THE ESTIMATION OF VITAMIN B_1 IN PHARMACEUTICAL PRODUCTS

BY J. J. DOHERTY, NORA CANE and F. WOKES

From the Ovaltine Research Laboratories, King's Langley, Herts.

Received July 28, 1955

BIOLOGICAL assays[1] of vitamin B_1 were described in the first addendum, and fluorimetric assays in the seventh addendum, to B.P. 1932. The fluorimetric method has been shown to give satisfactory agreement with biological and microbiological methods when applied to unmalted and malted cereals, malt extract, malt and oil, yeast and meat extracts[2,3,4,5]. Nevertheless, in the B.P. 1953, the fluorimetric method was replaced by a chemical precipitation method[6] claimed to be more accurate. This precipitation method was shown to give satisfactory agreement with the fluorimetric method on 4 samples in which different degrees of destruction of the vitamin had been produced by heating. No comparison was made with biological, microbiological or spectroscopic methods. The precipitation method requires much larger quantities of material than the fluorimetric method, and further investigation of the latter seemed desirable before eliminating it from pharmaceutical analysis, especially as it has proved so successful in food analysis. Our fluorimetric results on B.P. injections of vitamin B_1 have been checked by a spectrophotometric method which has enabled us to detect changes in the vitamin molecule which are not directly shown by the fluorimetric method.

METHODS

The fluorimetric method[5] employed was that recommended by the Vitamin Sub-committee of the Society of Public Analysts (now the Society for Analytical Chemistry,) but more correct evaluation of results was secured by using a modified fluorimeter[7] and adopting precautions suggested in a recent treatise[8]. These precautions included more exact measurement of blanks, which the highly sensitive fluorimeter made possible, and use of cuvettes calibrated by means of cross-over experiments. They enabled us to improve the accuracy of our fluorimetric measurements, the coefficient of variation being reduced below 1·0.

The gravimetric (chemical precipitation) method was that of Bessot[9] as used by Adamson and Handisyde[6]. Spectrophotometric estimations were made using the Beckman DU Photoelectric Spectrophotometer, allowance being made for irrelevant absorption as described below. A number of microbiological assays, using *Ochromonas malhamensis*, were kindly carried out for us by Dr. J. E. Ford of the National Institute for Research in Dairying.

EXPERIMENTAL

Spectroscopy of vitamin B_1. Spectroscopic methods offer promise for the assay of vitamin B_1 injections in which little irrelevant absorption is

caused by the other substances present. Our tests on a number of commercial samples showed them to be free from such substances, although the possibility remained of other samples containing interfering substances. Allowance for these might be made by the Morton-Stubbs method, provided that the specific absorption band is not too much distorted. However, difficulties arose because of the susceptibility of the absorption spectrum to slight changes in *p*H, which may explain why no spectrophotometric method of assaying vitamin B_1 has received official recognition, in spite of its apparent advantages of simplicity and accuracy. A detailed study of *p*H effects was therefore undertaken.

Effects of p*H.* Since attention was first drawn[10] to this effect, various workers[10,11,12,13,14] have explored the changes in the absorption spectrum of vitamin B_1 when the *p*H of the solution is raised from 1 to 7. The single peak at about 245 mμ seen in solutions at *p*H 1 to 3 gradually changes to 2 separate peaks at approximately 236 and 265 mμ with considerably lower extinctions. The greatest drop in extinction occurs at approximately 245 mμ. On bringing the *p*H back to about 1, the 2 peaks revert to the single one, but the reversibility may not be quite complete, since a slight flattening of the peak may occur[13].

An attempt was made[15] in 1946 to allow for the *p*H effect by measuring the extinction at 5 different wavelengths—250, 255, 260, 265, 270 mμ—and statistical examination of the results indicated some improvement in accuracy. However, the method, which had been developed using photographic techniques, does not seem to have been adapted to the more rapid and accurate photoelectric techniques which have since come into general use.

