THE POLAROGRAPHIC DETERMINATION OF RIBOFLAVINE IN COMPRESSED TABLETS AND AMPOULE SOLUTIONS

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SEVERAL methods are at present available for the estimation of riboflavine, including microbiological, fluorimetric, and spectrophotometric assay. These rather complex techniques have been used primarily for the determination of riboflavine in foods, where, along with many other substances, it is present in relatively small amounts. The U.S.P. XIV prescribes the fluorimetric and microbiological methods for the assay of riboflavine in compressed tablets. In view of the increasing medicinal importance of riboflavine, and the proposal to include compressed tablets containing it in the British Pharmacopœia, it seems desirable to have a simpler and more rapid method for its determination in such products. The U.S.P. XII included a colorimetric method of estimating riboflavine in compressed tablets which has the merit of simplicity, but which, in our experience, does not give good results.

Lingane and Davies[1] have drawn attention to the possibility of determining riboflavine polarographically. Their work was not extended to the estimation of riboflavine in natural products, or pharmaceutical preparations. The present investigation was prompted by the desire to find the most suitable conditions for the polarographic determination of riboflavine in compressed tablets. The determination of riboflavine in ampoule solutions was also investigated on a more limited scale. Some time was also devoted to the photo-decomposition of riboflavine. This latter portion of the work is not complete, but because of its pharmaceutical interest, some results obtained have been incorporated into this paper.

EXPERIMENTAL

A manually operated polarograph was employed. The cell was similar to one described by Kolthoff and Lingane,[2] and was constructed from a B.40 Pyrex glass joint. The capillary constants determined on open circuit conditions in 0·1 N potassium chloride solution were as follows: m. = 0·969 mg. sec.$^{-1}$, t. = 3·37 sec., where m. = the rate of mercury flow, and t. = the drop-time. Current was measured by means of a calibrated damped Cambridge "spot" galvanometer. All experiments were carried out at 25° C. Air was removed from the cell solution by passing cylinder nitrogen, purified by passage through a series of gas-washing bottles containing alkaline hydrosulphite solution. The potential of the dropping mercury electrode (d.m.e.) was adjusted by means of a tapped resistance network, forming a potential divider. In obtaining current-potential curves, potential increases of about 20 mV. were usually employed. All dropping mercury electrode potentials were measured against an immersion type saturated calomel electrode.

For the examination of the current-time relationship during the life of a single mercury drop, an Ultrascope Mark I cathode-ray oscilloscope was employed, in conjunction with an external time base of about 5 seconds traversing time. Though non-linear, this time base was found adequate. The cell current was amplified by means of a simple D.C. amplifier employing two EF 50 valves, constructed in the laboratory. The output of the amplifier was fed to the Y plates of the cathode-ray tube.

Sörensen's buffer solutions served as supporting electrolyte. For many of the determinations, urea was also present as a co-solvent for the riboflavine. In preparing the cell solutions, the usual technique was to dissolve the riboflavine, with the aid of gentle heat, in a 10 per cent. solution of urea in buffer solution. This solution was then made up to final volume with plain buffer solution, giving a solution of known urea content. For the removal of tablet base debris a porosity 4 sintered glass filter was chosen in preference to filter paper which adsorbs riboflavine.

Two batches of compressed tablets, and two batches of ampoules were examined. For both tablets and ampoules the different batches will be designated A and B. Tablets and ampoules of batch A were of one manufacture, and the batch B products of another. The tablets of batch A were uncoated; those of batch B had a deep orange coating. All the tablets were stated to contain 3 mg. of riboflavine. The ampoules contained 2 ml. of sterile solution of riboflavine, strength 5 mg./ml.

Results and Discussion

Riboflavine is only sparingly soluble in water at about *p*H 7. It is very much more soluble in acid and alkaline solvents, in which it may exist as a cation or an anion. It was shown by Brdička and Knobloch[3] that in acid solution, the riboflavine step is complicated by the presence of an anomalous fore-wave, later shown by Brdička[4] to be due to the adsorption of the reduction product on the surface of the mercury drop. This anomalous fore-wave was not present in the case of solutions of *p*H higher than about 6. In order to avoid possible complications due to these adsorption effects, it seemed desirable to employ solutions of *p*H greater than 6. In alkaline solution, however, riboflavine undergoes rapid photolysis. For these reasons, it was decided to work with approximately neutral solutions, and to employ urea to increase the solubility of riboflavine.

