The Assay of Riboflavin in Cereals and other Products

1. MICROBIOLOGICAL ASSAY

2. FLUOROMETRIC ASSAY

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(*Received 12 September 1942*)

1. MICROBIOLOGICAL ASSAY

The fluorometric methods of estimating riboflavin developed by Hodson & Norris [1939], Najjar [1941] and others have so far proved to be unsatisfactory in our hands when applied to cereals, for reasons referred to in Part 2. The microbiological method introduced by Snell & Strong [1939], however, with suitable modifications has been found to be satisfactory and expeditious.

EXPERIMENTAL

Organism. The organism used was *Lactobacillus helveticus* from Snell & Strong's original strain no. 7469 obtained from the American Type Culture Collection, Georgetown University, Washington, D.C.* A number of different strains of *L. helveticus* were tested for a quantitative response to riboflavin, but with the exception of a strain from Dr J. G. Davies of the National Institute for Research in Dairying, Reading, and which originated in U.S.A., none gave a quantitative response to riboflavin. The majority grew equally well in the absence of the vitamin, while others gave an 'all-or-none' reaction.

The technique employed for the growth of the organism and for the assay of riboflavin was similar to that employed by Snell & Strong with certain modifications which are described below.

Stock cultures of the organism were carried on a yeast-water glucose agar. The cultures were kept in a refrigerator at a temperature of approximately 4° and removed at monthly intervals.

Basal medium. The composition of the riboflavin-free basal medium for the determination of the vitamin was the same as that recommended by Snell & Strong, with, however, the further addition of asparagine, xylose, pantothenic and nicotinic acids. The composition of 250 ml. of the final medium, sufficient for 50 assay tubes was as follows:

50 ml. photolysed NaOH-treated peptone solution; 50 ml. cystine solution; 12·5 ml. of a 1 % asparagine solution; 5·0 g. glucose; 0·25 g. xylose; 2·5 ml. solution A;* 2·5 ml. solution B; 5 ml. yeast supplement and nicotinic and pantothenic acids so that every ml. of the medium contains 0·05 μg. of these two substances.

Media and extracts were always made with glass-distilled water; this precaution is essential.

Preparation of inoculum. The procedure described by Snell & Strong for the preparation of fresh batches of inoculum for assay purposes was not found to be satisfactory, as there was frequently heavy growth in the riboflavin-free blanks. On this account a fresh inoculum was always prepared from the stock agar culture for each set of determinations.

The presence of xylose in the medium has a two-fold action. In the first place the results between replicates were found to be more concordant whether direct titration with 0·1 *N* NaOH or a nephelometric method were employed for the actual determination, and in the second place, in the case of nephelometric determinations there was a larger 'spread' between concentration levels.

The fermentations were carried out in ordinary chemical or bacteriological test-tubes (18 × 150 mm.) and the technique described by Snell & Strong was strictly followed.

Two methods have been used to determine quantitatively the response of the organism to added riboflavin. In the first the turbidity produced by the growth of the bacteria is measured. This procedure is difficult with highly coloured extracts and special precautions have to be taken. In the second method the acid produced during growth is determined by direct titration with 0·1 *N* NaOH, using bromothymol blue as indicator.

If a nephelometric method be employed, then it is necessary on statistical grounds to have five repli-

* We are indebted to Dr J. C. Drummond for obtaining this culture from Washington.

* Solution A is composed of 25 g. K_2HPO_4 and 25 g. KH_2PO_4 dissolved in 250 ml. distilled water. Solution B contains 10 g. $MgSO_4.7H_2O$, 0·5 g. NaCl, 0·5 g. $FeSO_4.7H_2O$. 0·5 g. $MnSO_4.4H_2O$ dissolved in 250 ml. distilled water. Solution B tends to deposit a dark coloured precipitate upon long standing, so that the solution should be well shaken before use.

cates for each level of concentration. Snell & Strong consider that two are sufficient. This number, however, is too small and the results cannot be relied upon. Moreover, for the unknown solutions whose riboflavin content is to be determined there must also be at least three 'blanks' for each concentration level. These 'blanks' are inoculated in the same way as the other tubes and placed in a refrigerator so that bacterial growth is prevented. The readings for the blanks are subtracted from the values obtained for the tubes in which growth has taken place.

The estimation of acid formed during fermentation, however, was found to be the most convenient method. It is simpler and less cumbersome than a nephelometric method and fewer tubes are required for an assay. Here again, Snell & Strong recommend only two tubes for each concentration level. It is, however, from the practical standpoint, much more satisfactory to use three, and this number was always employed in the present investigation.

