FLUORIMETRIC AND MICROBIOLOGICAL ASSAYS OF RIBOFLAVINE IN MALTED PREPARATIONS

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In a previous communication[1] we described fluorimetric and microbiological assays of riboflavine in barley, malted barley and malt extract. Whilst the two assay methods gave the same general picture for increases in content of this vitamin during malting and brewing, certain discrepancies were encountered between the results given by the two methods. On 4 samples of barley and malted barley, the fluorimetric method gave lower results (72 to 89 per cent. of the microbiological result), the deviation on 3 of the samples being significant. On 4 samples of malt extract the fluorimetric result did not deviate significantly from the microbiological result. On a fifth sample of malt extract the mean fluorimetric result was 231 per cent. of the mean microbiological result, a highly significant difference. Extensive investigations have been carried out in our laboratories to explain these discrepancies, and show how they may be overcome by improvements in fluorimetric and chromatographic technique.

Elvidge[2] reported good recoveries (84 to 105 per cent.) of this vitamin from 5 more potent pharmaceutical preparations (an elixir, capsules and tablets) in which he estimated it fluorimetrically or spectrophotometrically. A detailed study of the spectrophotometric method was made in our laboratories[3]. Optical densities were recommended to be read at 267 or 375 mμ as well as at the maximum of 444 to 445 mμ recommended by Elvidge. The importance of controlling the pH was emphasised, and experiments described on the development of lumiflavine through the action of light on alkaline solutions of riboflavine. The possible interference of lumiflavine fluorescence has been one of the factors we have had to investigate in the fluorimetric work described below.

MICROBIOLOGICAL ASSAYS

The method as laid down by the Analytical Methods Sub-Committee of the Society of Public Analysts[4] was almost exactly followed. It has been described with comments in a previous paper[1]. Continued experience with the method has shown that *Lactobacillus helveticus* is not on the whole so easy to work with as, for example, *Lactobacillus arabinosus*, which is used for the assay of nicotinic acid and biotin. Difficulties are sometimes experienced in maintaining sub-cultures, and it would seem tha* the organism requires nutrilites whose nature is not necessarily exactly known, but which are present in some natural products. This is further indicated by the observation in recent months that there is a tendency to obtain less satisfactory linearity in the standard curve than in the curves for the assay samples.

The effect of the presence of fats and fatty acids in samples and

extracts undergoing assay has long been observed, although completely satisfactory explanations are not as yet forthcoming (see, for example, Kodicek and Worden[5], and Norris and Lynes[6]). The occurrence of fats as such in materials for assay need present little difficulty since the sample may be quantitatively pre-extracted with light petroleum in a Soxhlet extractor. The assay is then performed on the fat-free product, and the vitamin content calculated back to the original material. Where the fat or fatty acids occur in lipin combinations, the position is not so simple. Ordinary extraction as above removes little or none of the lipin, and resort to more drastic treatment involves loss of all or part of the vitamin. In these cases it has usually been satisfactory to extract the hydrolysed extracts with light petroleum or, better, ether.

' FLUORIMETRIC ASSAYS

We previously employed a modification of the fluorimetric methods of Rubin et al.[7] and of Hoffer et al.[8] kindly given us by Dr. A. J. Amos, Secretary of the Chemical Panel of the Vitamin Sub-Committee of the Society of Public Analysts. Whilst this method as adapted for use with the Spekker fluorimeter gave reasonable agreement with microbiological assays on some foods fairly rich in the vitamin and relatively free from interfering substances, the Panel members did not find it satisfactory for malted preparations and certain other foods in which more of these interfering substances were present. The final conclusion of the Panel was that this method, although showing distinct promise, was not capable of general application until a more sensitive fluorimeter giving greater reproducibility was available. The Panel devised a deflection instrument of the Cohen type, which provided about 4 times the sensitivity of the old Spekker Fluorimeter, but did not find this to be satisfactory. Our own investigation of this instrument shows it to be unsatisfactory for riboflavine assays because of its high instrumental blank (see Table I).

TABLE I
PERFORMANCE OF DIFFERENT FLUORIMETERS IN RIBOFLAVINE ASSAYS

Type of fluorimeter					Relative sensitivity	Instrumental blank as per cent. of F/1
Old Spekker	...	...	...	...	...	1	3
New ,,	...	...	...	...	...	7	10 to 15
Cohen type...	...	...	...	...	...	3 to 4	15
Electronic ...	...	...	...	...	...	63 to 73	1 to 2

Notes.—Relative sensitivities measured by deflections produced with F/1 using maximum sensitivity of each fluorimeter and the set-up of filters and cuvettes normally used in assays. Results given in comparison with Old Spekker as unity.
Instrumental blank determined with cuvette filled with distilled water and the usual set-up of filters.
Cohen type fluorimeter was prototype circulated to members of the Chemical Panel for critical examination, and was constructed to take test tubes instead of cuvettes.
Electronic fluorimeter used with photomultiplier at 78 volts per stage.