Isosbestic point(s). Our investigations show that as the *p*H of a vitamin B_1 solution is changed from 1 to 7, significant alterations in extinction occur at all the above 5 wavelengths. The most marked are at about 245 mμ. There is, however, no change in extinction at approximately 273 mμ over the whole *p*H range 1 to 9, and this establishes the presence of an isosbestic point (see Figs. 1 and 2) at approximately 273 mμ. Indications of the occurrence of such an isosbestic point have been provided by data published by several workers[13,14] but none of these seems to have mentioned its existence or attempted to use it in spectrophotometric assays.

We have used this isosbestic point to detect any changes in irrelevant absorption which may occur when the *p*H of the solution is altered from 1 to 7. As will be seen from Figures 1 and 2, this alteration produces a marked change in *E* 245 mμ but no change in *E* 273 mμ for the pure vitamin. Any change in *E* 273 mμ which does occur in our extracts when the *p*H is raised from 1 to 7 must be due to irrelevant absorption, for which due allowance can be made, so that the corrected change in *E* 245 mμ becomes directly proportional to the concentration of the vitamin.

Spectrophotometric method. Our spectrophotometric assays of vitamin B_1 injections are made as follows. A stock solution containing about 0·25 mg./ml. of vitamin B_1 is prepared by diluting with 0·1N hydrochloric acid a known volume (about 1 ml.) from an ampoule to a known volume

(e.g., 100 ml. for 1 ml. of a 25 mg./ml. injection.) This stock solution is stored in a cool dark place and used on the day it is prepared. An aliquot of 2 ml. of this stock solution is diluted to 50 ml. with 0·1N hydrochloric acid, giving a dilution containing about 10 μg/ml., which in a 1 cm. cell is suitable for measurement of extinction in a photoelectric spectrophotometer.

Another aliquot of 2 ml. is diluted to 50 ml. with the B.P. phosphate buffer *p*H 7, and similarly examined. The extinctions needed for the assay are only those at 245 and 273 mμ, but it is helpful to take readings also at every 5 mμ between 240 and 290 mμ to elucidate the general shape of the absorption curves.

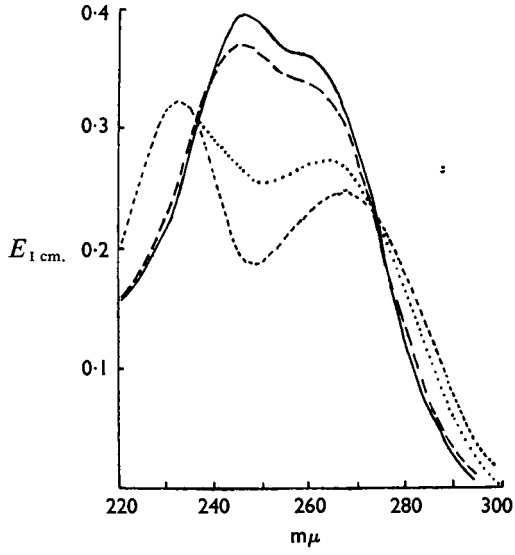

FIG. 1. Absorption spectra of vitamin B_1 solutions (10 μg./ml.), at — *p*H 1, — — *p*H 3·9, · · · · *p*H 5·4, — — — *p*H 6·9.

To calculate the vitamin B_1 content, it is first necessary to correct the observed change in *E* 245 mμ for any alteration in that extinction caused by the *p*H change on the irrelevant absorption, as indicated by a change in *E* 273 mμ. In our experience with B.P. injections of vitamin B_1, if there is any such correction needed, it is very slight. The corrected change in *E* 245 mμ is then directly proportional to the vitamin B_1 content, an increase of 0·1 in *E* 245 mμ produced by changing the *p*H from 7 to 1, being equivalent to approximately 5 μg. anhydrous vitamin B_1 per ml.