The Effect of Urea and Pyridine on the Polarographic Behaviour of Riboflavine.—In order to determine the effect of urea on the polarographic reduction of riboflavine, a series of solutions containing varying amounts of urea with the same concentration of riboflavine was prepared and examined polarographically. No variation of wave height was observed, nor was there any shift of the half wave potential (— 465 mV.). It may be inferred from these results that close compound formation between riboflavine and urea does not occur; such compound formation would almost certainly be accompanied by a shift of half-wave potential, and a lowering of the diffusion coefficient of the reducible substance, leading

to a diminution of wave height. No reduction of urea was observed over the range of potential covered, i.e., up to — 1·8 V.

Another point of interest was to examine the effect of urea on the adsorption of riboflavine on the mercury drop. It has been shown by Kaye and Stonehill[5] that the addition of ethanol to solutions of acridine, by increasing the solubility of the latter, and its reduction products, prevented the adsorption of electro-active material on the mercury drop surface, and enabled a normal polarographic wave to be obtained. These workers also found that a normal polarogram was obtainable when a limited amount of adsorption of electro-active material occurred, and that the best indication of adsorption on the dropping mercury electrode was the form of the current–time relationship during the life of a single mercury drop. It might therefore be supposed that urea, by increasing the solubility of riboflavine, would have a similar effect in preventing

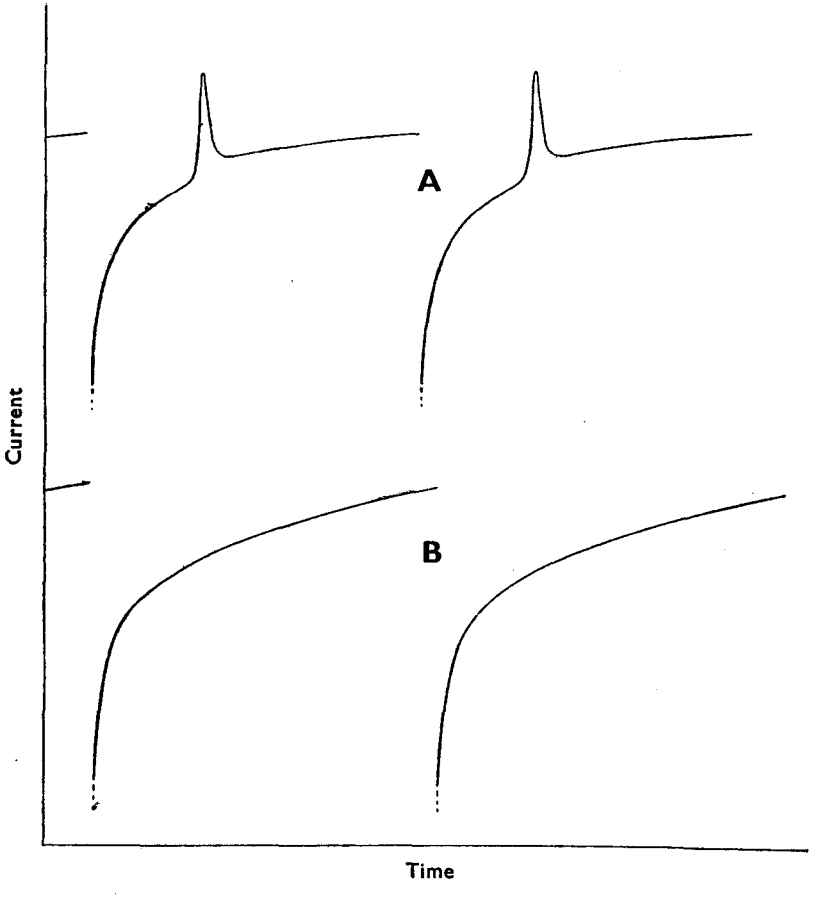


FIG. 1. Time-current relationships during the reduction of riboflavine.
A. With or without addition of urea.
B. With addition of pyridine.

