# THE DETERMINATION OF RIBOFLAVINE IN PHARMACEUTICAL PRODUCTS

BY L. BREALEY AND D. A. ELVIDGE

*From the Physical Assay Division, Standards Department, Boots Pure Drug Co. Ltd.*

Received June 26, 1956

A VARIETY of methods for the determination of riboflavine in pharmaceutical preparations are available to the analyst. Apart from the microbiological method, which suffers from the disadvantage of being time consuming, a number of procedures involving physical properties of the vitamin are well known. Probably the most widely used of these is the measurement of the fluorescence of riboflavine solutions at known pH values and this forms the basis of the official U.S.P. method. In this country, however, the technique has fallen into some disrepute, largely we believe because of the lack of sensitivity of commercially-available fluorimeters and consequently the microbiological method is favoured in the B.P.C. for all but the simplest preparations.

One of the intrinsic difficulties of the fluorimetric method is that high results may be obtained unless the riboflavine is in a pure state. To overcome this difficulty Conner and Straub[1] adsorb the riboflavine on a natural earth and elute it with a mixture of pyridine and acetic acid. Any remaining interference is then destroyed by treatment with potassium permanganate which does not attack riboflavine. The procedure is tedious and other workers[2,3] have introduced a dithionite treatment to reduce the riboflavine to a non-fluorescent form. Any residual fluorescence is caused by interfering substances which are stable and is subtracted from the total fluorescence. Polarographic methods have been described for the determination of riboflavine in simple solutions and tablets[4,5], but lack the flexibility necessary in a modern pharmaceutical laboratory where highly complex mixtures are often submitted for assay. This limitation also applies to the direct spectrophotometric determination[5,6], which otherwise has the advantage of speed and accuracy.

There exists, however, a general spectrophotometric technique which sometimes makes it possible to determine a single component in a complex mixture. If it is possible to perform a chemical reaction on a mixture containing more than one absorbing substance such that the absorption characteristics of one component only is altered, then it is a simple matter to determine that component by its difference spectrum. It seemed possible that the method could be applied to the determination of riboflavine because of the ease with which it undergoes reduction.

Reduction of riboflavine with dithionite has been widely used in fluorimetry and is included in the U.S.P. method. This reduction converts the riboflavine to its leuco-form which is non-fluorescent in the visible region; the absorption band at 445 m$\mu$ which gives rise to the fluorescence is also affected.

During preliminary investigations into the possibility of using this

reduction as the basis of a difference spectrophotometric method for the determination of riboflavine in complex mixtures, it soon became clear that the reduction is sufficiently vigorous to affect other materials often present. This applied particularly to dyestuffs. Hodson and Norris[2] in their fluorimetric method were able to make use of the oxidation reaction from the leuco-form back to riboflavine and it was found that a very mild oxidation was specific for riboflavine in all the pharmaceutical products examined.

## EXPERIMENTAL

*Materials and apparatus.* Riboflavine—as supplied by the Medical Research Council.

Dithionite (sodium hydrosulphite) of reagent grade.

Buffer solutions of pH 2·0, prepared from 0·2 M KCl and 0·2 N HCl; of pH 4·0, 6·0 and 8·0 prepared from 0·2 M $KH_2PO_4$ and suitable quantities of $H_3PO_4$ or NaOH.

All spectrophotometric measurements were made on either the Unicam SP500 spectrophotometer or the Unicam SP600. When fluorimetric methods were used for comparison purposes the instrument was one built in this laboratory. This fluorimeter is extremely sensitive and will be described elsewhere.

It is well known that the absorption of riboflavine varies with hydrogen ion concentration[7] and it was considered necessary to investigate the reactions at different pH values. For these determinations a standard solution containing 100 μg./ml. riboflavine was diluted with the appropriate buffer to give final solutions containing 10 μg./ml.

The variations in spectral characteristics over the range pH 2·0 to pH 8·0 were found to be negligible, the data obtained being given in Table I. This agrees substantially with the reports of others.