Attempts have been made to overcome the difficulty by developing a more sensitive fluorimeter which would permit the use of more selective filters and thus eliminate non-specific fluorescence in other parts of the spectrum. A prototype of the new Spekker fluorimeter (Hilger and

Watts) was found to possess still greater sensitivity, but its instrumental blank was still rather high. This blank has since been considerably reduced by improvements in the cuvette housing and by the use of black glass lids for the cuvettes. The improved instrument may, in our opinion, provide valuable service in riboflavine assays, especially if it is modified as we have recommended to take the new electron photomultipliers. We have been experimenting for over 2 years on the use of the latter to replace barrier layer photocells, and have constructed what is probably the most sensitive fluorimeter so far described in this country. Full details of this electronic fluorimeter were given to the Society of Public Analysts[9]. It employs as photoelectric detector either the RCA 1.P.21 or RCA 1.P.28 photo-tube or suitable British equivalents with a specially designed power pack and stable galvanometer. Table I shows that the sensitivity with the photomultiplier at 78 volts per stage is 63 to 73 times that of the old Spekker fluorimeter, and the instrumental blank has been brought quite low. It has given satisfaction during over a year's constant use, many of the results in this paper having been obtained with it.

FLUORIMETRIC METHOD

All chemicals to be of Reagent or similar quality. Pyridine to be freshly redistilled.

Extraction and hydrolysis. Weigh a sample containing about 25 to 35 μg. of riboflavine. Add 50 ml. of 50 per cent. v/v concentrated hydrochloric acid and heat in a boiling water bath for 20 minutes. Cool. Make up to 100 ml. with distilled water. Filter.

Oxidation. To 30 ml. of extract adjusted to pH 4 to 4·5 with saturated solution of sodium acetate, add 1 ml. of 4 per cent. potassium permanganate solution. If the pink colour does not persist for 1 minute, add further 1 ml. quantities of permanganate solution until it does. Remove excess of permanganate by adding hydrogen peroxide (3 per cent.) drop by drop. Adjust the pH to 4·5. Add sufficient pyridine to bring the concentration of pyridine to 1 per cent.

Adsorption and elution. Prepare an adsorption column of special fuller's earth 8 to 10 cm. long.* Wash the column with about 25 ml. of 2 per cent. acetic acid solution, followed by 15 ml. of water. Pour the whole of the oxidised extract through the column. Wash the column with 1 per cent. pyridine, examining in ultra-violet light, until the yellow band due to riboflavine is clearly defined on the column. Elute with solvent (20 per cent. pyridine in 2 per cent. acetic acid), collecting the yellow fluorescent eluate. Dilute to 50 ml. with solvent.

Fluorimetry. Dilute a suitable aliquot of eluate with solvent to produce 25 ml. dilution containing about 0·15 μg. riboflavine/ml. (= U). To another equal aliquot of eluate add 2 ml. of riboflavine standard (0·5 μg./ml.) in solvent and dilupte with solvent to 25 ml. (= UR). Take 5 ml. of riboflavine standard (0·5 μg./ml.) and dilute to 25 ml. (= S).

* Florisil brand 60/100 mesh (Wilkens-Anderson. Co., Chicago) has been found suitable.

Measure the fluorescence of U, UR, S and solvent only (= SB) against F/4 (0·25 μg. fluorescein/ml. in phosphate buffer pH 7) separately in the same cuvette, using as primary filter Wratten 47 protected by H503 or other suitable heat-resisting filter, and as secondary filter Chance's orange OY2 or other suitable filter.

Calculation. Convert all densities into antilogarithms. Take reciprocals of antilog. densities for solutions weaker than the F/4 standard. Let the results be U, UR, S and SB. Subtract SB from U, UR and S to obtain net fluorescence for each. Then, assuming that the net fluorescence is proportional to the concentration

$$\text{concentration of riboflavine in the U dilution} = \frac{(S-SB) \times \text{concentration of riboflavine in dilution of standard}}{U-SB}$$

$$\text{concentration of riboflavine in the UR dilution} = \frac{(S-SB) \times \text{concentration of riboflavine in dilution of standard}}{UR-SB}$$

Calculate the concentration of riboflavine in the sample using the data for weight of sample taken and volume of aliquot of eluate taken. The result can be corrected for percentage recovery of the riboflavine added to UR by multiplying by $\frac{100}{\text{percentage recovery}}$.

Calibration of Fluorimeter. The usual method of calibrating a fluorimeter is to measure the fluorescence of a series of solutions, of which the strongest has 4 to 8 times the concentration of the weakest. If the fluorescence plotted against the concentration gives a reasonably straight line, the linearity of response is considered satisfactory over the given concentration range, which may be rather narrow. Thus in a recent paper[10] a calibration curve for riboflavine was given based only on 4 solutions ranging from 0·06 to 0·24 μg./ml.

Our experience of riboflavine assays has led us to believe that calibration should be carried out over a much wider concentration range, including especially more dilute solutions to permit accurate evaluation of the blank. Moreover, we find it preferable to evaluate the linearity of response not visually from a calibration curve, but mathematically by calculating the ratio of net fluorescence to concentration of riboflavine, which should remain constant if the response is strictly linear. Table II gives details of one of our calibration experiments with the electronic fluorimeter. Column 2 in the Table gives the mean densities of a series of riboflavine solutions ranging from 1 down to 1/32 μg./ml. (approximately 0·03 μg./ml.) as compared with fluorescein standard F/4. Column 3 gives the antilogs. of these densities and Column 4 the reciprocals of these antilogs. for the more dilute riboflavine solutions of which the fluorescence was weaker than that of the fluorescein standard, so that the zero was set on the riboflavine. Column 5 gives the *gross* fluorescence of each riboflavine solution as percentage of that of the fluorescein standard, and Column 6 gives the corresponding *net* fluorescence, calculated by deducting from the gross fluorescence the

fluorescence of the solvent only in the same cuvette ("solvent blank"). Column 7 gives the ratio of the net fluorescence to the concentration, and should be constant. Column 8 shows the percentage deviation of this F/C ratio from the average for the 6 riboflavine solutions. The

TABLE II

LINEARITY OF RESPONSE OF SPEKKER FLUORIMETER USING R.C.A.1.P.28 ELECTRON
PHOTOMULTIPLIER

Calibration with riboflavine solutions at pH 5·7 matched against fluorescein standard F/4 (¼ μg./ml.)
using Wratten 39 and H503 primary filters and Chance's orange OY2 secondary filter.