adsorption of the latter, and possibly its reduction product on the mercury drop. To test this supposition, the current–time relationships during the reduction of riboflavine (4×10^{-4} M) in *p*H 7·38 buffer solution in the presence and absence of urea were examined on the oscilloscope, and the tracings photographed. The current–time relationship during the life-time of a single drop was of the form shown in Figure 1A. The sudden surge of current, which occurs early in the life of the mercury drop and is probably due to the adsorption of oxidant at the mercury surface, may clearly be seen. The form of this current–time relationship was not altered by the presence of up to 25 per cent. of urea in the solution, showing that urea does not affect the adsorption of electro-active material on the drop surface. Another experiment with a solution of riboflavine in a buffer-pyridine mixture showed that the inclusion of 20 per cent. of pyridine, which is a good solvent of riboflavine, eliminated entirely the adsorption of electro-active material on the mercury drop, and enabled a normal current–time curve to be obtained (Fig. 1B). Measurements showed that the inclusion of the somewhat basic pyridine in the solution did not significantly alter the *p*H. The elimination of adsorption was not therefore due to the existence of riboflavine in a different ionic state in the pyridine-buffer mixture. These results show that the reactions of urea and pyridine as co-solvents with riboflavine are quite different. Since the limited amount of adsorption occurring at *p*H 7·38 was found not to cause any distortion of the polarogram, and because of the objectionable nature of pyridine, it was decided to employ urea as an aid in the preparation of tablet solutions for polarographic assay.

The diffusion current constant ($\frac{i_d}{c}$) for riboflavine was found by Lingane and Davies (*loc. cit.*) to vary slightly with *p*H, and it was therefore important to know how the addition of small amounts of urea affected the *p*H of the buffer solution used. That this effect was little if any, was already apparent from the independence of the half-wave potential on the presence of urea; any alteration of *p*H would be accompanied by a corresponding shift of the $E_{\frac{1}{2}}$ value, since hydrogen ions are involved in the electro-reduction. Measurements with a glass electrode *p*H meter confirmed this.

Having found the inclusion of urea in the solution to be free from objection, it was decided to employ a standard base solution consisting of Sörensen's phosphate buffer solution, *p*H 7·38, with the addition of 2 per cent. of urea. A satisfactory method was to dissolve the riboflavine in 10 ml. of buffer solution containing 10 per cent. of urea, followed by adjustment to volume with plain buffer solution. Using this technique, a series of solutions containing 1×10^{-4} M. to 6×10^{-4} M. of a B.P. sample of riboflavine, dried over sulphuric acid, was prepared and polarograms obtained. The residual current was obtained for the buffer-urea mixture, and subtracted from the measured wave height to obtain corrected values of the latter for different concentrations of riboflavine. The relationship between i_d (the limiting value of the diffusion

current), and concentration was found to be linear. In order to assess the accuracy with which the diffusion current could be measured, several determinations were made using the same solution. For riboflavine concentrations of 10^{-4} M to 10^{-3} M the accuracy of measurements was $\pm$ 2 per cent.

The effect of soluble tablet excipients on diffusion current.—A variety of materials is likely to be encountered in compressed tablets, and it is important to know if any of the ingredients present in commercially produced tablets would affect the polarographic behaviour of riboflavine. It might be expected, for example, that acacia and similar substances, by increasing the viscosity of the solution slightly, would lower the value of the riboflavine diffusion coefficient and therefore the diffusion current, which is governed by the diffusion coefficient in accordance with the Ilkovič equation $\bar{i}_d = 605\ n\ D^{\frac{1}{2}}\ C\ m^{\frac{2}{3}}\ t^{\frac{1}{6}}$; where $\bar{i}_d$ = diffusion current in microamperes; C = concentration of reducible substance in millimols per litre; n = number of electrons involved in the electro-reduction; D = diffusion coefficient; t = drop time; and m = rate of mercury flow in mg./sec.$^{-1}$ In the case of coloured sugar-coated tablets there is the additional possibility of electro-reducible dyes being present in the coating. To decide these points, a tablet from batch A was disintegrated in 10 per cent. urea solution in *p*H 7·38 buffer, and the riboflavine dissolved. The solution was clarified as described and adjusted to 50 ml. The amount of riboflavine was determined polarographically. 3 mg. of riboflavine, accurately weighed, was then dissolved in the solution, and the total riboflavine content obtained. The difference between the two determinations gave the added amount of riboflavine as 3·07 mg., showing any soluble component of the tablet base to be without effect. A second experiment with orange-coloured sugar-coated tablets from batch B gave a similar result. To determine if the orange dye in the coating was reducible, the dye coat was dissolved in 10 per cent. urea in buffer solution (the tablet removed before any riboflavine dissolved) adjusted to volume with plain buffer and assayed polarographically. The dye was found to be non-reducible over the potential range covered.