TABLE I

VARIATION OF RIBOFLAVINE SPECTRA WITH pH

| pH | $\lambda_{max.}$ | $E$ (1 per cent. 1 cm.) | $\lambda_{max.}$ | $E$ (1 per cent. 1 cm.) | $\lambda_{max.}$ | $E$ (1 per cent. 1 cm.) | $\lambda_{max.}$ | $E$ (1 per cent. 1 cm.) |
|---|---|---|---|---|---|---|---|---|
| 2·0 | 223 | 802 | 267 | 829 | 374 | 265 | 445 | 306 |
| 4·0 | 223 | 807 | 267 | 830 | 374 | 268 | 445 | 308 |
| 6·0 | 223 | 797 | 267 | 833 | 374 | 270 | 445 | 307 |
| 8·0 | 223 | 788 | 267 | 838 | 374 | 268 | 445 | 308 |

*Reduction of riboflavine.* From a spectrophotometric point of view, therefore, there is a wide pH range in which the absorption or riboflavine may be measured. The effect of dithionite on the spectrum of riboflavine was measured at all four pH values. Since dithionite decomposes in aqueous solution with products which absorb below 400 mμ, the spectrum of the leuco-riboflavine between 220 mμ and 400 mμ is not easily measured accurately and the spectrum shown in Figure 1 is typical. However, since the final method adopted makes use of the maximum extinction at 445 mμ where the dithionite decomposition products do not absorb, the spectrum below 400 mμ is of academic interest only. The leuco-riboflavine was formed by the addition of 0·1 ml. of the freshly prepared

TABLE II

REOXIDATION OF LEUCO-RIBOFLAVINE AT VARIOUS pH VALUES

| pH | 223 mμ | | | 267 mμ | | | 374 mμ | | | 445 mμ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $E_i$ | $E_f$ | R | $E_i$ | $E_f$ | R | $E_i$ | $E_f$ | R | $E_i$ | $E_f$ | R |
| 2·0 | 802 | 880 | 110 | 829 | 892 | 107 | 265 | 265 | 100 | 306 | 300 | 98 |
| 4·0 | 807 | 795 | 99 | 830 | 820 | 99 | 268 | 257 | 96 | 308 | 304 | 99 |
| 6·0 | 797 | 852 | 107 | 833 | 825 | 99 | 270 | 260 | 96 | 307 | 300 | 98 |
| 8·0 | 788 | 900 | 114 | 838 | 820 | 98 | 268 | 264 | 98 | 308 | 300 | 98 |

R = per cent. recovery.

TABLE III

EFFECT OF TIME OF AERATION ON RIBOFLAVINE SPECTRA

| Time of aeration (min.) | $\lambda_{max.}$ | $E$ (1 per cent. 1 cm.) | $\lambda_{max.}$ | $E$ (1 per cent. 1 cm.) | $\lambda_{max.}$ | $E$ (1 per cent. 1 cm.) | $\lambda_{max.}$ | $E$ (1 per cent. 1 cm.) | Per cent. recovery at 445 mμ |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 223 | 797 | 267 | 833 | 374 | 270 | 445 | 307 | — |
| 1 | 223 | 825 | 267 | 830 | 374 | 274 | 445 | 303 | 98·7 |
| 5 | 223 | 810 | 267 | 830 | 374 | 269 | 445 | 302 | 98·4 |
| 10 | 223 | 805 | 267 | 828 | 374 | 270 | 445 | 303 | 98·7 |
| 20 | 223 | 820 | 267 | 830 | 374 | 269 | 445 | 300 | 97·8 |
| 30 | 223 | 828 | 267 | 842 | 374 | 272 | 445 | 307 | 100·0 |

dithionite solution (1 per cent. in water) to 5 ml. of the riboflavine solution used in the first experiment. The spectra were measured immediately using a blank of the appropriate buffer solution (5 ml.) to which 0·1 ml. of 1 per cent. dithionite had been added.

The general shape of the absorption spectra of the leuco-riboflavine does not vary much with pH. The maximum at about 250 mμ occurs at all pH values from 2·0 to 8·0 and the two absorption bands at 374 mμ and 445 mμ present in riboflavine are eliminated by dithionite treatment.

*Reoxidation of leuco-riboflavine.* On aeration of a solution of leuco-riboflavine the yellow colour re-appears and is shown to have the same spectrum as the original riboflavine.