Concentration of riboflavine μg./ml.	Mean density against F/4	Antilog. density (AD)	$\frac{1}{AD}$	Fluorescence as percentage of F/4	Net F	$\frac{F}{C}$	Percentage deviation of F/C from mean
1	2	3	4	5	6	7	8
Fluorescin at zero :—							
1	·387	2·438	—	243·8	237	237	0·2
½	·049	1·258	—	125·8	119	238	0·7
Riboflavine at zero:—							
¼	·188	1·542	·649	64·9	58·2	233	1·5
⅛	·436	2·729	·366	36·6	29·9	239	1·1
1/16	·665	4·624	·216	21·6	14·9	238	0·7
1/32	·855	7·161	·140	14·0	7·3	234	1·1
Solvent blank ...	1·175	14·96	·067	6·7	—	—	—

Net F calculated by deducting solvent blank from gross fluorescence.

greatest deviation is 1·5 per cent. and the mean deviation 0·9 per cent. In further calibration experiments the mean deviation was 0·4 and 0·2 per cent. (see Table III). This compares favourably with a mean devia-

TABLE III

EFFECT OF PRIMARY FILTERS ON DEGREE OF LINEARITY, SENSITIVITY AND INSTRUMENTAL
BLANK OF ELECTRONIC FLUORIMETER

Primary filter	Relative sensitivity as F/C ratio	Solvent blank as percentage of F/4	Sensitivity solvent blank	Average percentage deviaion from linearity
Woods	6·67	18·3	0·37	0·4
Wratten 39	2·37	6·7	0·35	0·7
,, 47	1·82	3·6	0·51	0·2

tion of 0·7 per cent. in the calibration data for 4 fluorescein solutions ranging from 1·25 to 6 μg./ml. published by Cohen[11] in 1935, and not unsatisfactorily with the mean deviation of about 0·2 per cent. in data published on 5 fluorescein solutions by Umberger and La Mer[12] in 1945, especially considering that fluorescein solutions are much more stable than riboflavine solutions.

Choice of primary and secondary filters. Table III also shows the effect of using different primary filters, and thus varying the nature of the incident light beam, with the object of reducing the magnitude of the solvent blank, which influences the deviation from linearity. When the Wood's glass filter supplied with Spekker fluorimeters was replaced

by a Wratten 39 filter (placed behind the H503 heat-resisting filter used with the Wood's) the solvent blank was reduced by about two-thirds, but the relative sensitivity was also reduced in similar degree, so that the sensitivity/solvent blank ratio was scarcely altered. The mean percentage deviation from linearity was increased from 0·4 to 0·7, so there did not appear to be any advantage in using the Wratten 39 as primary filter. With the Wratten 47 filter the decrease in the solvent blank was greater than the decrease in sensitivity, the sensitivity/solvent blank ratio being raised to 0·51. Moreover, the mean percentage deviation from linearity was reduced to the low figure of 0·2. Further calibration experiments with pure riboflavine solutions confirmed that the Wratten 47 filter gave better results than Wood's glass, and did not show the Wratten 47A filter to possess any distinct advantage over the Wratten 47. This justifies our choice of the Wratten 47 as primary filter. Figure 1 compares the transmission curves of these filters with the absorption and fluorescence spectra of riboflavine. If the fluorescence of riboflavine were due mainly to light absorbed above 400 mμ, as previous evidence[3] suggests, then the Wratten 39 or 47 filters should give higher results than the Wood's glass filter, which transmits practically nothing between 400 and 700 mμ. In fact, however, the Wood's glass gives similar results to the Wratten 39 or 47 as primary filter, suggesting that some of the fluorescence may be due to light absorbed below 400 mμ. (We have encountered some variation between different samples of filters. The data given in Figure 1 were obtained on the actual filters used in our assays.)
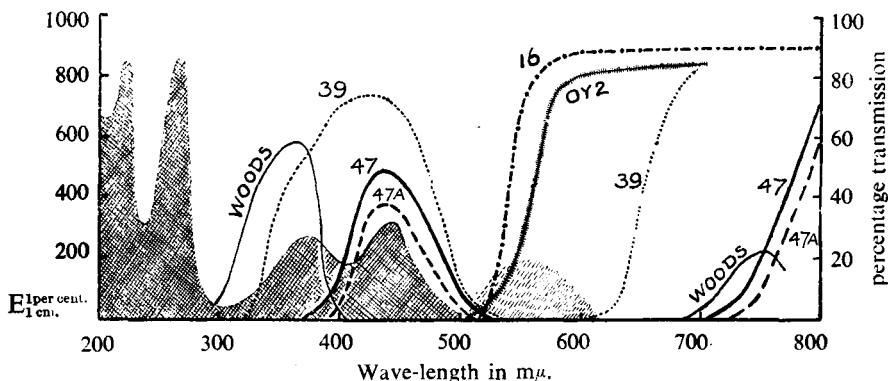

FIG. 1. Transmission curves of primary (Wood's, Wratten 39, 47, 47A) and secondary (Wratten 16, Chance's OY2) filters used in riboflavine fluorimetric assays, as compared with absorption and fluorescence spectra of the vitamin. The heavily shaded area between 200 and 500 mμ represents absorption spectrum under the given experimental conditions, and the lightly shaded area between 500 and 620 mμ indicates approximate position of fluorescence spectrum with maximum at about 570 mμ.

