of the two tubes shows the greater fluorescence. It is inadvisable to look for longer than 3 sec. at a time. Reverse the position of the tubes and confirm the finding. Repeat this procedure after each addition of the control solution until a match is obtained. Finally, add a small excess of control and confirm that the fluorescence is then slightly greater.

F. *Differentiation of cocarboxylase and aneurin*

The above method gives the total vitamin B_1 both as free aneurin and cocarboxylase. Most cereals contain little or no cocarboxylase. Meat products and yeasts contain both. To estimate them differentially proceed as follows. First prepare an extract as directed in paragraph A above (amount as specified), omitting the digestion. Transfer the extract plus residue at about *p*H 4, to a conical flask. Immerse in boiling water for 5 min. (to inactivate phosphatases) and cool to room temperature. Add a drop of acid or alkali as necessary to bring the reaction to *p*H 4, adjust to a convenient volume (e.g. 10–25 ml.). Determine the free aneurin on a portion of the centrifuged extract as described above (B to E), but without the preliminary digestion. Digest the remaining portion with takadiastase and papain as before, and estimate the aneurin content again. The difference between the values before and after digestion gives the amount of cocarboxylase.

G. *Preparation of aneurin standard*

The standard solution of vitamin B_1 can be conveniently prepared from 'Benerva' ampoules by diluting a measured amount with water and acidifying with a drop of conc. HCl. Prepare, by such means, a stock solution containing

[1] The mercury vapour lamp should be completely enclosed in a light-proof case, save only for the small horizontal window of Wood's glass between it and the experimental tubes; otherwise the room should be completely dark.

20 μg. of aneurin per ml. Dilute a portion of this to a concentration of 4 μg. per ml. and use 1 ml. of the latter for the 'control' (paragraph C). Keep the stock solution in a refrigerator. The strength of the stock solution should be checked occasionally by comparing with a preparation of aneurin of known purity, preferably a specimen of the international standard.[1] The stock solution can be kept in the refrigerator for a few days, after which it should be replaced by a freshly prepared supply.[2]

H. *Calculation*

Let g = wt. of sample taken, g.
V = total vol. of extract after digestion, ml.
r = ratio of the vol. of extract after preliminary washing with *iso*butanol, to vol. before washing.
v = vol. of final extract used for oxidation.
x = vol. of thiochrome standard required, ml.
y = amount of aneurin taken for oxidation in diluted standard, μg.

Then aneurin content of the sample $= \frac{xy}{10} \times \frac{V}{v} \times \frac{r}{g}$ μg. per g.

Example. A specimen weighing 2 g. was taken for analysis. The total volume of extract after digestion was 25 ml.; the ratio of volumes after and before washing with *iso*butanol was 5·5/5; the volume of the extract used for oxidation was 1 ml.; the volume of thiochrome standard used in the matching was 1·5 ml., and the amount of aneurin standard taken for oxidation was 4 μg. Thus, the amount of aneurin in the sample $= \frac{1{\cdot}5 \times 4}{10} \times \frac{25}{1} \times \frac{5{\cdot}5}{5} \times \frac{1}{2} = 8{\cdot}3$ μg. per g.

Routine method

In routine work when large numbers of samples have to be analysed together, much effort can be saved by having a battery of cylindrical separating funnels which can be fitted on to a special rack and shaken simultaneously, so that operations C and D can be done very expeditiously.[3]

Under ordinary working conditions about five separate digests can be conveniently examined in 1½ hr. by one worker, or 20 in a day, while with the 'battery' equipment just mentioned, the number can be still further increased and the labour reduced.

Modified procedure for some special products

The procedure described above should suffice for all the more important carriers of vitamin B_1, including cereals, yeast and yeast derivatives, meat products etc. A fuller study of fruits and vegetables is still in progress, but no difficulty has been experienced with any of those so far examined, and the thiochrome values found for them agree well with those determined biologically (see below). When working with new types of products, probably the only two problems likely to arise are those relating to the preliminary digestion and to

[1] It is worth noting that the international standard preparation, at first described as the chloride-hydrochloride-monohydrate, is in reality nearly anhydrous. By definition, the activity of this preparation is 333,333 I.U. per g. Hence the activity of the chemically pure chloride-hydrochloride-monohydrate, as available commercially and corresponding with the specification of the 'Third Addendum to the British Pharmacopoeia, 1932 (1940)' is 320,000 I.U. per g., i.e. a difference of about 4%, or almost within the experimental error of the method at its best.