Comparison of the Polarographic and U.S.P. colorimetric methods.—The U.S.P. XII colorimetric method consists of comparing the colour of a filtered tablet solution with that of two standard solutions containing 20 per cent. less and 20 per cent. more than the amount of riboflavine expected to be present in the tablets. The comparisons are made in matched tubes. Using this method the riboflavine content of a batch A tablet was found to be about 2·4 mg. Polarographic analysis of the same tablet solution gave the result as 2·01 mg. In the colorimetric method it was found difficult to distinguish colour difference between the solutions used when the comparison was carried out in tubes, and different workers failed to agree about the colour matching. In order to reduce the subjective errors of this method a Duboscq colorimeter was employed to determine the riboflavine content of a number of tablets. The same tablet solutions were then estimated polarographically, and the two sets of results compared (Table I).

Some difficulty was experienced when using the Duboscq colorimeter, the tablet solutions appearing slightly opalescent. This imparted a slightly grey appearance to the solution in the colorimeter. It was found impossible to clarify the solutions perfectly using a porosity 4 sintered glass filter. The rate of filtration, moreover, was extremely slow. All the colorimetric results were considerably lower than those obtained polarographically. To determine to what extent this was due to imperfect clarification of the tablet solutions, a further series of comparisons of the two methods was made using carefully weighed samples of pure riboflavine. The results are given in Table II.

TABLE I

UNCOATED RIBOFLAVINE TABLETS 3 MG., BATCH A

Colorimetric method mg.	Polarographic method mg.
2·01	2·46
2·08	2·40
1·75	2·37
1·84	2·07
1·38	1·73

TABLE II

Weight of sample used mg.	Result of colorimetric determination mg.	Result of polarographic determination mg.
3·8	4·43	3·76
4·03	4·50	4·13
3·67	3·89	3·76

In the case of the weighed samples of pure riboflavine, the colorimetric results are higher than the polarographic. It is evident that the presence of minute amounts of suspended material from the tablet base exerts a marked influence on the results obtained colorimetrically. In addition to this difficulty, subjective errors were apparent in using the Duboscq colorimeter. The results obtained by different observers differed considerably.

The effect of suspended tablet debris on polarographic analysis.—As stated above, the effect of minute amounts of suspended tablet debris on the colorimetric determination was appreciable. Since clarification through a No. 4 sintered glass filter was always tedious, it was decided to examine the effect of the presence of tablet debris during the polarographic determination of riboflavine, with a view to eliminating altogether the clarification process. Accordingly, an uncoated 3-mg. tablet from batch A was disintegrated in 10 per cent. urea solution in *p*H 7·38 buffer, care being taken to dissolve all the riboflavine. The solution was adjusted to 50 ml. and placed in the cell without clarification. After bubbling nitrogen through the liquid, rapid settling of the larger particles occurred, leaving a translucent supernatant liquid. Current-potential readings were obtained. 3 mg. of riboflavine was then dissolved in the liquid, and a second determination made. The difference between the two diffusion currents enabled the amount of added riboflavine, as determined in the presence of tablet debris, to be calculated. The experiment was repeated with a sugar-coated tablet from batch B. The uncoated tablet

batch A showed 3·08 mg. of added riboflavine, estimated in the presence of tablet debris and the coated tablet batch B showed 2·95 mg. These results show that the presence of suspended tablet debris has little influence on the polarographic determination of riboflavine. A possible objection to the technique is that the tablet debris, by displacing a small volume of liquid, will affect the concentration of dissolved riboflavine. Experiments showed that the average volume occupied by the debris from both batch A and batch B tablets was approximately 0·05 ml. The volume of the solution plus tablet debris was 50 ml. so that the error introduced by leaving the tablet debris in the solution is less than 0·2 per cent.—considerably less than the errors involved in measuring the diffusion current. Using this technique the remaider of the tablets in batch B (sugar-coated) were assayed. The results are given in Table III.