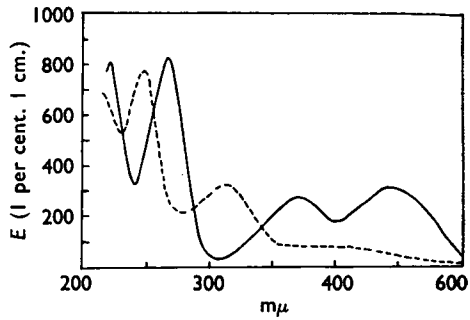


FIG. 1. Reduction of riboflavine with dithionite.

—— Riboflavine.
– – – Leuco-riboflavine.

Riboflavine solutions at pH 2·0, 4·0, 6·0 and 8·0 containing 10 μg./ml. were reduced with dithionite and then reoxidised by aerating for 1 minute. The aeration was carried out in a small tube into which a sintered glass filter stick was inverted, and after aeration the solutions were examined spectrophotometrically. Maxima were obtained at 223, 267, 374 and 445 mμ and the $E$ (1 per cent. 1 cm.) values are shown in Table II.

The "recovery" of riboflavine in Table II is expressed as $E_f/E_i \times 100$, where $E_f$ is the $E$ (1 per cent. 1 cm.) value of the aerated solution and

$E_1$ is the $E$ (1 per cent. 1 cm.) value of the original solution. It will be seen that excellent results are obtained at 267, 374 and 445 m$\mu$, in particular at the last wavelength. The variable results at 223 m$\mu$ may be attributed to either incomplete removal of dithionite decomposition products or absorption of oxygen.

*Effect of time of aeration on the spectra of riboflavine.* Aeration of reduced riboflavine solutions might have a possible detrimental effect if too prolonged, and the effect of aeration time was studied on a solution containing 10 $\mu$g./ml. Periods of aeration between 1 and 30 minutes gave the results shown in Table III. Reoxidation is complete after 1 minute and no decomposition occurs by aerating for periods up to 30 minutes. The aeration was carried out in diffuse daylight at room temperature.

## Application to Pharmaceutical Materials

The spectrophotometric properties of riboflavine enable its determination to be carried out in two main types of preparation. In a first group, where there is no interference from other coloured materials a simple measurement of the maximum at 445 m$\mu$ in a suitable buffer solution suffices. In a second group, where other materials are present, including dyes and pigments, the change in spectrophotometric characteristics on addition of dithionite and on reoxidation can be measured and compared with the change under similar conditions of a pure riboflavine solution. However, each pharmaceutical preparation must be treated on its own merit and sufficient work must be carried out to ensure that only the riboflavine is being estimated. We have found that in many cases where artificial colouring matters are added these are reduced by dithionite but are not reoxidised by aeration. A number of preparations have been examined by the spectrophotometric and by either microbiological or fluorimetric methods. The details are given below.

(*a*) *Direct spectrophotometric method.* Sufficient sample was dissolved or diluted in buffer solution of pH 4·0 to give a concentration of between 10 and 20 $\mu$g./ml. riboflavine. The extinction at 445 m$\mu$ was measured in a 1 cm. cell on the Unicam SP500 or SP600. The concentration of riboflavine was calculated using an $E$ (1 per cent. 1 cm.) value at 445 m$\mu$ of 308 for pure riboflavine.

(*b*) *Direct fluorimetric method.* Sufficient sample was dissolved or diluted in buffer solution of pH 4·0 to give a concentration of between 0·05 and 0$\mu$1 $\mu$g./ml. riboflavine. The fluorescence was measured using an OX1 primary filter and an OY13 and OG3 as secondary filters. A solution of 0·1 $\mu$g./ml. pure riboflavine in buffer pH 4·0 was used as a standard. This method was used only for the simplest types of sample.

(*c*) *Dithionite spectrophotometric method.* Solutions were prepared as for the direct spectrophotometric method. The extinctions at 445 m$\mu$ were measured after addition of 0·1 ml. of 5 per cent. dithionite to 20 ml. of solution and allowing to stand for 1 minute, and also after aeration of the dithionite treated solution for 1 minute. The difference between

the last two readings was compared with that obtained for pure riboflavine under the same conditions, and the riboflavine content calculated using a value of 264 for the difference in $E$ (1 per cent. 1 cm.) at 445 m$\mu$ between riboflavine and leuco-riboflavine.