[2] Comparisons with preformed thiochrome are not recommended, it being obviously more accurate to treat the vitamin B_1 standard of reference in the same way as the unknown.

[3] Stopcocks should be sealed by moistening with a drop of water (*not* grease).

interference from pigments or non-specific fluorescing substances. In case of any doubt it is useful to add a known amount of the vitamin to the unknown and test the proportion recovered. Only rarely, when there is obstinate interference from pigments or non-specific fluorescence, should it be necessary to have recourse to preliminary adsorption of the vitamin to free it from these other substances. This seems to be best avoided when possible because of the danger of incomplete adsorption or elution. But, in actual practice, almost the only difficulty in matching which we have encountered when using the above methods has been with deeply pigmented autoclaved products, e.g. alkali-autoclaved marmite. Inadequate extraction or digestion is perhaps a more serious source of error. It should be specially guarded against with desiccated products which seem liable to prove exceptionally resistant to liberation of the vitamin (see further below).

Biological comparisons

Probably the most searching test of the accuracy of a chemical method is to check the results against those obtained by biological assays. We have carried out systematic comparisons of this kind on most classes of foodstuffs, including yeast and yeast extracts, cereals (whole grains, flours, breads, biscuits, etc.), fruits and vegetables, dairy products, fish and meat.

The majority of the biological tests were done by the bradycardia method [Drury & Harris, 1930; Birch & Harris, 1934; Leong & Harris, 1937; Baker & Wright, 1938], the advantages of which have been discussed by several recent investigators [e.g. Todd & Bergel, 1937; van Veen, 1937; Karrer & Kubli, 1937; Pedersen, 1938; Lunde, 1938; Lunde *et al.* 1938; 1939; Leong, 1939, 1, 2; Pannekoek-Westenburg & van Veen, 1939; Yang & Platt, 1939]. Frequently, as a further check, other biological methods were used in addition, viz. cure of convulsions in rats, or growth rates of rats [methods as in Birch & Harris, 1934; Harris, 1941].

In all the biological tests the procedure was to feed at least two and generally three levels of the 'unknown' and three of the standard (crystalline aneurin chloride hydrochloride), with generally about six animals at each level. A dose-response curve for the standard was constructed for each separate experiment, and the amounts of vitamin B_1 present in the various doses of the 'unknown' were read off the curve from the corresponding average responses. The accuracy of the bradycardia method under such conditions is such that the standard error of the mean is about ± 5–10% [Leong & Harris, 1937; Lunde *et al.* 1938; 1939; Pannekoek-Westenburg & van Veen, 1939; Harris, 1941]. A similar order of accuracy attaches to the two other methods used [Harris, 1941].

Summaries of the experimental data are to be found in Table 9. Values entered on the same line indicate that the comparison has been made on the identical specimen. It will be seen that, for the sake of further cross-reference, a few additional values are given, either where different biological methods have been compared on the same specimen without direct chemical check or where the chemical or biological results are useful for indicating the range of variations to be expected for a particular product or class of foodstuffs.

The products examined cover the widest possible range of activities for known foodstuffs, from the very high figure of 190–200 I.U. per g. for a dried yeast of special potency down to such negligible values as 0·2 I.U. per g. or less for such products as white bread, herring, egg white. The parallelism between the chemical and biological values seems highly satisfactory, and no serious discrepancy is apparent for any single class of the foodstuffs so far examined.

Table 9. *Comparison of chemical and biological results. Entries arranged by groups, roughly in descending order of activities*