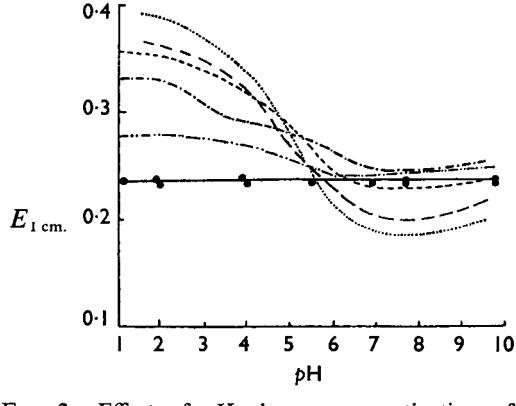

FIG. 2. Effect of *p*H changes on extinction of vitamin B_1 solutions (10 μg./ml.) at

· · · · ·	250 mμ	— · —	265 mμ
— —	255 mμ	— · · —	270 mμ
— — — —	260 mμ	———	273 mμ

Our spectroscopic data also indicate that under certain conditions, there may be another isosbestic point at approximately 236 mμ. Such an additional point could be of

great value in supplementing the check on irrelevant absorption provided by the other isosbestic point at approximately 273 mμ. However, we have not yet established this second isosbestic point with certainty. Some variations which we have observed in it are probably due to factors connected with *p*H changes which affect the molecular structure and may be responsible for slight distortions of the peaks at approximately 236 mμ and 265 mμ in neutral solution and at approximately 245 mμ in strongly acid solution.

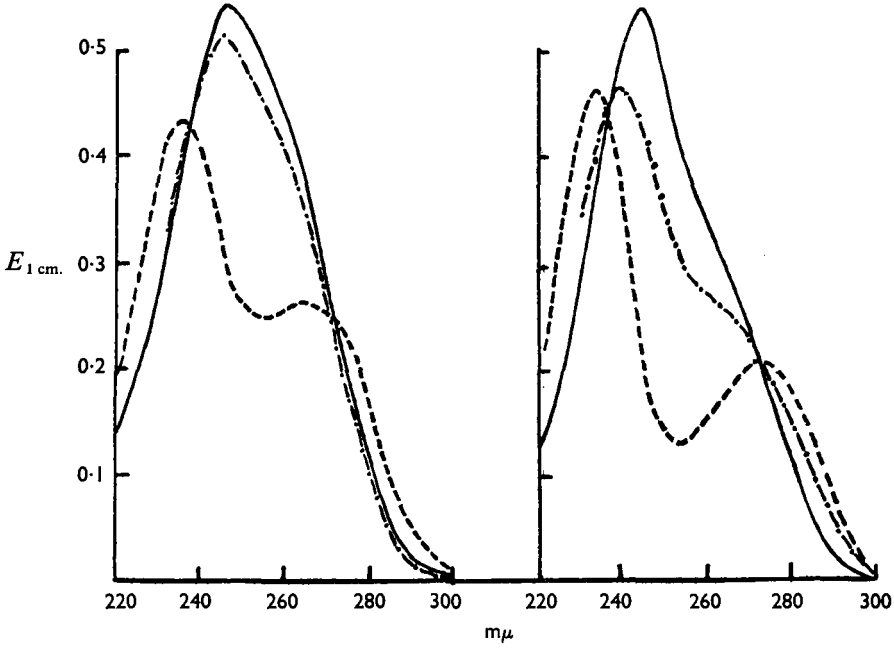

FIG. 3. Absorption spectra of pyrimidine derivatives, 2-methyl-4-amino-5-hydroxymethylpyrimidine (A) and 2-methyl-4-amino-5-aminomethylpyrimidine dihydrochloride monohydrate (B), at —— *p*H 1, — · — *p*H 5, – – – – *p*H 7. Aqueous solutions of 6·7 and 10·3 μg./ml. respectively.

If this spectrophotometric method is applied to materials in which the vitamin B_1 has been broken down into its pyrimidine and thiazole components, complications may arise because of the fact that the spectra of the mixed components closely resemble[13] those of the unaltered vitamin. Moreover, pyrimidine derivatives show with alterations in *p*H a series of changes similar to those given by vitamin B_1 (see Fig. 3.) However, the spectra of thiazole derivatives, whilst changing with *p*H, do not show an isosbestic point. (See Fig. 4.) We therefore do not consider our spectrophotometric method suitable for the assay of vitamin B_1 in preparations in which there has been extensive destruction of the vitamin, e.g., by excessive heating. However, in our experience, the method gives reliable results on injections subjected to any reasonable sterilisation or storage conditions.