(*d*) *Microbiological method.* The method employed *Lactobacillus casei* as the micro-organism.

## RESULTS

*Preparations containing no interfering materials.* A number of tablets and solids were examined by spectrophotometric method (*a*), and the fluorimetric method (*b*), the dithionite treatment being omitted. The results are given in Table IV.

TABLE IV

RIBOFLAVINE CONTENT OF SIMPLE TABLETS AND SOLIDS

| Sample No. | Theory | Found | |
|---|---|---|---|
| | | Method (*a*) | Method (*b*) |
| T1 | 1·0 mg./tab. | 0·880<br>0·876 | 0·88 |
| T2 | 1·0 ,, | 1·01<br>1·01 | 1·03 |
| T3 | 3·0 ,, | 2·93<br>2·97 | 2·96 |
| T4 | 1·0 ,, | 0·985<br>0·991 | 1·01 |
| T5 | 1·0 ,, | 1·07<br>1·07 | 1·01 |
| V1 | 35·6 µg./g. | 35·5<br>35·0 | 35·0 |

TABLE V

RIBOFLAVINE CONTENT OF TABLETS WITH ADDED DYES

| Sample No. | Theory mg./tab. | Found | | |
|---|---|---|---|---|
| | | Method (*c*) mg./tab. | Method (*b*) mg./tab. | Method (*d*) mg./tab. |
| P1* | 0·50–0·69 | 0·530<br>0·536 | 0·54 | 0·54 |
| P2 | .. | 0·630<br>0·633 | 0·55 | 0·63 |
| P3* | .. | 0·570<br>0·578 | 0·52 | 0·53 |
| P4 | .. | 0·610<br>0·608 | 0·55 | 0·63 |
| P5 | 0·50 | 0·664<br>0·672 | 0·58 | 0·50 |
| P6 | .. | 0·558<br>0·570 | 0·56 | 0·51 |

*Preparations containing added dyes.* These included tablets, capsules and liquid vitamin preparations. As all the samples contained added dyes method (*c*) was used.

*Tablets.* The tablets were of two types, one containing riboflavine, vitamins A, $B_1$, C and D, the other riboflavine, vitamin $B_1$ and strychnine. In each case the coating material contained a dye and it was possible to determine the riboflavine before and after coating. The tablets were shaken with buffer solution until complete disintegration occurred, made up to a suitable volume and filtered. A typical aqueous extract is shown in Figure 2. It will be seen that the reoxidised solution shows an almost identical curve with the original (Fig. 2 (*a*)) for the uncoated tablets but there is a considerable difference for the coated tablets (Fig. 2 (*b*)). The tablets were also assayed by the microbiological method. The results are shown in Table V. Samples with an asterisk were uncoated.

*Multivitamin capsules.* Refluxing with buffer solution at pH 4·0 to ensure complete solution of riboflavine resulted in the gelatin coating also being dissolved. As this incorporated a dye method (*c*) was employed. A typical set of spectra are shown in Figure 3 (*a*). Here again the extraneous absorption is seen to be considerable. The difference between the reoxidised and reduced solutions is shown in Figure 3 (*b*). This compares favourably with that for pure riboflavine. The results