Ref. no.*	Material		Vitamin B_1, I.U. per g. Thiochrome method	Bradycardia method	Growth method 14 days	Growth method 21 days	Cure of convulsions
	YEASTS AND YEAST PRODUCTS:						
1	Yeast, high potency, dried		200	191	—	—	—
2	„ high potency (74% H_2O)		57	66	—	—	—
3	„ high potency		67	—	—	—	—
4	„ *torula utilis*		7·5–9·0	—	—	—	—
5	„ brewers' (Guinness)		12	—	—	—	—
6	„ brewers' (dried, for test)		—	18·8	—	20	19·3
7	Yeast extract, 'M', stored		4·8	5·6	—	—	—
8	„ 'M', retail		4·6	7–8	7	6·3	5·1
9	„ 'M', retail		7·0	8·9	—	—	—
11	„ 'M', special		—	8·3	—	9·1	8·7
12	„ 'Y', retail		8·0	—	9·7	9·1	6·6
13	„ 'Y', wholesale		13·4	13·4	—	—	—
	PORK AND BACON:						
14	Pork, raw, *dried powder*		7·67	9·0	—	—	—
15	„ raw, *dried powder*		8·07	7·6	—	—	—
16	„ roast, *dried powder*		5·13	4·7	—	—	—
17	Bacon		4·0	3·7	—	—	—
	CEREALS:						
	WHEAT AND WHEAT PRODUCTS:						
18	Wheat germ, proprietary		9·5	8·4–9·6	—	—	—
19	„ special		—	7·0	—	7·2	6·9
20	„ retail		—	6·0	—	—	—
21	Middlings		—	4·5	—	—	—
22	Bran		—	3·6	—	—	—
23	Whole meals (different varieties and countries	specimen 1	1·7	1·73	—	—	—
24	„ of origin)	specimen 2	1·3	1·27	—	—	—
25	„	specimen 3	1·3	1·20	—	—	—
26	„	specimen 4	1·1	1·48	—	—	—
27	„	specimen 5	1·3	1·35	—	—	—
28	„	specimen 6	1·1	1·23	—	—	—
29	„	specimen 7	1·0	1·0–1·1	—	—	—
30	„	specimen 8	1·2	1·2–1·4	—	—	—
31	„	specimen 9	1·0	1·0–1·3	—	—	—
32	Whole meals (same variety, different soil	specimen *a*	0·97	—	—	—	—
33	„ conditions)	specimen *b*	1·3	—	—	—	—
34	„	specimen *c*	0·87	—	—	—	—
35	„	specimen *d*	1·2	—	—	—	—
36	„	specimen *e*	1·0	—	—	—	—
37	Flour, wholemeal, commercial 1		1·2	—	—	—	—
38	„ wholemeal, commercial 2		—	1·5	—	—	—
39	„ 'Hovis'		—	1·2, 1·3	—	—	—
40	„ white		—	0·2	—	—	—
41	„ straight run, bleached		0·29	—	—	—	—
42	„ straight run, unbleached		0·26	—	—	—	—
43	Bread, white, retail, various		0·22–0·24	—	—	—	—
44	„ white, fortified, formula 1		0·44	0·48	—	—	—
45	„ white, fortified, formula 2		0·4	0·47	—	—	—
45*a*	„ white, fortified, dried crumb, formula 1		0·74	0·77	—	—	—
45*b*	„ white, fortified, dried crumb, formula 2		0·60	0·61	—	—	—
46	„ yeast-enriched (Proprietary)		0·7	—	—	—	—
47	„ yeast-enriched (1·65 g./100 g.)		1·42	1·45	—	—	—
48	„ yeast-enriched (1·20 g./100 g.)		1·49	1·47	—	—	—
49	„ yeast-enriched (0·53 g./100 g.)		0·56	0·61	—	—	—
50	„ wheatmeal, dried crumb		1·08	1·1	—	—	—
51	„ wheatmeal, dried crumb, special formula		0·98	1·1	—	—	—
52	„ whole wheat, *dried powder*		0·80, 0·87	1·31	—	—	—
53	Wheatmeal biscuits, raw materials (1)		0·67	0·78	—	—	—
54	„ raw materials (2)		0·80	0·74	—	—	—
55	„ baked with $(NH_4)_2CO_3$, experimental (1)		0·67	0·71	—	—	—
56	„ baked with $(NH_4)_2CO_3$, experimental (2)		0·60	0·56	—	—	—
57	„ baked with $(NH_4)_2CO_3$, commercial (1)		0·70, 0·75	0·77	—	—	—
58	„ baked with $(NH_4)_2CO_3$, commercial (2)		0·60, 0·62	0·92	—	—	—
59	„ baked with $(NH_4)_2CO_3$, commercial (3)		0·99	1·0	—	—	—
60	„ baked with $NaHCO_3$ and $(NH_4)_2CO_3$ experimental (1)		0·25	0·17	—	—	—

Table 9 (*cont.*)