TABLE III

Corrected wave height (galvanometer scale readings)	Riboflavine content mg.
2·0	2·60
2·26	2·95
1·94	2·52
2·35	3·09
1·95	2·54
2·08	2·70
2·13	2·77
2·26	2·95
2·20	2·88
2·32	3·03

Estimation of riboflavine in ampoule solutions.—2 batches of ampoules of different manufacture were examined. The ampoules were stated to contain 10 mg. of riboflavine in 2 ml. of solution. Using a method similar to that employed for compressed tablets, it was found that the solutions in both batches of ampoules contained nothing which affected the riboflavine diffusion current, or significantly affected the *p*H of the cell solution. The solutions for examination were prepared by taking 1 ml. of the ampoule solution and making up to 50 ml. with *p*H 7·38 buffer solution. The amount of riboflavine present was estimated both polarographically and colorimetrically, using a Duboscq colorimeter. The results for ampoules of batch A are given in Table IV.

TABLE IV

Polarographic result mg./ml.	Colorimetric result mg./ml.
2·26	2·75
2·45	2·63
2·26	2·58
2·58	2·68
2·69	2·75

As was found previously for samples of pure riboflavine, the colorimetric results are considerably higher than the polarographic. Several of the ampoules of batch A showed a black deposit, which dispersed on shaking; this might account for the serious shortage of riboflavine in the ampoules. There was also a slight difference in the colour quality of the diluted ampoule solution, and the standard solution employed for the colorimetric determination. The contents of the ampoules of batch B were also examined by the two techniques. The colour of the solution diluted with *p*H 7·38 buffer, was orange yellow, compared with the greenish-yellow colour of the standard solution prepared for the colorimetric assay. It was found quite impossible to obtain any reliable results by colorimetry. The polarographic results for 4 of the ampoules of batch B were:—4·97, 4·98, 5·12, 5·02 mg./ml.

The riboflavine content of the ampoules was thus in accordance with

the statement of strength on the label. No black deposit or other signs of deterioration were observed.

The effect of light on the polarographic behaviour of riboflavine.— Solutions of riboflavine are usually regarded as being unstable to light, especially if alkaline. Since the water bath used for maintaining the contents of the cell at 25° C. was heated by two carbon filament lamps, it was important to know if decomposition was likely to occur during the determination. To determine this, a 4×10^{-4} M solution of riboflavine was prepared in *p*H 7·38 buffer using urea as co-solvent. The urea concentration in the final solution was 2 per cent. Current-potential curves were obtained both before and at intervals during exposure to a 60-watt lamp, the maximum time of exposure being 44 hours. The resulting polarograms showed a slight but quite definite modification after exposure of the solution to light, this consisting of a more rapid decrease in the slope of the curve towards the crest of the wave, suggesting the formation of a second and ill-defined wave. There was also a very small reduction of the total wave height. Further experiments showed that exposure to the light of the heating lamps, in the water bath, for a moderate period of time produced no polarographically detectable change in the solution.

In view of the possibility of using the polarographic method to detect and to estimate the photolytic products from riboflavine, a series of experiments with an alkaline solution of riboflavine was undertaken, photolysis being much more rapid at high *p*H. Riboflavine was dissolved in *p*H 11 buffer solution and determined polarographically, the solution being protected from light. The solution was then divided into two portions, one being exposed to the light of a 60-watt lamp under standard conditions, and the other stored in the dark. Current-potential curves for both solutions were obtained periodically. A few hours exposure to light resulted in the appearance of a second wave with a half-wave potential more negative than that of the main riboflavine wave. Continued exposure to light caused an increase in the height of the second wave, and a corresponding decrease of the main riboflavine wave. It seemed likely that the new substance was lumiflavine. To confirm this a small sample of the solution was taken, neutralised with hydrochloric acid and extracted with chloroform, in which lumiflavine is soluble. The separated chloroform layer was yellow. The chloroform layer was distilled off, and the residue taken up in *p*H 11 buffer solution. This yielded a single polarographic wave, of half-wave potential identical with that of the second wave described above. Continued exposure of the solution to light resulted in the appearance of a third wave, at still more negative potential, and a corresponding decrease in the height of the lumiflavine wave, indicating a further breakdown of the lumiflavine, and the formation of a third reducible substance. These changes are shown in Figure 2. The points of inflection at the top of the third wave in polarograms 5 to 9 are probably due to incipient maxima. Figure 3 shows how the diffusion currents, and hence the concentrations of riboflavine, lumiflavine, and its breakdown product alter with time.