Turning to secondary filters, these should transmit radiation corresponding to the fluorescence spectrum of riboflavine but exclude other radiation, either from non-specific fluorescence or from exciting light reflected from the lid and sides of the cuvette[13]. Until the fluorescence

spectra of the interfering substances have been determined it is not pos-sible to give a completely satisfactory theoretical basis for the selection of the secondary filters to be used in assays on malted preparations. However, since these preparations can exhibit marked non-specific blue fluorescence, it seems desirable to use a secondary filter which does not transmit blue light. Theoretically this requirement is fulfilled either by the Chance's OY2 orange filter, which we have mainly used during the last 4 years, or by the Wratten 16 which Kodicek and Wang recently recommended. In practice the exclusion of the blue fluorescence is not complete. For exclusion of reflected exciting light either the OY2 or the Wratten 16 filter is unsuitable for use with the Wratten 39 as primary filter, since the latter lets through light between 600 and 700 mµ, which is also transmitted by these secondary filters. This disadvantage can be overcome by using Wratten 47 or 47A as primary filter. The com-binations of 47 primary with OY2 secondary, and of 47A primary with 16 secondary filters seem on a theoretical basis to be the best so far devised, and have given in our hands as good analytical results as any other combination of British or American filters we have tested, includ-ing the Lumetron 575. However, even the best filters may not provide effective correction when there is a considerable amount of interfering substance present. Table IV shows the effect of using different filter

TABLE IV

EFFECT OF DIFFERENT FILTER COMBINATIONS ON INTERFERENCE OF NON-SPECIFIC BLUE FLUORESCENCE IN MALTED PREPARATIONS

Filters				Net deflections (cm) given by		Percentage interference of blue fluorescence
Primary			Secondary	Blue non-specific	Riboflavine	
Wood's	...	...	...	OY2	3·35	14·7	23
39	...	...	...	...	,,	17·2	72·4	24
47	...	...	...	...	,,	5·7	26·3	22
47A	...	...	...	...	,,	2·6	11·3	23
Wood's	...	...	...	16	4·9	22·5	22
39	...	...	...	...	,,	25·0	127·0	28
47	...	...	...	...	,,	8·2	43·0	19
47A	...	...	...	...	,,	3·5	18·1	19
47A cal.	...	...	...	,,	3·4	15·9	21

Note.—Each of the above primary filters was protected by H503 filter, except the 47A Cal. where the H503 was replaced by a Calorex heat resisting filter. The numbers of filters refer to Wratten gelatin filters, except OY2 which is a Chance's glass filter.

combinations on a mixture of riboflavine with some of the non-specific blue fluorescent material obtained from malted preparations as described below. Using OY2 as secondary filter, no appreciable improvement in results was obtained by replacing the Wood's glass by 39, 47 or 47A primary filters. When Wratten 16 was used as secondary filter, the use of Wratten 39 as primary filter led to less satisfactory results.

Destruction of riboflavine to provide blanks. Various procedures have

been tried for destroying the riboflavine in extracts, assuming that the residual fluorescence correctly represents the non-specific fluorescence of the extracts. We have found the following objections to these procedures : —

(a) *Sodium hydrosulphite* destroys the non-specific fluorescence as well as that of riboflavine. Whilst the latter is more readily destroyed, attempts to control the destructive process by estimating the fluorescence at different stages are baulked by the fact that sodium hydrosulphite absorbs some of the rays needed to produce fluorescence in riboflavine[10].

(b) *Irradiation* under carefully controlled conditions will destroy riboflavine[3], but may produce lumiflavine, the blue fluorescence of which can interfere with the fluorimetric measurements. The method is also very tedious.

(c) *Strong alkali* (*p*H above 11) will destroy riboflavine in absence of light, but the shift in *p*H reduces the fluorescence of any undestroyed riboflavine and makes it difficult to follow and control the destructive process, which otherwise can destroy non-specific fluorescence.

(d) *Stannous chloride* destroys not only riboflavine, but also non-specific fluorescence.

Removal of interfering substances. The alternative to removal of riboflavine is the removal of the interfering substances. Attempts have been made to effect this in various ways with the following results : —

(a) *Decolorisation with a fixed proportion of potassium permanganate,* followed by bleaching with peroxide, has been recommended by some workers. We found that this often led to high results, especially with malted preparations. Table V summarises some of our results on the use of permanganate and of different filters to correct for the presence of inter-

TABLE V

FLUORIMETRIC ESTIMATION OF RIBOFLAVINE IN MALT EXTRACT—CORRECTION FOR
INTERFERING SUBSTANCES BY PERMANGANATE TREATMENT AND BY USE OF FILTERS
MEAN FLUORIMETRIC RESULTS STATED AS PERCENTAGE OF MEAN MICROBIOLOGICAL
RESULTS