Ref. no.*	Material	Vitamin B_1, I.U. per g. Thiochrome method	Bradycardia method	Growth method 14 days	Growth method 21 days	Cure of convulsions
	WHEAT AND WHEAT PRODUCTS (*cont.*):					
61	Wheatmeal biscuits, baked with $NaHCO_3$ and $(NH_4)_2CO_3$ experimental (2)	0·30	0·29	—	—	—
62	„ baked with $NaHCO_3$ commercial	0·3	≷0·3	0·34	0·37	—
62*a*	„ baked with yeast	1·4	—	—	—	—
	OATS:					
63	Whole grain	1·8	—	—	—	—
63*a*	Prepared oats, various specimens	1·7–1·8	—	—	—	—
	BARLEY:					
64	Meal, varying soil conditions	0·43–0·67	—	—	—	—
65	Pearl, retail (1)	0·44	0·62	—	—	—
66	„ retail (2)	0·44	0·41	—	—	—
67	„ retail, various specimens	0·41–0·48	—	—	—	—
	FRUITS AND VEGETABLES:					
68	Spinach, dried powder	1·53	1·69	—	—	—
69	Orange juice, partly evaporated	0·66	0·64	—	—	—
70	„ dried	5·4	—	—	—	—
72	„ fresh	0·17	—	—	—	—
73	Potato, dried powder	0·57	1·03	—	—	—
74	„ Majestic	0·3	0·50	—	—	—
75	„ Majestic	0·32	0·39	—	—	—
77	„ King Edward	0·28	—	—	—	—
79	Grass	0·52	—	—	—	—
80	Brussels sprouts	0·28	—	—	—	—
81	Cauliflower	0·26	0·34	—	—	—
	DAIRY PRODUCTS:					
82	Eggs (hen), yolk, raw, dried	2·35	2·73	—	—	—
83	„ yolk, raw, fresh	0·50	—	—	—	—
84	„ yolk, raw, fresh	0·50	—	—	—	—
85	„ yolk, raw, fresh	0·54	—	—	—	—
86	„ whole, raw	0·30	—	—	—	—
87	„ whole, vacuum ice-dried, reconst.	0·30	—	—	—	—
88	„ white, cooked	0·05	<0·1	—	—	—
89	Milk (cow's) powder, skimmed	0·85	0·89	—	—	—
90	„ fresh	0·15	—	—	—	—
91	„ fresh	0·12	—	—	—	—
92	„ fresh	0·11	—	—	—	—
93	„ fresh	0·10	—	—	—	—
94	„ colostrum	0·15	—	—	—	—
95	„ colostrum	0·09	—	—	—	—
	LIVER:					
96	Beef, roasted, dried powder	0·7	1·27	—	—	—
97	„ raw, dried powder	0·93–1·1	1·67–1·74	—	—	—
98	Sheep, raw, fresh (4 samples)	0·65–1·4	—	—	—	—
99	Calf, raw, fresh (6 samples)	0·38–0·75	—	—	—	—
	See also: Pork and bacon.					
	FISH PRODUCTS:					
100	Fish 'drip', concentrated	0·30	0·41	—	—	—
101	Herring, muscle, fresh, raw	0·03	0·04	—	—	—
	CANNED FOODS:					
102	Bacon, canned	—	1·13	—	—	—
103	Ham, sliced, after storage	—	0·48	—	—	—
104	Meat and vegetable rations	—	0·22	—	—	—
105	„ „	—	0·15	—	—	—
106	„ „	—	<0·10	—	—	—
107	„ „	—	<0·10	—	—	—
108	Meat, preserved	—	0	—	—	—
109	Potatoes, canned	—	0·12	—	—	—
110	Beetroot, canned	—	0·12	—	—	—
111	Carrots, canned	—	0·17	—	—	—

* Reference numbers in column 1 relate to corresponding entries in Fig. 1.

Table 10. *Agreement between chemical and biological values. Statistical analysis*

				No. of cases	% of total no.
Total no. of specimens compared				55	100
Chemical and biological results differing by			0–10%	32	58
,,	,,	,,	10–20 ,,	10	18
,,	,,	,,	20–30 ,,	6 (*a*)	11
,,	,,	,,	>30 ,,	7 (*b*)	13
,,	,,	,,	0–10 ,,	32	58
,,	,,	,,	0–15 ,,	41	75
,,	,,	,,	0–30 ,,	48 (*a*)	87
,,	,,	,,	>30 ,,	7 (*b*)	13

(*a*) Of the 6 cases where there was a difference of 20–30% between the chemical and biological values, all but 2 were low in vitamin B_1 (<1 I.U. per g.).