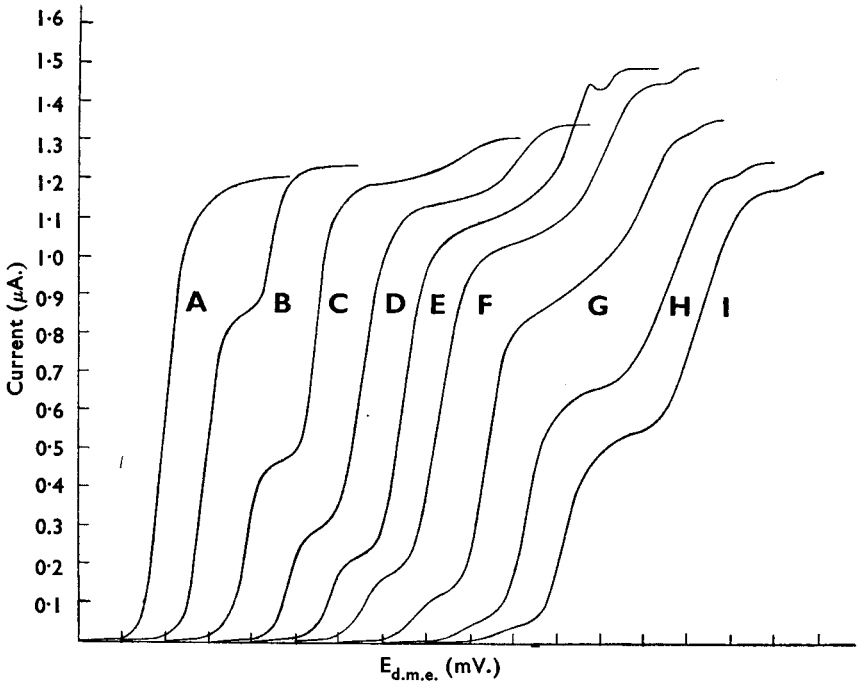


FIG. 2. Current potential curves for a solution of riboflavine exposed to light. A. Zero. B. 18 hours. C. 68 hours. D. 92 hours. E. 108 hours. F. 144 hours. G. 255 hours. H. 473 hours. I. 947 hours.
The potential scale is marked off at 100 mV. intervals. Each curve commences at — 400 mV.

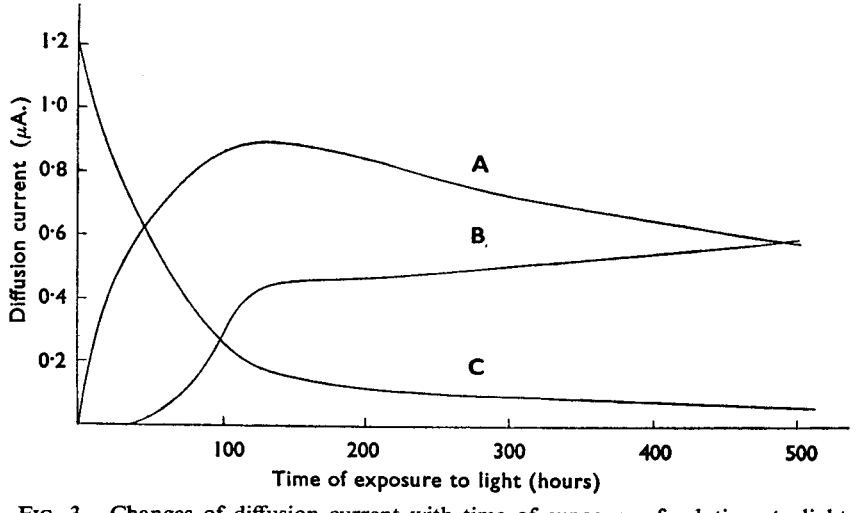
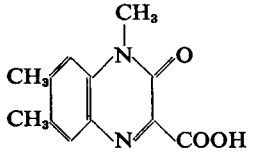


FIG. 3. Changes of diffusion current with time of exposure of solutions to light.
A. Lumiflavine.
B. Breakdown product of lumiflavine.
C. Riboflavine.