Filters		New malt extract		Old malt extract	
Primary	Secondary	No Potassium permanganate	Potassium permanganate	No Potassium permanganate	Potassium permanganate
Woods	OY2	668 —	— 310	1317 —	— 502
Woods + HR ...	OY2	740 —	— 307	1566 —	— 474
Wratten 47 + HR...	OY2	496 —	— 114	1094 —	— 199
Wratten 47A + HR	OY2	570 —	— 203	1223 —	— 184
Wratten 47A + HR	Wratten 16	568 —	— 109	1144 —	— 183

Notes.—" New " malt extract was about 6 months old ; " Old " malt extract about 3 years old. Both had been stored at room temperature. HR filter = Chance's H503. OY2 = Chance's orange No. 3. The extracts were all made with N/10 hydrochloric acid for ¼ hour in boiling water-bath. No Florisil treatment was used.

fering substances. It shows that in the absence of permanganate treatment the fluorimetric results were all much too high, and were very little affected by the use of different filters. When permanganate was used the results were all much lower. On a fairly new malt extract they were still too high with the usual set-up of Wood's and OY2 filters, but were brought much closer to the microbiological result by using Wratten 47 or 47A as primary filter. On a 3-year-old malt extract containing much more of the interfering substances the Wood's plus OY2 filters gave much too high results, and the use of the heat-resisting HR filter made no appreciable difference, showing that there was no interference from infra-red rays. Again, the use of Wratten 47 or 47A filters brought the results lower, but they were still considerably higher than the microbiological results and we had to resort to Florisil treatment to obtain satisfactory agreement. The combination of filters recommended by Kodicek and Wang was of similar efficiency to the combination we had been using. When we applied the full method of these workers, including their filters, we found that the preliminary washing of the extract with chloroform did not make the results satisfactory. As we had found the interfering blue fluorescence of malted preparations by no means completely soluble in chloroform, we were not surprised at the failure of the chloroform treatment to provide complete correction. On following Kodicek and Wang's method as closely as possible we found that on our malted preparations it gave rather too high a pH (about 5) for optimal decolorisation. A pH of about 4 gives better results.

(b) *Effect of varying amount of permanganate used.* Permanganate has been used by many previous workers to eliminate interfering substances in riboflavine assays. However, none of these seems to have provided information about the effect of varying the amount of permanganate used. Most workers seem to rely on using a fixed proportion. Kodicek and Wang[10] have recognised the need for varying the amount of permanganate with different materials, but we have found their dropwise addition of permanganate very tedious with our malted preparations. Table VI gives some of our results showing that, whereas a dried yeast

TABLE VI

EFFECT OF VARYING AMOUNT OF PERMANGANATE IN RIBOFLAVINE ASSAYS USING
DIFFERENT PRIMARY FILTERS

MEAN FLUORIMETRIC RESULTS STATED AS PERCENTAGE OF MEAN MICROBIOLOGICAL
RESULTS

Volume of potassium permanganate solution added to aliquot ml.	Primary filter used		
	Woods	Wratten 39	Wratten 47
Yeast sample—			
0	397	177	169
1	116	102	92
3	59	54	56
Malted food—			
3	165	150	131
9	98	96	103

sample needed only 1 ml. of the 4 per cent. permanganate per aliquot to give satisfactory results, and 3 ml. was far too much, a malted food sample needed 9 ml. per aliquot. Some of our old malt extracts required much more than 9 ml. Table VI shows that when too little permanganate has been used, the results can be considerably improved by using more specific filters to eliminate interfering fluorescence. The results in the Table were obtained using Florisil to remove interfering substances as described in our method above and show that it is equally essential to use the right amount of permanganate. If this is done, the choice of more selective filter combinations becomes less important.

(c) *Phase separation.* Our aqueous extracts were shaken with various immiscible organic solvents under the ultra-violet lamp to detect whether separation of the riboflavine fluorescence from non-specific fluorescence was being effected. With amyl alcohol, chloroform, ether and light petroleum, slight separation occurred, but could not be made complete. With benzene, diacetone alcohol and isobutyl alcohol no separation was detected.

(d) *Chromatographic separation.* Elvidge[2], using the Connor and Straub[14] method, examined a number of grades of fuller's earth and selected P.A. of the Fuller's Earth Union as the most suitable. We tested a sample of this grade, but did not find it to give satisfactory purification of our materials. Other samples of fuller's earth, also of alumina and of Decalso, likewise proved unsatisfactory. We then tried Florisil, which has been largely employed by American workers, and found that it removed some, but not all, of the non-specific fluorescence when used as they recommend[15]. Observations under the ultra-violet lamp showed that riboflavine is adsorbed more strongly than the interfering substances. After numerous trials we found that elution with 1 per cent. aqueous pyridine could separate most of the interfering substances, the procedure being controlled under the lamp. 2 per cent. pyridine was seen to elute riboflavine almost as rapidly as it eluted the interfering substances, so that complete separation could not be effected. We found that the adsorption of riboflavine on Florisil was equally effective between pH 1 and pH 6. Contrary to American workers[15], we did not find the adsorbed riboflavine to be removed by washing the column with large volumes of water. Elution with 20 per cent. pyridine in 2 per cent. acetic acid gave recoveries ranging from 90 to 100 per cent.