(*b*) Of the 7 cases with a difference greater than 30%, all were low in vitamin B_1 (<1 I.U. per g.), or were desiccated products for which the method of extraction employed was probably inadequate.

Excluding desiccated products and foods with negligible vitamin B_1 ($\leqslant$0·3 I.U. per g.) the statistical analysis is as follows:

				No. of cases	% of total no.
Total no. of specimens compared				39	100
Chemical and biological results differing by			0–10%	27	69
,,	,,	,,	10–20 ,,	8	21
,,	,,	,,	20–30 ,,	3	7·7
,,	,,	,,	>30 ,,	1	2·5
,,	,,	,,	0–10 ,,	27	69
,,	,,	,,	0–15 ,,	34	87
,,	,,	,,	0–30 ,,	38	97·5
,,	,,	,,	>30 ,,	1	2·5

In 75% of cases the chemical and biological tests agreed within an error of 15%, or in 87% of cases within 30%. Fuller statistical data are given in Table 10.

It seems significant that in virtually all instances where the difference between the chemical and biological results was at all serious (>15%) the food was either relatively poor in the vitamin—so that an appreciable error was only to be expected—or else was a desiccated product for which the usual method of extraction was inadequate. Again, some experimental fluctuation is inevitable in all biological methods; and, in fact, in several instances a repetition of the tests, using if necessary a larger number of animals, has given more complete agreement. We have to recognize, however, that where there has been any divergence between the chemical and biological results it has almost invariably been in the direction of the former being lower, and the latter higher.[1] The principal reason for this, we believe, is that the known sources of error in the chemical method—particularly incomplete extraction and digestion—tend to cause low figures if not adequately controlled. On several occasions we have traced unsatisfactory recoveries to the use of faulty technique for the extraction or digestion. Another partial explanation is that in the biological method, fat and protein or other ingredient may have a vitamin B_1-sparing action, so that the antineuritic potency of a food is not necessarily strictly identical with its vitamin B_1 content. Further work is needed on this latter point. It may for example prove to be advisable, when testing biologically foodstuffs poor in the vitamin but rich in fat or protein, to prepare an extract for dosing. However, these minor irregularities do not affect the validity of our main conclusion.

[1] The difference between the chemical and biological values was, as an average, −6%, or as a median −4 to −5% (excluding desiccated products and foods containing negligible vitamin B_1, $\leqslant$0·3 I.U. per g.); or average −9%, and median −8 (if all determinations are included).