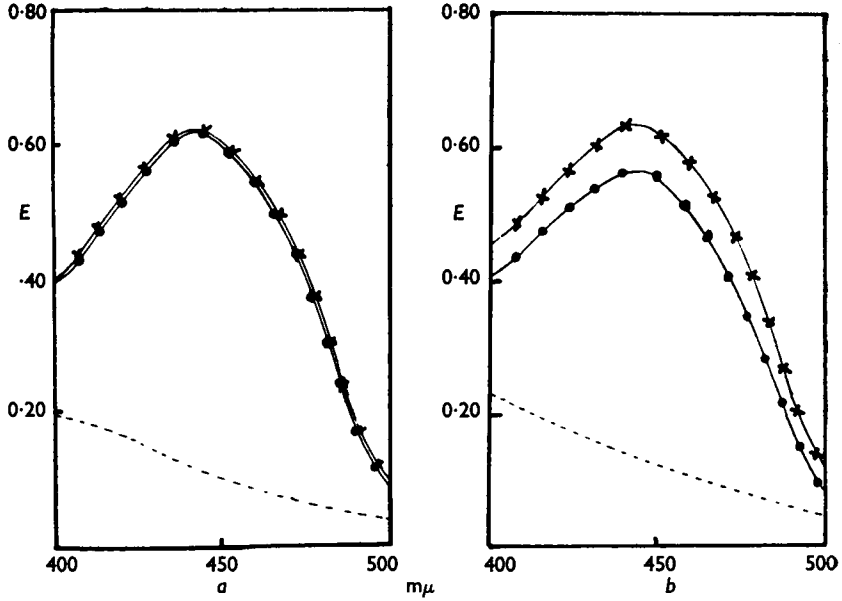


FIG. 2. (*a*) Extract of uncoated tablets. (*b*) Extract of coated tablets.
×——× Gross. - - - - After $Na_2S_2O_4$. ●——● After reoxidation.

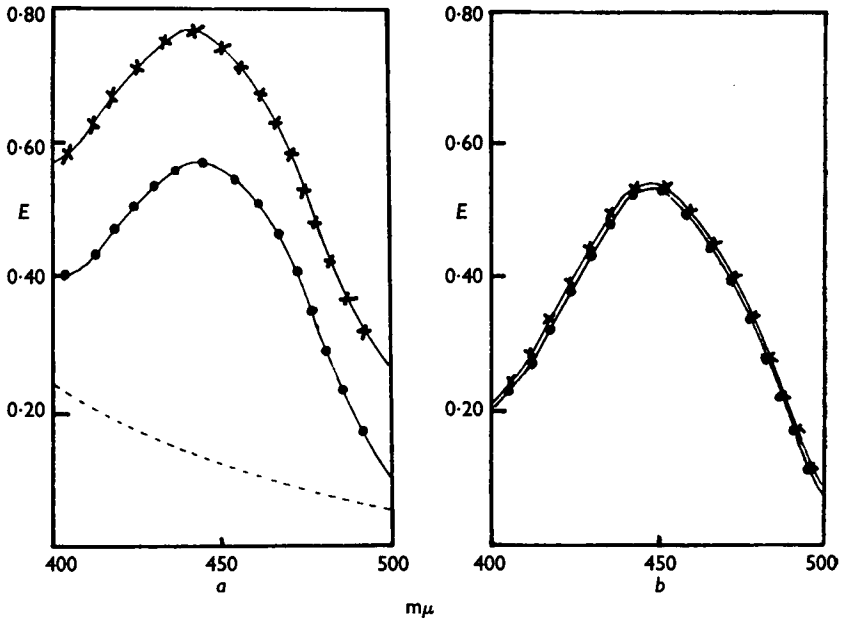


FIG. 3*a*. Extract of multivitamin capsules.
×——× Gross.
- - - - After $Na_2S_2O_4$.
●——● After reoxidation.

FIG. 3*b*. Difference of spectra for capsules and pure riboflavine.
×——× Reoxidised minus leuco for capsules.
●——● Reoxidised minus leuco for riboflavine.

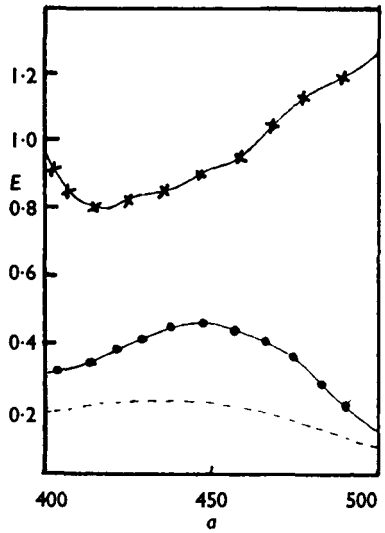


FIG. 4*a*. Liquid vitamin preparation.

×——× Gross.
- - - - After $Na_2S_2O_4$.
●——● After reoxidation.

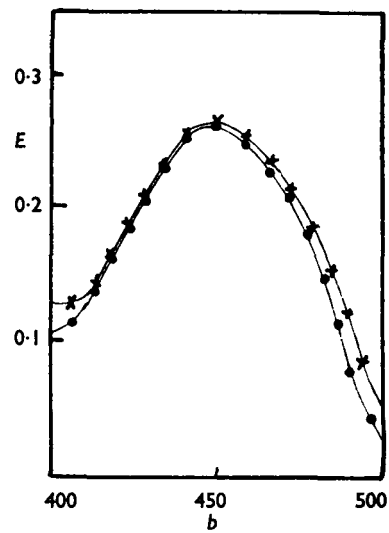


FIG. 4*b*. Difference spectra for liquid preparation and pure riboflavine.