Sulphite splitting of vitamin B_1. Whilst our work was in progress, a paper was published by Somogyi[13], describing a spectrophotometric method of assaying vitamin B_1 by measuring the increase in E 250 mμ produced by heating to 100° C. with sodium pyrosulphite. This method gave good agreement with the fluorimetric method when applied to simple aqueous solutions of vitamin B_1, but was not found to be completely reliable for more complex materials, possibly because the sulphite treatment had altered the irrelevant absorption at 250 mμ. It is of interest to note that Somogyi's published data showed isosbestic points at approximately 235 mμ and 265 mμ which might perhaps be used to correct for this action on the irrelevant absorption and thus improve Somogyi's method.

Using our spectrophotometric method, we have assayed vitamin B_1 in a number of B.P. injections. Our replicate assays show an average coefficient of variation of 0·51 ± 0·3 which compares favourably with that previously obtained with the M.R.C. spectroscopic method[15] when applied to solutions of pure vitamin B_1. We therefore used this spectrophotometric method, in comparison with fluorimetric and precipitation methods, in a series of experiments on the effect of heat and light on vitamin B_1.

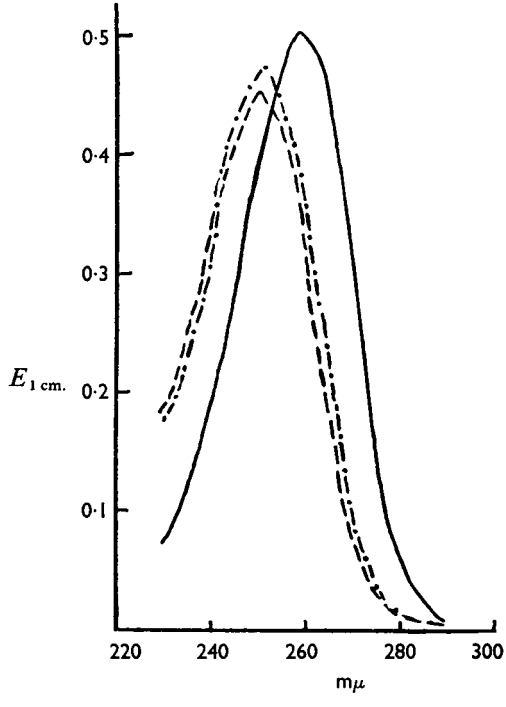

FIG. 4. Absorption spectra of 4-methyl-5-hydroxyethylthiazole at —— *p*H 1, —·— *p*H 5, — — *p*H 7. Aqueous solutions of 16·2 μg./ml.

Effect of heat on vitamin B_1. Experiments were carried out at 3 different temperatures—70° C. (in a hot air oven,) 100° C. (in a boiling water bath) and 112 to 115° C. (in an autoclave.) The vitamin B_1 solutions ranged from 1 to 30 mg./ml., but were all at the same approximate *p*H (3·7) and were contained in sealed ampoules such as are used for injections. The results are summarised in Figure 5, in which a logarithmic time scale is used to enable long term storage results also to be included, and the values are all shown as percentages of the respective initial strengths, which are indicated in the legend. As the mean fluorimetric result was 101 ± 1·9 per cent, and the mean spectrophotometric result was 105 ± 2·1 per cent. of the mean gravimetric method, we considered it justifiable to include in the figure results obtained by all 3 methods.

The rate of loss at 100° C. was slower than was observed by Adamson and Handisyde, perhaps because of a difference in *p*H or other experimental conditions. The *p*H of their solutions was not stated. However, our conditions, though they may have differed from those of these workers, were still such as might be encountered in general practice, and hence we were glad to confirm their finding that the fluorimetric results agreed with the gravimetric results.