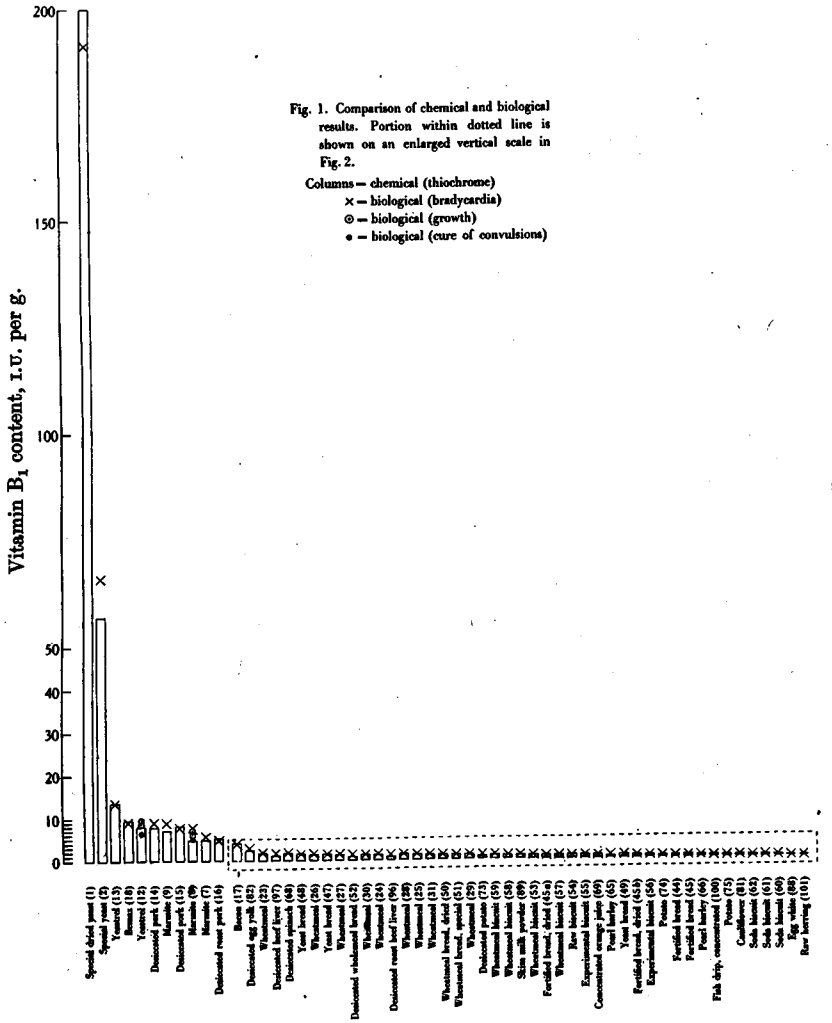

Fig. 1. Comparison of chemical and biological results. Portion within dotted line is shown on an enlarged vertical scale in Fig. 2.

Columns — chemical (thiochrome)
× — biological (bradycardia)
⊙ — biological (growth)
• — biological (cure of convulsions)

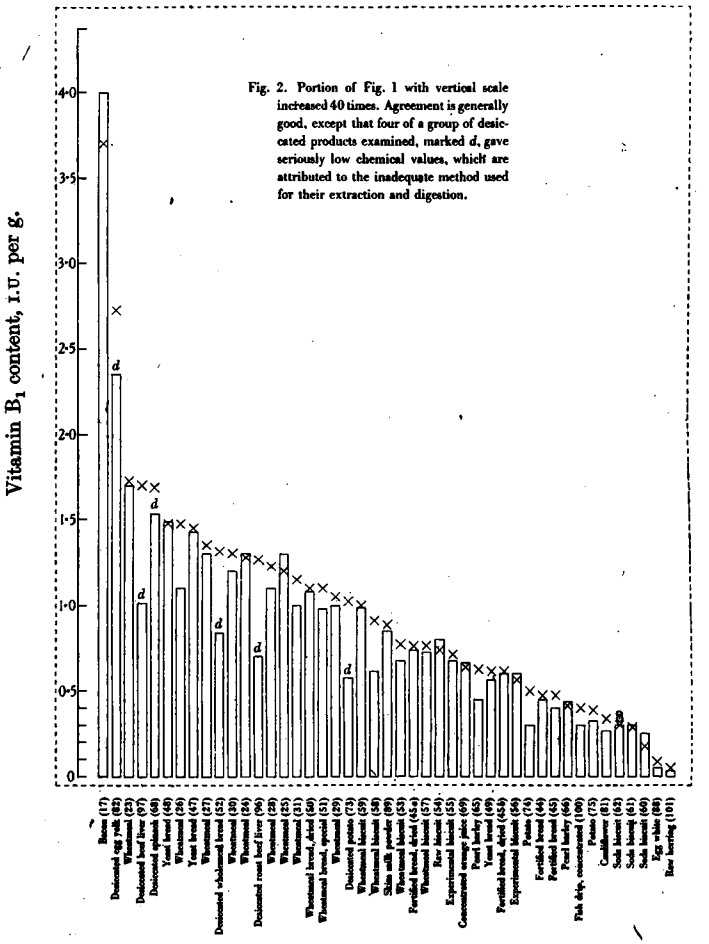

Fig. 2. Portion of Fig. 1 with vertical scale increased 40 times. Agreement is generally good, except that four of a group of dessicated products examined, marked *d*, gave seriously low chemical values, which are attributed to the inadequate method used for their extraction and digestion.

The degree of parallelism between chemical and biological findings is perhaps best shown graphically (Figs. 1 and 2). The conclusion seems justified that the improved chemical method, obviating as it does the various sources of error mentioned in the early part of the paper, does give a reliable measure of the vitamin B_1 present in foodstuffs and other animal or vegetable tissues.

Summary

1. A comprehensive study has been made of the numerous sources of error encountered when the thiochrome reaction is applied to the quantitative estimation of vitamin B_1 in foodstuffs and animal and vegetable tissues.