×——× Reoxidised minus leuco for mixture.
●——● Reoxidised minus leuco for riboflavine.

of three samples examined by spectrophotometric and microbiological methods are given in Table VI.

*Liquid vitamin preparations.* A typical sample is shown in Figure 4 (*a*). The extraneous absorption is considerable but after reduction and reoxidation a curve similar to that for pure riboflavine is obtained. Figure 4 (*b*) shows the difference curve for the sample and pure riboflavine. Results obtained by spectrophotometric and microbiological methods are given in Table VII.

*Naturally occurring materials.* Probably the most important natural source of riboflavine is yeast. Unlike synthetic vitamin preparations the chemical properties of the non-riboflavine pigments are unknown and it is virtually impossible to estimate the effect of reduction and reoxidation on these pigments.

TABLE VI

RIBOFLAVINE CONTENT OF MULTIVITAMIN CAPSULES

| Sample No. | Theory mg./capsule | Found | |
|---|---|---|---|
| | | Method (*c*) mg./capsule | Method (*d*) mg./capsule |
| CV1 | 0·425–0·575 | 0·57 | 0·55 |
| CV2 | | 0·55 | 0·54 |
| CV3 | | 0·52 | 0·50 |

TABLE VII

RIBOFLAVINE CONTENT OF LIQUID VITAMIN PREPARATION

| Sample No. | Theory μg./ml. | Method (*c*) μg./ml. | Method (*d*) μg./ml. |
|---|---|---|---|
| E1 | 47 | 44·3<br>45·3 | 46·7 |
| J1 | 375 | 268 | 288 |

Yeast was hydrolysed by refluxing for 1 hour with a mixture of acetone and 0·2 N $H_2SO_4$ (1 : 1). After adjusting to a suitable volume with

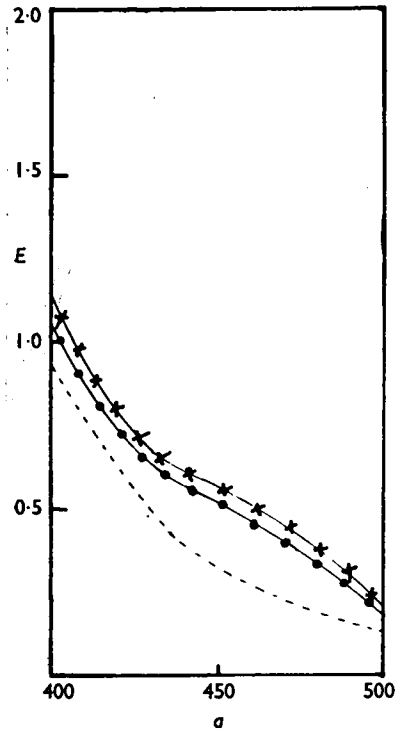


FIG. 5*a*. Extract of yeast.

×——× Gross.
- - - - After $Na_2S_2O_4$.
●——● After reoxidation.

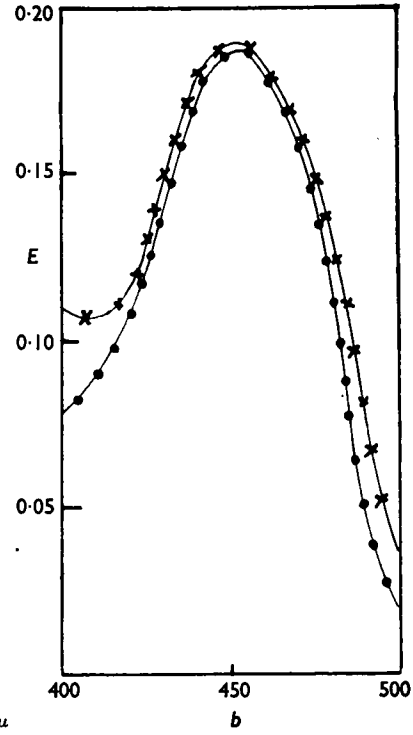


FIG. 5*b*. Difference spectra for yeast and pure riboflavine.