For the actual titration a comparator was used. The procedure adopted was the same as that described by Cole [1928] for the estimation of amino-acids. Instead of the contents of the tube being washed into a conical flask and then titrated to *p*H 6·8 using a colour-comparison flask to obtain the necessary end-point as Snell & Strong recommend, the titration was carried out in the fermentation tube itself. A great saving of time is effected by this procedure, and the end-point is clearer and more easily determined than with a colour-comparison flask.

With this method the titration is carried out as follows:

A buffer solution of *p*H 6·8 is prepared by mixing 50 ml. of a 0·2*M* solution of KH_2PO_4 with the equivalent of 23·65 ml. of 0·2*N* NaOH, and diluting to 200 ml. 20 ml. of this solution is then pipetted into a test-tube (18 × 150 mm.) and 20 drops of bromothymol blue indicator added. 20 ml. of distilled water is pipetted into another tube. Two of the tubes of which the acid content is to be determined are now taken and to one is added 10 drops of indicator and the tubes are then arranged in the comparator as shown in the following diagram:

A	B
C	D

Tube A contains distilled water and tube B buffer solution + indicator. Tubes C and D contain the assay solutions and to C indicator is added. No indicator is added to D.

Tube C is now titrated with 0·1*N* NaOH, and for every ml. or part of a ml. of standard alkali added an extra drop of indicator is also added, while to tube D the same amount of standard alkali is run in from the burette. The titration is completed when the colours seen through C and D exactly match.

Preparation of extracts. It has been shown by Scott, Randall & Hessel [1941] that starch has a marked effect in stimulating the growth of *L. helveticus*, and as cereals contain a large amount of starch, this must first be destroyed in the extract before the estimation is carried out. Scott *et al.* used taka-diastase for this purpose. In the present investigation, however, taka-diastase was found to be unsatisfactory. The various samples of the enzyme that were tested showed a marked stimulatory action over other hydrolysing agents, such as ptyalin, H_2SO_4 and HCl. The method of extraction that was eventually adopted was to hydrolyse suitable weighed samples of material with 0·25*N* HCl for 15 min. at 15 lb. pressure in an autoclave. For cooked wheaten products, such as bread, the time of hydrolysis must be increased to 30 min. Nevertheless, hydrolysis with ptyalin in place of taka-diastase or refluxing for 1 hr. with 0·25*N* H_2SO_4 [Hodson & Norris, 1939] or with 0·25*N* HCl were all found to give the same results as autoclaving for 15 min. Autoclaving of the material was adopted simply because it was found to be more convenient. After hydrolysis the *p*H of the extract was adjusted to 6·8 with NaOH and made up to the requisite volume. For the great majority of cereals it was found that a 5 g. sample of the finely ground material hydrolysed with 50 ml. of 0·25*N* HCl and made up to 500 ml. was a convenient quantity for assay. For substances with a low riboflavin content, such as patent flours, 10 g. samples were used. When a high riboflavin content was suspected, e.g. germ, 1 g. samples were used. A convenient rule to follow is to have the extract of such a strength that it contains a total of approximately 10–12 μg. of riboflavin. For each assay three different levels of concentration must be used. The results should not differ among themselves by more than 10 %, and the mean of the values is taken as the riboflavin content. In the present investigation the levels of concentration taken were, 2, 3 and 4 ml. of the extract.

The results from the different methods of extraction that were employed are given in Table 1.

Table 1

Sample	Riboflavin content μg./g.
National Straight Run flour (75 % extraction)	
1. Hydrolysis with ptyalin	0·75
2. Refluxed with 0·25*N* H_2SO_4 for 1 hr.	0·75
3. Refluxed with 0·25*N* HCl for 1 hr.	0·80
4. Autoclaved at 15 lb. pressure for 15 min. with 0·25*N* HCl	0·80
5. Hydrolysed with taka-diastase	1·15

Fats, like starch, exert a stimulating effect upon the growth of *L. helveticus*, and substances with a high fat content, such as soybean, maize, germ and milk, must first be defatted before an

extract is made. Cereals with a high fat content are best defatted by extraction in a Soxhlet extractor with light petroleum. Milk should be separated on a centrifuge and the serum shaken up in a separating funnel with ether. Light petroleum is not a suitable fat solvent with milk, as the mixture tends to form an emulsion.

The stimulating effects of fats on *L. helveticus* are shown by the results in Table 2.

Table 2

Sample	Riboflavin content μg./g.
Milk	
1. Raw	1·95
2. Separated	1·66
3. Separated and extracted with petrol ether	1·66
4. Separated and extracted with ether	1·45

EXPERIMENTAL RESULTS

The results of a number of determinations on different varieties of wheat, various fractions of the wheat grain, wheaten flours and other cereals are given in Table 3; figures are based on the normal water contents of the products.