Riboflavine in malt extract. On studying the history of the 5 malt extracts for which fluorimetric and microbiological results were given in our paper[1], we found that the extract on which the fluorimetric method gave a much higher result than the microbiological method was in fact an old one. This led us to compare the fluorimetric and microbiological results on a series of malt extracts of different known ages, all stored at room temperature. The results plotted in Figure 2 showed a definite tendency for the fluorimetric/microbiological ratio to increase as the extracts become older. Assuming that a difference of 20 per

cent. between the two methods was significant, then in 8 extracts less than 2 months old, not one showed a significant difference between the two methods, in 8 extracts 40 to 130 weeks old, 7 gave a fluorimetric result significantly higher than the microbiological result, the average fluorimetric/microbiological ratio being 1·78 and in 7 extracts 3½ to 4½ years old the fluorimetric result was always significantly higher than the microbiological result and the average fluorimetric/microbiological ratio was 2·64. (The fluorimetric result on one of this last 7 was too high to include in Figure 2.)

We confirmed these findings by assaying fluorimetrically and microbiologically at intervals the riboflavine in malt extracts stored under known conditions. Figure 3 gives typical data showing a steady rise in
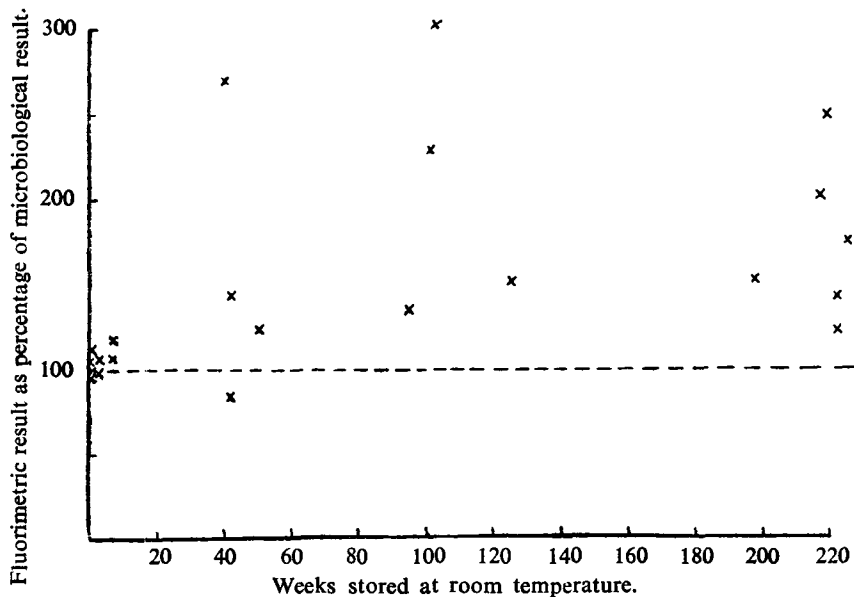

FIG. 2. Riboflavine assays on malt extract—relation between fluorimetric and microbiological results after different periods of storage.

the fluorimetric result and a steady fall in the microbiological result during 2½ years' storage at room temperatures of a malt extract fortified with riboflavine. Similar results are being obtained with unfortified malt extract and malted preparations, but, as the riboflavine in these seems to be more stable, longer storage periods are needed for the microbiological method to show a definite loss of the vitamin.

Non-specific fluorescence in malt extract. When using the ultra-violet lamp to control our purification procedures we discovered that old malt extracts exhibit a marked blue fluorescence which is taken up by the pyridine/acetic solvent for the riboflavine, and cannot be efficiently separated from the latter by organic solvents, e.g., benzene, chloroform, ether. This blue fluorescence is much less marked in new malt extracts. It can be developed by oxidation of the malt extract (e.g., with cold

potassium permanganate), a procedure which also develops it in aqueous extracts of barley, oats and yeast. Stronger acid extracts of these exhibit, before oxidation, a non-specific yellow fluorescence which seems not to be due to riboflavine since it is only feebly absorbed by Florisil, and is converted into a blue fluorescence by permanganate oxidation. These observations indicated that the blue fluorescence is not due to lumichrome. The spectrum of the blue fluorescence has been approximately determined using the technique described by one of us (F.W.N.)[6]. Comparison of this fluorescence spectrum and that of riboflavine with the transmission curves of various secondary filters indicated that the Wratten 47 should give the greatest degree of selectivity, but could not be expected to provide completely satisfactory correction for the non-specific fluorescence. Such correction was ensured by applying the chromatographic technique described above.
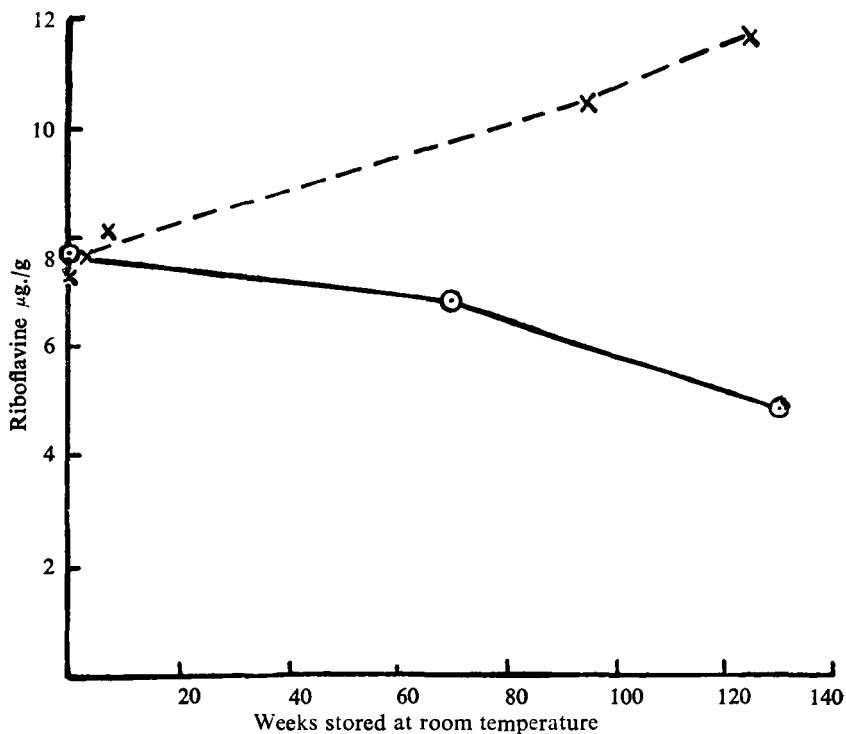