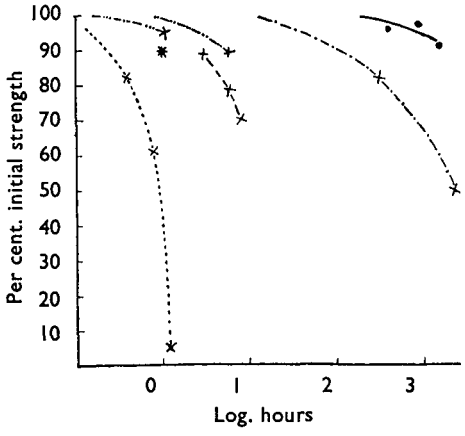

FIG. 5. Effect of heat and light on vitamin B_1. Results on injection solutions at room temperature in sunlight indicated thus ●——●.
Results on injection solutions at—
70° C. in dark indicated thus ×—·—×.
100° C. " " " " ×—··—×.
112 to 115° C. in dark indicated thus ×—···—×.
100° C. (Adamson and Handisyde) indicated thus ×——×.
100° C. (Beadle *et al.*) indicated thus *.
Results on baked vitamin B_1 indicated thus ×------×.
Ordinates indicate vitamin B_1 content as per cent. of initial content. Abscissæ indicate duration of treatment in hours on log. scale.

Comparison of the rate of loss at 70° C. with that at 100° C. indicated a very low temperature coefficient. However, the solution stored at 70° C. developed a yellow colour, which first became visible within 2 or 3 days storage, and steadily increased in intensity, being so marked at the end of a week that the ampoules would certainly not have been accepted for clinical use. As a loss of only 9 per cent. was indicated by the physico-chemical methods, we had the injections assayed microbiologically which showed no larger losses. Thus, although the yellow colour suggested serious deterioration, this was not confirmed by any of the 4 assay methods employed.

Effect of light on vitamin B_1. Although the experiments at 70° C. had been carried out in a closed hot air oven, there was a possibility that during the prolonged storage light might have penetrated the solutions and caused development of yellow colour. We therefore carried out a number of experiments in which the ampoules were fastened by transparent tape to the upper part of a window facing south west and hence exposed to sunlight for much of the day. The results, which are also summarised in Figure 5, indicated a lower rate of loss than in the solutions stored at 70° C., but the difference was much less than would have been expected from the temperature coefficient between 70° C. and 100° C. This suggested some destructive action by light. Again there was marked development of yellow colour and also pungent odour, signs of definite deterioration.

Effect of baking on vitamin B_1. In the above experiments the losses of

vitamin B_1 were not very great, and in some instances were not much larger than the experimental error. In order to test the different assay methods more critically, we decided to subject the vitamin to the drastic conditions of baking, such as are applied to dietetic products in which it may be present. A diabetic bread, for example, may be heated to 180° C. to 190° C. for 60 minutes. A number of experiments were carried out in which a known quantity of the vitamin, dissolved in 0·1N hydrochloric acid, was thoroughly mixed with "filter aid,"* and the mixture placed in an evaporating basin covered with a clock glass and passed through an automatic gas heated oven for 25 minutes at approximately 230° C. The baked material, after cooling, was extracted with 0·1N hydrochloric acid, filtered through No. 3 sintered glass and then through No. 3 Whatman, and the vitamin in the filtrate was assayed by the 3 methods.

Control experiments on unbaked mixtures showed that extraction of the vitamin was not quite complete, the recovery ranging from 95 to 98 per cent. Due allowance was made for these small losses when calculating the percentage of vitamin destroyed by the baking. The net results thus obtained indicated by the fluorimetric method an average loss of 30·1 per cent. and by the gravimetric method an average loss of 34·8 per cent. in 4 experiments each of 25 minutes heating. The spectrophotometric method indicated an average loss of only 16·9 per cent. in the same experiments. The absorption curves revealed breakdown of the molecule which was probably responsible for the low results. In a further experiment with more prolonged heating there was good agreement between the average fluorimetric and gravimetric results, which indicated losses of 41·0 and 38·3 per cent. respectively, although the gravimetric result was now higher than the fluorimetric result, and the precipitate looked rather abnormal. Spectrophotometric results indicated a lower loss of 20·2 per cent. Finally, very drastic baking (3 periods of 25 minutes) was employed in an attempt to destroy most of the vitamin. The fluorimetric results indicated an average loss of 94·8 per cent., but the gravimetric results indicated an average loss of only 60·4 per cent., and the precipitate was quite abnormal, consisting of brown particles in place of the usual fine white powder.