2. An accurate procedure, based on the method of Wang & Harris [1939] for urine, has been worked out. Special features include (*a*) a preliminary process of extraction (varying according to the nature of the product), (*b*) digestion with papain and takadiastase (for conversion of cocarboxylase into aneurin, breaking down proteins, polysaccharides, etc. and removing inhibitors), (*c*) washing of the digest with *iso*butanol (to remove interfering substances), (*d*) omission of adsorption (which in our experience caused variable losses), (*e*) conversion into thiochrome in presence of methanol, and with addition of the $K_4Fe(CN)_6$ before the NaOH (in order to protect against loss by oxidation), (*f*) washing of the thiochrome layer with water (to permit accurate differentiation between cocarboxylase and free vitamin B_1, and to eliminate non-specific fluorescent substances and pigments), (*g*) visual comparison of fluorescence with the aid of light-filters and blank controls (to detect differences of tint).

3. To test the reliability of the method, systematic comparisons with biological tests have been carried out on over 50 foodstuffs, including wheat-meals and flours, other cereals, breads and biscuits, yeast preparations, meat and animal tissues, dairy products, and a few vegetables, fruits and fish. The agreement has been good, the products examined covering the wide range of activities of 200–0·03 I.U. per g. In 75% of instances the biological and chemical values differed by less than 15%, or in 87% of instances by less than 30%; the larger percentage errors were found only with foods poor in the vitamin, or with a group of desiccated products which were difficult to extract. Where there were detectable differences the trend was for the chemical values to be lower than the biological rather than *vice versa* (average difference $= -6\%$).

4. With 0·1 μg. of aneurin per ml. chemical results can be duplicated with an error of about $\pm 5\%$ and with 0·03 μg. per ml. with an error of $\pm 10\%$. The procedure is easy to work, and upwards of 20 specimens can be examined in a working day.

5. Erroneously low values given by certain other modifications of the thiochrome test are explained.

REFERENCES

Baker & Wright (1938). *Biochem. J.* **32**, 2156.
Birch & Harris (1934). *Biochem. J.* **28**, 602.
Drury & Harris (1930). *Chem. Ind.* **49**, 851.
Harris (1941). *Bull. Hlth Org. L.o.N.*
—— & Wang (1941). *Biochem. J.* **35**, 1068.
Hennessy & Cerecedo (1939). *J. Amer. chem. Soc.* **61**, 179.
Houston, Kon & Thompson (1940). *J. Dairy Res.* **11**, 183.
Jansen (1936). *Rec. trav. chim. Pays-Bas*, **55**, 1046.
Jowett (1940). *Biochem. J.* **34**, 1348.

Karrer & Kubli (1937). *Helv. chim. Acta*, **20**, 369.
Kinnersley & Peters (1934). *Biochem. J.* **28**, 667.
—— —— (1938). *Biochem. J.* **32**, 1516.
Leong (1939, 1). *Biochem. J.* **33**, 1394.
—— (1939, 2). *Biochem. J.* **33**, 1397.
—— & Harris (1937). *Biochem. J.* **31**, 672.
Lunde (1938). *Nord. med. Tidsskr.* **15**, 444.
—— Kringstad & Olsen (1938). *Norske Videnskaps-Akad. Oslo*, **1**, Mat. Naturv. Klasse, No. 7.
—— —— —— (1939). *Nord. med.* **3**, 2533.
Melnick & Field (1939). *J. biol. Chem.* **127**, 505, 515, 531.
Pannekoek-Westenburg & van Veen (1939). *Geneesk. Tijdschr. Ned.-Ind.* **79**, 2891.
Pedersen (1938). *Dansk Tidsskr. Farm.* **12**, 137.
Prebluda & McCollum (1939). *J. biol. Chem.* **127**, 495.
Pyke (1939). *J. Soc. chem. Ind., Lond.*, **58**, 338.
Ritsert (1938). *Dtsch. med. Wschr.* **64**, 481.
Todd & Bergel (1937). *J. chem. Soc.* p. 364.
van Veen (1937). *Meded. Dienst Volksgezondh. Ned.-Ind.* **26**, 300.
Wang (1941). Ph.D. Thesis. University of Cambridge.
—— & Harris (1939). *Biochem. J.* **33**, 1356.
Westenbrink & Goudsmit (1938). *Enzymologia*, **5**, 307.
—— & Jansen (1938). *Acta brev. neerl. Physiol.* **8**, 119.
Wiegland (1938). *Arch. neerl. Physiol.* **23**, 281, 312.
Yang & Platt (1939). *Chin. J. Physiol.* **14**, 259.