×——× Reoxidised minus leuco for yeast.
●——● Reoxidised minus leuco for riboflavine.

buffer solution at pH 4·0 the solution was centrifuged and filtered. A typical set of spectra are shown in Figure 5. Although only general absorption is obtained even after reoxidation (Fig. 5 (*a*)), the difference curve (Fig. 5 (*b*)) is similar to that for pure riboflavine. The results obtained on a number of samples by the spectrophotometric and microbiological methods are given in Table VIII.

TABLE VIII

RIBOFLAVINE CONTENT OF YEAST

| Sample No. | Method (*c*) μg./g. | Method (*d*) μg. g. |
|---|---|---|
| Y1 | 54·1<br>55·3 | 53·0 |
| Y2 | 64·4<br>65·0 | 53·5 |
| Y3 | 55·5<br>55·3 | 54·3 |
| Y4 | 64·8<br>65·2 | 56·3 |
| Y5 | 57·4<br>57·2 | 53·0 |
| Y6 | 65·7<br>61·4 | 55·0 |
| Y7 | 59·0 | 57·5 |
| Y8 | 60·8 | 66·8 |
| Y9 | 58·8 | 58·5 |
| Y10 | 51·5 | 57·0 |

## SUMMARY

1. A spectrophotometric method has been described for the determination of riboflavine in pharmaceutical materials. It has a number of advantages over the present official methods.

2. It is much more rapid than the microbiological method.

3. By taking a series of spectrophotometric readings over the range 400 mμ to 500 mμ it can be shown that only riboflavine is determined because the difference curve will, in this case, be identical with that of pure riboflavine. In this respect it is more specific than most fluorimetric methods.

4. The method is more flexible than fluorimetric procedures in that the pH range over which it is applicable is very wide.

5. It is not necessary to prepare a standard riboflavine solution for each determination. Once the absorption values have been determined for the working conditions they can be applied with only very occasional checks because extinction readings are absolute and not comparative.

6. The results obtained on the samples of yeast are in general sufficiently close to those of the microbiological method to suggest that the spectrophotometric procedure could in due course replace the microbiological method in the B.P.C.

7. The principal shortcoming of the method lies in its poorer sensitivity than the fluorimetric technique, but this is not important in the assay of pharmaceutical products.

We are grateful to Miss F. N. Mulholland for the microbiological determinations.

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## DISCUSSION

The paper was presented by MR. L. BREALEY.

DR. G. E. FOSTER (Dartford) said he had tried the suggested method and found it satisfactory for multivitamin capsules. He asked if the authors had applied their method to the assay of malt extract.

DR. F. WOKES (King's Langley) referred to the authors' statement that the fluorimetric technique had fallen into some disrepute because of the lack of sensitivity of commercially available fluorimeters. The riboflavine content of the various preparations examined was much higher than the figures he had published. He had made some calculations from the Tables giving the comparison of the microbiological and fluorimetric methods, and had found the latter to be on average 99·7 per cent. of the microbiological assay as compared with 108 per cent. for the spectrophotometric method. Therefore the data did suggest that the fluorimetric method was as accurate as the proposed spectrophotometric method.

MR. S. G. E. STEVENS (London) said that certain difficulties had arisen in the accurate determination of the riboflavine in animal feed substances.

It appeared that where certain grass meals were used, materials were being extracted which did not readily reduce, and they interfered with the fluorimetric determination of the re-oxidised riboflavine. On occasions it had been necessary to make use of the technique of adding known amounts of riboflavine to those extracts and to make a number of recovery experiments. He asked whether the authors had encountered this difficulty and had carried out recovery experiments.

MR. L. BREALEY, in reply, said that the method had not been used for malt extracts. The difficulty about using the method for materials low in riboflavine was in securing a solution of sufficient concentration to obtain good spectrophotometric readings. That was why the work had been limited to materials of relatively high vitamin content. The fluorimetric results were good because commercial instruments had not been used. They had built their own instrument and it was much more sensitive than the generally available commercial equipment. There were many arguments for and against the procedure of recovery experiments, but they had not made any such experiments.