Discussion

The main purpose of this investigation has been to compare the fluorimetric method of assaying vitamin B_1 with the present official gravimetric method. The accuracy and specificity of the latter is not disputed, as far as general pharmaceutical analysis is concerned, although its reliability may be diminished when it is applied to products which have been subjected to prolonged baking. The gravimetric method has, however, one serious disadvantage—that it requires a minimum of about 25 mg. of the vitamin for each assay, and therefore would not be applicable to single ampoules of low potency injections. The fluorimetric method is far more sensitive, measuring as little as 2 or 3 μg. of the vitamin, in a much shorter

* Dicalcite special speedflow obtained from F. W. Berk & Co., Ltd.

time, provided that a carefully calibrated fluorimeter is available. Our fluorimetric results have shown good agreement with our gravimetric results on materials which have been subjected to different degrees of exposure to heat and light covering practically all possibilities in normal pharmaceutical practice, and we therefore think that the *specificity* of the fluorimetric method need not be questioned. The *accuracy* of this method must now be considered.

Adamson and Handisyde rejected the fluorimetric method in 1948 because of lack of accuracy, stating that it cannot be relied upon to give results of much greater precision than $\pm$ 5 per cent. As far as assays of vitamin B_1 tablets are concerned, this may well be true for the method published[16] from these laboratories in 1945, which as then described was less accurate, though more sensitive, than the gravimetric method described by Adamson and Handisyde in 1948. Its accuracy might indeed be considerably improved by recent advances in fluorimetric techniques, particularly for overcoming quenching difficulties.

Turning to fluorimetric assays of vitamin B_1 in solutions for injection, the accuracy of these was studied by the Aneurine Panel of the Society of Public Analysts (now the Society for Analytical Chemistry) by means of calibration experiments with quinine solutions against solutions of thiochrome obtained from aneurine, as well as by collaborative assays on aneurine solutions circulated to different laboratories. The results obtained in half a dozen well-known laboratories, specified in the Panel's report[5], indicated that the coefficient of variation in such assays usually lay between 1·0 and 1·5 and could be reduced to 0·5 to 1·0 by utilising cross-over tests to eliminate errors due to differences between different cuvettes. We therefore think that for assays of vitamin B_1 in injections, the fluorimetric method, with suitable modifications, might well be employed *not* to replace the gravimetric method, but as an supplement to it, especially for examining small amounts of material for which the gravimetric method is unsuitable. In many pharmaceutical laboratories use could thus be made of fluorimeters available for other purposes.

The alternative use of the spectrophotometric method, based on the decrease in E 245 mμ produced by lowering the *p*H of the solution from 7 to 1, might also be considered for the assay of vitamin B_1 in injections. The spectrophotometric results can thus provide a useful check on the gravimetric results, in instances where a fluorimeter may not be available.

Although spectrophotometric methods have at present only limited practical applications, they are of some theoretical interest because of the isosbestic points which have been revealed. These may help to elucidate the changes which take place when the vitamin B_1 molecule is exposed to heat and light.

Finally, our results provide a certain amount of new information on the effect of heat and light on the vitamin. When they are correlated, as far as is possible, with the findings of other workers, they help to make a more complete picture of this phenomenon which is of importance in pharmaceutical and food problems.

Summary

1. A spectroscopic study of vitamin B_1 over the *p*H range 1 to 9 has revealed the existence of an isosbestic point at approximately 273 mμ which can be used as a check on the production or destruction of irrelevant absorption when the *p*H is lowered from 7 to 1. This lowering of *p*H produces a rise in E 245 mμ which for anhydrous aneurine is approximately 0·1 for 5 μg/ml., a factor employed in a new spectrophotometric assay method.