Table 3

Sample	Riboflavin content μg./g.
1. Wheat, English	1·9–2·65
2. Wheat, Manitoba	2·7–2·9
3. Wheat, Australian	2·4–2·65
4. Wheat, Russian	3·7
5. Wheat, Testa	3·9–5·8
6. Wheat, Aleurone layer	6·3–7·55
7. Wheat, Germ. Commercial sample 1	11·35
8. Wheat, Germ. Commercial sample 2	12·85
9. Wheat, Germ. Commercial sample 3	9·7
10. Flour, A roll	0·4–0·5
11. Flour, C roll	0·5–0·6
12. Flour, National Straight Run (75% extraction)	0·75–1·25
13. Flour, National Wheatmeal (85% extraction)	1·7–2·1
14. Barley	2·3
15. Rye	2·65
16. Maize	2·3–2·7
17. Soybean	3·0–4·9

The riboflavin content of samples of baker's yeast and brewer's yeast was also determined.

	Riboflavin content μg./g.
Baker's yeast	23·0
Brewer's yeast	66·0

The possible destruction of riboflavin by baking was investigated. A control loaf and a loaf prepared from the same flour to which 2 μg./g. of synthetic riboflavin had been added were assayed. The crumb was air dried in the dark and in both cases had a moisture content of 6·7 % (Table 4).

Table 4

	Riboflavin content μg./g.
1. Control loaf	2·85
2. Loaf + 2 μg./g. added riboflavin	4·80
3. Recovered riboflavin	1·95

It will be seen from the results in Table 4 that there is no significant destruction of riboflavin in the baking process.

A survey of the riboflavin content of different milling fractions of an English wheat which was specially milled by our colleague Mr A. G. Simpson gave the results in Table 5.

Table 5

Sample; English wheat (dry milled, moisture content 14·0%)

	Milling fraction %	Riboflavin content μg./g.	Contribution of riboflavin to Straight Run flour %	Contribution of riboflavin in whole wheat %
Break and scratch flours	23·4	0·9	18·8	11·1
Semolina reduction flour	7·2	0·5	3·5	2·1
Middlings (dunst reduction flour 1)	43·6	0·6	23·2	13·7
Middlings (dunst reduction flour 2)	5·4	4·4	22·7	13·4
Middlings (dunst reduction offals, retreated flour)	1·9	6·2	10·7	6·3
Bran and scratch offals, retreated flour	4·1	5·8	21·5	12·7
Straight Run flour (Calculated value)	85·6	1·5 (1·32)	100·4	59·3
Bran	6·0	6·9	—	18·0
Pollards	5·0	6·3	—	13·6
Fine offals	3·4	6·3	—	9·4
Whole wheat (deter.)	100·0	2·4	—	100·3
Whole wheat (calc.)	—	2·3	—	—

This wheat was milled in the laboratory by a special technique designed to give the maximum amount of white flour. It differs from that which can be normally manufactured in commercial practice.

2. FLUOROMETRIC ASSAY

Two major difficulties have been encountered in this estimation when carried out by any of the previously published fluorimetric methods: (1) the presence of colloidal matter and/or fine suspended particles in the final riboflavin solution as prepared for visual or photoelectric assessment of its fluorescence; this

material cannot be removed by centrifuging or normal filtration and its effect in absorbing and scattering the blue exciting light can easily be so great as to mask completely any fluorescence; (2) the initial low concentration of riboflavin in wheat and the majority of the products thereof which causes the fluorescence of final solutions derived from such materials to be too small to be measurable with any degree of accuracy.

The first of these difficulties was overcome by recourse to the procedure of Najjar [1941] in which the riboflavin is extracted by a non-aqueous medium; by this means all suspended material is left in the aqueous layer.

The second difficulty is of a more serious nature—adsorption of the riboflavin from a fairly large quantity of a wheat extract, followed by elution by means of a small volume of liquid was considered to be most worthy of trial as also by this means various interfering fluorescent materials present in the extract might be eliminated.

Freshly precipitated lead sulphide (as recommended by Najjar for urines) was found to be unsatisfactory for wheat or flour extracts; as also was English fuller's earth, even of grades specially selected for adsorption purposes. Through the kindness of Dr B. S. Platt opportunity was afforded of trying out an American fuller's earth of the 'white' variety ('Superfiltrol') and this has proved satisfactory.

The method which follows has been elaborated around the 'Spekker' fluorimeter but may be adapted readily to other types of fluorimeters.