Although the production of lumiflavine from riboflavine in alkaline solution is primarily a photo-chemical process, we have no evidence suggesting that the further break-down of lumiflavine depends on exposure to light. The solution stored in the dark was examined periodically. Polarograms showed a gradual loss of riboflavine, but at a much slower rate than in the case of the solution exposed to light. Disappearance of riboflavine was accompanied by the formation of another reducible substance which, from its half-wave potential, appeared to be identical with the break-down product of lumiflavine present in the solution exposed to light. No lumiflavine could be detected in the solution stored in the dark. It seems probable therefore that this break-down product has the structure:—



which is known to be formed by the action of alkali on both riboflavine and lumiflavine (Kuhn and Rudy[6]). It is interesting to note that the polarographic evidence of the existence of this further break-down product supports the observations of Daglish, Baxter, and Wokes,[7] who, from evidence obtained spectroscopically, suggested the existence in alkaline riboflavine solutions of some decomposition product other than lumiflavine.

The small, ill-defined wave, noted on polarograms obtained after exposure of an approximately neutral solution to light, is probably not due to the presence of lumiflavine, since its $E_{\frac{1}{2}}$ value was only about 60 m. more negative than that of the main riboflavine wave. It may be attributed to the presence of a small amount of lumichrome, which is formed from riboflavine by irradiation in neutral or acid solutions.

The polarograms of Figure 2 show that the *total* wave height at first increases with the progressive decomposition of riboflavine. This is to be expected, since the decomposition products, of smaller molecular weight than the parent substance, would have larger diffusion coefficients. After 144 hours exposure to light, however, the total wave height decreases. The most likely explanation of this is that further decomposition occurs, yielding products which are not detectable polarographically.

This study by the polarographic method of the decomposition of riboflavine is incomplete, and further investigation is in progress. The results obtained so far show that polarography is suitable for the detection of some of the break-down products of riboflavine. None of these products was found in the compressed tablets and ampoule solutions examined.

SUMMARY

1. Conditions suitable for the polarographic estimation of riboflavine in compressed tablets have been determined.

2. Polarography has been found to be more reliable than the simple colorimetric procedure of the U.S.P. XII. It is more rapid than the fluorimetric, microbiological, and spectrophotometric methods of riboflavine estimation.

3. Two batches of commercially produced tablets, and two of ampoules have been examined. One batch of tablets and one of ampoules were found to be seriously deficient in riboflavine. There exists a need for more stringent control of such products.

4. Some decomposition products of riboflavine may readily be detected and estimated polarographically. The decomposition of riboflavine in alkaline solution, when exposed to light and also when stored in the dark, has been followed polarographically. The results are in agreement with those of spectroscopy.

5. The inclusion of pyridine in a solution of riboflavine at *p*H 7·38 has been found to prevent adsorption of electro-active material at the surface of the mercury drop.

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DISCUSSION

The paper was presented by MISS S. FOWLER.

DR. F. WOKES (King's Langley) suggested that the authors might in their future work obtain more information from spectrophotometrical results. He pointed out that they had given results in respect of two samples of tablets and ampoules only, and on the basis of those results had found one deficiency. The samples might be deficient, but as soon as possible more information should be obtained on a much wider range of samples. What were the actual wavelengths at which the authors radiated their riboflavine?

MR. T. D. WHITTET (London) asked whether the authors had tried the possible effect of nicotinamide in their determinations as he had used nicotinamide and urea together to solubilise riboflavine.

MR. M. DOMBROW (London) said that the authors mentioned that an anomalous fore-wave which occurred early in the life of the drop was probably due to the absorption of oxygen on the mercury surface. Early in the paper there was a reference to the work of Brdička in which it was suggested that the anomalous fore-wave occurring was due to absorption of the reduced material, in particular the leuco or semi-quinone form. There appeared to be some discrepancy in that connection and it would be interesting to have some information concerning evidence that the absorption was due to the oxidant and not to the reductant. With regard to the statement that the reactions of urea and pyridine as co-solvents for riboflavine were different due to the fact that in the presence of urea the anomalous fore-wave occurred, while in the presence of pyridine it was dissipated, had they different actions as co-solvents?

MISS S. FOWLER, in reply, said that the polarographic method was adopted because it had the merit of simplicity in the preparation of solutions and in the actual reading of the final results. With regard to the comparison with the spectrophotometric method, facilities were now available for comparison with that method and work would be carried out. Nicotinamide had not been used as a solvent for riboflavine, but it would be borne in mind. With regard to the standard conditions for radiation, light was provided from a 60 watt lamp in which there would not be a great deal of ultra-violet light. Their aim had been to produce such substances as lumiflavine by some artificial means as quickly as possible. She was not in a position to say whether it was the oxidant or reductant product which was absorbed. The assertion that the actions of urea and pyridine as solvents were different was based on spectroscopic evidence.