2. Results obtained by this new spectrophotometric method have been in good agreement with those obtained by the fluorimetric and gravimetric methods on B.P. injections of vitamin B_1 before and after subjection in various degrees to heat and light. The latter causes development of a yellow colour which may be due to breakdown of the vitamin, although this is not detected by the fluorimetric, spectrophotometric or gravimetric methods, or by the microbiological method using *Ochromonas malhamensis*.

3. Baking vitamin B_1 at about 230° C. produces breakdown of the molecule as evidenced by the distorted absorption spectra which invalidate the spectrophotometric assay. The fluorimetric and gravimetric results are in agreement for short period baking (e.g., 25 minutes,) but as baking is prolonged the gravimetric method gives too high results, that is, too small losses.

4. On the basis of these findings it is suggested that the present official vitamin B_1 assay for injections be supplemented by an optional alternative method, preferably fluorimetric or failing that, a spectrophotometric method as outlined above.

5. The results on the rate of destruction of vitamin B_1 by heat and light enlarge the picture obtained by previous workers.

We are indebted to Dr. A. R. Moss of Roche Products, Ltd., for specimens of pyrimidine derivatives and samples of vitamin B_1 injections, to Mr. D. C. M. Adamson of Glaxo Laboratories for a supply of silicotungstic acid and to Dr. J. E. Ford for carrying out microbiological assays on our experimental samples.

References

1. Coward, *The Biological Standardisation of the Vitamins*, London, 1947.
2. Accessory Food Factors Committee, *Biochem. J.*, 1943, **37**, 433.
3. Wokes and Organ, *Biochem. J.*, 1943, **37**, 259.
4. Organ, Duncan, Westbrook and Wokes, *Quart. J. Pharm. Pharmacol.*, 1943, **16**, 275.
5. Society of Public Analysts, *Analyst*, 1951, **76**, 127.
6. Adamson and Handisyde, *Quart. J. Pharm. Pharmacol.*, 1948, **21**, 370.
7. Wokes and Slaughter, *Analyst*, 1949, **74**, 624.
8. Bowen and Wokes, *Fluorescence of Solutions*, London, 1953.
9. Bessot, *J. Pharm. Chim.*, 1940, Ser. 9, **1**, 281.
10. Peters and Philpot, *Proc. Roy. Soc. Lond.*, 1953, **113B**, 48.
11. Holiday, *Biochem. J.*, 1953, **29**, 718.
12. Loofbourow, *Vitamins and Hormones*, 1945, **1**, 109, New York.
13. Somogyi, *Bull. Schweiz. Akad, Med. Wiss.*, 1954, **10**, 446.
14. Beadle, Greenwood and Kraybill, *J. biol. Chem.*, 1943, **149**, 339.
15. Holiday, Irwin, Gillam, Morton, Moore and Wang, *Quart. J. Pharm. Pharmacol.*, 1946, **19**, 155.
16. James and Wokes, *ibid.*, 1945, **18**, 258.

DISCUSSION

The paper was presented by DR. F. WOKES.

DR. G. E. FOSTER (Dartford) said that in the present method of estimating vitamin B_1 by means of silicotungstic acid, the factor varied with each batch of reagent, and he determined the factor for each batch of silicotungstic acid, using one particular batch of aneurine hydrochloride.

DR. D. C. GARRATT (Nottingham) asked whether the authors suggested that an alternative method should be included in the Pharmacopœia, because for the official preparations the silicotungstic acid method was sufficiently accurate.

MR. WHITTET (London) said that in his experience discolouration of vitamin B_1 did not appear to indicate loss of activity.

DR. F. WOKES, in reply, said that only one batch of silicotungstic acid was used. The work had shown that the gravimetric method was entirely satisfactory for the injections, even when they had been subjected to much more drastic storage and exposure to light than would occur in normal practice, but it had a serious disadvantage in that it required a minimum of 50 mg. of vitamin B_1. Despite the deep yellow colour produced in ampoules after exposure to sunlight for months, there was no evidence of decomposition of the vitamin itself. He was gratified that Mr. Whittet confirmed this finding.