FIG. 3. Effect of storage on riboflavine content of malt extract as measured fluorimetrically X— —X and microbiologically ⊙—⊙

Effect of varying the primary filter. Our experiments with pure riboflavine solutions described in the early part of this paper showed that, as " primary filter," Wratten 47 placed behind a heat-resisting filter might be expected to give the best results. This expectation was confirmed in assays on a series of malted preparations and other foods. The average results, summarised in Table VII, showed that after adopting all the above precautions and carefully carrying out the purification

procedures, the fluorimetric results on all the samples averaged 126 per cent. of the microbiological results when using Wood's glass, 107 per cent. when using Wratten 39, and 105 per cent. when using Wratten 47. The variability in results, as indicated by the standard deviation of the mean, was also lowest with the Wratten 47 filter.

Application of improved method to old malt extracts. As a final check on our improved method we applied it, using the Wratten 47 as primary filter, to a series of old malt extracts stored under known conditions for considerable periods of time. With our previous fluorimetric method these would have given results very much higher than the microbiological results. It will be seen from Table VIII that our improved method gave on this series of old malt extracts fluorimetric results in satisfactory agreement with microbiological results, and seems to have overcome the discrepancies earlier encountered by ourselves and other workers. (The table also gives details of the fluorimetric and microbiological results

TABLE VII

EFFECT OF USING DIFFERENT PRIMARY FILTERS IN FLUORIMETRIC RIBOFLAVINE ASSAYS

Sample	Fluorimetric result as percentage of microbiological result using as primary filter behind heat-resisting filter		
	Wood's glass	Wratten 39	Wratten 47
Malt and soya food	98	97	103
Malt and soya food	174	129	122
Dried yeast	115	102	92
Flour, fortified	124	101	108
Malt yeast preparation	103	90	94
Malt food A	111	102	99
,, ,, B	103	98	100
,, ,, C	114	109	106
Malt extract	198	138	121
Means	126	107	105
Standard deviation of mean	10·5	5·2	3·6

TABLE VIII

FLUORIMETRIC AND MICROBIOLOGICAL ASSAYS OF RIBOFLAVINE IN MALTED PREPARATIONS AND OTHER FOODS

Sample	Riboflavine μg./g.		Fluorimetric result as percentage of microbiological
	Fluorimetric	Microbiological	
Malt extract 7½ years old	3·93	3·90	101
,, ,, 7 ,, ...	4·37	4·0	109
,, ,, 7 ,, ...	3·65	3·60	102
,, ,, 4 ,, (1)	2·5	2·4	104
,, ,, 4 ,, ...	3·15	3·10	102
,, ,, 3 ,, ...	4·3	4·0	108
,, ,, 3 ,, ...	5·91	5·8	102
,, ,, ½ ,, (fortified)	14·2	15·1	94
Malted preparation (2)	11·8	11·5	103
,, ,, (3)	18·5	17·1	108
,, ,, (3)	23·0	23·1	100
,, ,, (3)	25·0	23·8	105
Dried yeast (4)	94·1	102·0	92
Flour, fortified (4)	3·68	3·4	108

Notes.—(1) Sample prepared for the Analytical Methods Sub-Committee of the Society of Public Analysts. (2) Prepared from malted cereals and soya for clinical trials in this country. (3) Samples of baby foods. (4) Samples obtained from the Association of Vitamin Chemists, Chicago.
  The average coefficient of variation for a single fluorimetric assay was 4·6 in 13 consecutive assays, and for a single microbiological assay was 5·6 in 35 consecutive assays.

on some samples mentioned in the previous table). We therefore think that the fluorimetric method we have described in this paper can be relied upon to give satisfactory results on different foods, including malted preparations, with which difficulties have previously been encountered.

DISCUSSION

We have found that in devising baby foods based on malted cereals and soya the raw materials can provide the baby's requirements of aneurine and nicotinic acid, but the content of riboflavine is more critical. By far the greater part of this vitamin in such foods comes from the malted barley, and as we have previously shown, its content of riboflavine can vary widely according to the efficiency of malting. Hence the particular need for riboflavine assays on malted products. Several years' experience with microbiological assays of riboflavine has led us to believe that these can measure the riboflavine value of a food more accurately and much more conveniently than biological assays. However, in the routine control of daily batches of food products it is not always possible to wait for the results of microbiological assays, and here fluorimetric assays are valuable.