EXPERIMENTAL

20 g. of finely ground wheat, flour or dried bread (or quantities of milling offals containing approx. 20–50 μg. of riboflavin) are taken in a 250 ml. flask and are suspended with shaking in 100 ml. of 0·25 N H_2SO_4. Two further flasks are made up in the same way but with the addition of 10 and 20 μg. respectively of riboflavin, conveniently added as 1 and 2 ml. respectively of a 10 μg./ml. standard solution. The flasks are plugged with cotton wool and are autoclaved for 15 min. at 15 lb. pressure. (In order to ensure complete hydrolysis of pre-cooked wheaten products such as bread, it is advisable to prolong autoclaving to 30 min.—see Part 1.)

After cooling, the digests are adjusted to approx. pH 5 by the dropwise addition of 30 % NaOH, using brom-cresol green as an external indicator. They are adjusted to a convenient volume (125 ml.) and are filtered through a no. 5 Whatman paper using suction. The filtrate should at this stage be crystal clear.

Three 50 ml. conical centrifuge tubes are prepared, each containing 0·5 g. ($\pm$ 5 mg.) of 'Superfiltrol' and 45 ml. aliquots of each digest are added to them. The fuller's earth is then maintained in suspension for 5 min. by brisk mechanical stirring, a fine glass rod bent at the tip being convenient for the purpose. After centrifuging, the supernatant liquor is carefully poured off and discarded and approx. 20 ml. of distilled water is added to each tube. After agitation for 2 min. in order to wash thoroughly the earth the tubes are again centrifuged and the washing water decanted off. The tubes are then allowed to drain in an inverted position for a few minutes, after which all traces of water adhering to the inside of the tube are wiped away as far as possible with a clean glass cloth.

Elution of adsorbed riboflavin is carried out by the addition of 8 ml. of a reagent with the following composition: 9 vol. of water, 5 vol. of pyridine (redistilled) and 2 vol. of glacial acetic acid. Mechanical stirring is again employed for 5 min., after which the tubes are centrifuged at slow speed for 5 min.

The eluate from each tube is decanted as completely as possible into suitable glass-stoppered flasks or cylinders, with a total capacity of not less than 33–34 ml. (25 ml. measuring cylinders are usually of this total capacity and are convenient for the purpose.)

To each vessel is added 3 drops of 4 % $KMnO_4$ solution; it is mixed and allowed to stand for at least 1 min. to oxidize interfering substances. Two drops of 10 vol. H_2O_2 will then be found sufficient to decolorize the solutions.

5 g. of anhydrous Na_2SO_4 and 20 ml. of n-butyl alcohol are added to each vessel, the vessels are immersed in a water bath to raise the temperature of their contents to about 35–40°, and are then shaken vigorously for 2 min. After cooling, the whole of the supernatant layers, together with as little of the aqueous layers as possible, are decanted into centrifuge tubes and are spun at slow speed for 5–10 min. It will then be found that sufficient of the butyl-alcohol pyridine layer, now perfectly clear, may be decanted to fill the fluorimeter cell. Measurement of fluorescence is made using blue activating light (Wratten no. 47 filter) and an orange filter (Chances) in front of the photocell. A convenient standard green fluorescing solution is a 1 μg./ml. solution of sodium fluorescein in weak alkali.

Blank determinations on each sample are carried out after destruction of the riboflavin contained therein by exposure to the unscreened radiation of a mercury-vapour arc or to direct sunlight for 1–2 hr. according to the method of Najjar.

INTERPRETATION OF RESULTS

Within a limited range of concentrations, Beers' law is obeyed by solutions of riboflavin. As the drum of the 'Spekker' fluorimeter is calibrated logarithmically, a linear ratio is obtained between drum reading and concentration over this range.

Under the conditions outlined above it is found that this linearity of ratio holds, and therefore a straight line may be drawn relating the fluorescences of the three flour extracts to their added riboflavin contents. The 'blanks' of these three extracts will be identical within the limits of experimental error, and this 'blank' figure is used on the extrapolation of the graph from which may be read the amount of riboflavin present in the total quantity of wheat, flour, bread, etc., which was taken in the first place.

In Table 6 are given some results which have been obtained by the method. They include a number of samples which have been assayed by the microbiological method and which appear in Tables 3 and 4.

Table 6

Sample	Riboflavin content μg./g.
1. Wheats, English (1 in Table 3)	1·7–2·3
2. Wheat, mixed grist	2·4
3. Germ (8 in Table 3)	14·5
4. Bran (6·8 μg./g. by microbiological method)	7·4
5. National Wheatmeal sample (*a*)	1·5
6. National Wheatmeal sample (*b*)	2·0
7. Control loaf (1 in Table 4)	2·6
8. Control loaf + 2 μg./g. added riboflavin)	4·4

Duplicate estimations by the method on different days have given results differing on all occasions by less than ± 10 %.