With foods of high riboflavine content (e.g., liver, milk, yeast) good agreement with microbiological results can be provided by fluorimetric assays without resorting to chromatography for removal of interfering substances[16]. These, however, may need treatment with a reducing agent such as sodium hydrosulphite or stannous chloride. These workers then shook their extracts vigorously with air to regenerate riboflavine, but this procedure was not favoured by subsequent workers.

With foods of lower riboflavine content, including unmalted or malted cereals, more efficient purification has been found necessary. Decolorisation with permanganate, as applied to lyochromes by Koschara[17] in 1935, was employed on foods by Connor and Straub[14], who also used supersorb, a brand of fuller's earth as adsorbent, and pyridine and acetic as eluant. Amongst other adsorbents tried have been lead sulphide[18], superfiltrol[19] and Florisil[20], the two latter being special brands of fuller's earth. Several workers[4,16] have obtained satisfactory results without using adsorbents, but not on malted preparations. Difficulties in obtaining reproducible results have been reported[19]. We could not find any record of other workers using the ultra-violet lamp to check their purification procedures, and detecting the non-specific blue fluorescence we have found in malted preparations. The fact that this fluorescence may be developed by permanganate emphasises the need for caution in the use of this reagent. The low solubility of the blue fluorescent substance in chloroform show it not to be lumichrome. It is most effectively been recommended for purifying extracts[10].

SUMMARY

1. Using a more sensitive fluorimeter than has previously been described in this country, a method is given for estimating riboflavine in malted preparations which gives much better agreement with microbiological assays than had previously been obtained.

2. These preparations when oxidised with permanganate during the purification procedure exhibit a non-specific blue fluorescence which interferes with the fluorimetric assay. In old samples of malt extract this blue fluorescence is quite marked before permanganate treatment. The behaviour of the fluorescent substance with Florisil and its low solubility in chloroform show it not to be lumichrome. It is most effectively separated from riboflavine by adsorption on Florisil and careful elution with 1 per cent. pyridine, the process being observed continuously under the ultra-violet lamp.

3. Spectroscopic studies indicated Wratten 47 as a satisfactory primary filter. Comparison of fluorimetric and microbiological results confirmed this.

4. When calculating the results of riboflavine fluorimetric assays a method based on the assumption that the *net* fluorescence is proportional to the concentration, and using solvent blanks to determine the net fluorescence, is preferable to the method more usual in this country of using calibration curves obtained by plotting *gross* fluorescence against concentration.

We are indebted to Mr. E. J. Bowen, F.R.S., for advice, to Miss Janet Horsford and Mr. R. Evans for technical assistance, to Mr. G. Slaughter for the fluorescence spectrum of riboflavine, to Messrs. Hilger and Watts, Ltd., and Messrs. Kodak, Ltd., for data on the transmission of filters.

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DISCUSSION

The two papers dealing with malted preparations were discussed together.

The first was read by Dr. F. Wokes, the second by Miss C. Klatzkin.

THE CHAIRMAN said that the diastatic value introduced in the B.P., 1948, was equivalent to a Lintner value of 50, which in these days could hardly

be regarded as a moderate diastatic value. Before the war, when Canadian high diastatic malt was available, it was quite easy to obtain such values, but now with English barley, it was not possible to do so. In the Report published by the Pharmacopœia Commission before the war a standard of 15° Lintner was recommended. Unfortunately, owing to an oversight for which he must take the major responsibility, the method given in the B.P. was equivalent to a diastatic value of 50, but even a standard of 15° Lintner was not obtainable in these days with any malt, except in rare samples. The same applied to the nitrogen figure. With English malt, unless it was possible to select samples, a figure of 4·5 per cent. was quite often not attainable.

MR. G. E. SHAW (Runcorn) asked whether the term " soluble protein " was used to denote a more or less denatured protein. Had the authors any evidence for the presence of animal protein factor in a malt extract, and, if so, did it develop during gemination? The percentage of protein required in a semi-synthetic diet was very different if there was a sufficient amount of animal protein factor present. Had the authors tried paper partition chromatography for the estimation of the riboflavine?

DR. F. WOKES, in reply, said that the diastatic value of malted barley depended on the barley which was available, and Canadian barley was higher in this respect than English barley; they had had no difficulty in obtaining diastatic values and protein contents considerably higher than the minima. On the question of the solubility test for the protein, they took the American method, which was a recognised one for solid soluble protein.

He was not quite clear what was meant by the term " animal protein factor " as applied to vegetable protein. The suggestion had been made that germination might develop this so-called factor, and it was true that there did seem to be some increase in the biological value of the proteins not only of barley but of other malted cereals after they had been malted. Whether that was due to the production of an animal protein factor, or whether it was due to the destruction of inhibitors of the type of the inhibitor in soya, he did not know.

MISS C. KLATZKIN, who also replied, said that they had not done any paper partition chromatography, because they had aimed at a quantitative method. With regard to the second yellow field as well as the blue fluorescence, they had evidence in malt extract, and more particularly in yeast, of a second yellow fluorescent material which was not riboflavine.

THE CHAIRMAN asked why the authors used the B.P.C. 1923 method for diastatic value in place of the official method.

DR. F. WOKES said that they had used it in the past and had simply continued to do so. They had done more work with the B.P.C. method than with the Lintner test.

THE CHAIRMAN, in bringing the meetings to a close, said that it only remained for him to ask the members to express their thanks to those who had contributed papers to the Conference. The Science Sessions had been exceedingly well attended, and the discussions had been most valuable.