DISCUSSION

The fluorometric method as outlined above is essentially a modification of that of Najjar. No assumption is made that adsorption or elution of riboflavin is complete—the method depends upon keeping volumes and quantities of reagents, etc., constant as between fortified and unfortified extracts, and the use of a standard solution of riboflavin eliminates calculations on such points, which would otherwise be necessary. This method was chosen as being simpler and more expeditious than adopting the directly quantitative system of multiple adsorptions and elutions, though the occasional adoption of such a procedure provides a useful check.

It is found that whereas for one product of fairly constant composition (e.g. National Wheatmeal flour) the gradient of the fluorescence curve is reasonably constant (and consequently it may be found possible, with the sacrifice of a little accuracy, to make riboflavin additions to occasional samples only), this gradient varies markedly from one wheat or milling product to another, and needs to be separately determined on each sample.

For this reason, and despite all efforts which have been made to simplify the fluorometric technique with a view to making it as expeditious as possible, it must be admitted that it is in our experience a very much more laborious and time-consuming method than the microbiological.

The values found by both methods in the present investigation for the riboflavin content of different cereals show that there are considerably greater amounts of this vitamin present in these products (about 10 times) than is indicated by the literature [Fixsen & Roscoe, *Nutr. Abstr. Rev.* 1939–40]. Recently, Andrews, Boyd & Terry [1942] have published figures for the riboflavin content of wheat and wheaten products, as well as other cereals, obtained by the Snell & Strong microbiological method. With the exception of patent flour, the values they obtained are all much lower (approx. half) than those now reported. One explanation for these low figures is that their extracts were too concentrated (5–10 g. made up to 100 ml.). In our experience there is always a falling off in estimated riboflavin if the extract be too concentrated. There is also the further possibility that the *p*H of the extracts was not correctly adjusted. If the *p*H of the extracts be lower than 6·6 the riboflavin values will be found to be too low. Kemmerer [1942] has reported the results of 18 collaborators in a comparison between the fluorometric and microbiological method for the assay of riboflavin in alfalfa meal, wheat and flour. Good agreement was obtained between the various collaborators for alfalfa meal by either method, but extremely wide variations were obtained for wheat and flour, whichever method was used. For example, the values for wheat by the fluorometric method varied between 0·53 and 2·6 μg./g. and with the microbiological method the variations were 0·78–2·7 μg./g., while for flour both fluorometric and microbiological methods gave a range of 0·15–1·5 μg./g. Such extremely wide variations in results between different workers are difficult to understand. In our experience with the microbiological method fluctuations of the order reported by Kemmerer were never encountered.

That the values reported here are reliable is shown firstly by the fact that the determined and calculated values obtained from the different milling samples (see Table 5 above) agree within experimental error. Secondly, the values obtained for the same samples of cow's milk obtained by one of us (E. C. B.-W.) working with the microbiological method and by Dr S. K. Kon of the National Institute for Research in Dairying working independently at Reading with the fluorometric method, agree within experimental error (see Table 7).

Table 7

Sample	Riboflavin content (μg./g.)	
Cow's milk	Microbiological method	Fluorometric method
1	1·6	1·5
2	1·4	1·32
3	1·5	1·4

SUMMARY

The microbiological method of Snell & Strong and the fluorometric method of Najjar for the assay of riboflavin have been improved and modified with particular reference to cereals and cereal products. Agreement between the results obtained by the two methods is good, and figures are presented covering a wide range of such products.

The microbiological method is preferred as it is more rapid than the fluorometric, and requires less material and no specialized apparatus.

We are greatly indebted to Miss Marjory Stephenson for much helpful advice and criticism.

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The Effect of Salts on the Formation of Protein Complexes during Heat Denaturation

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(*Received 22 September 1942*)

The separation of floccules from solutions of proteins denatured by heat is the final and most obvious stage in a process of aggregation. The factors controlling flocculation have been studied in detail by many workers, but little attention has been given to their effects on earlier stages of aggregation. If the heating is insufficient, or other conditions are unsuitable to cause flocculation, stable solutions of heat-changed proteins are obtained. When solutions containing more than one protein are heated in such conditions, aggregation may take place between particles of different proteins, when stable solutions of protein complexes are produced. Such complexes may have properties different from those of either the unheated mixture of proteins or the individual proteins heated separately. For example, such complexes are formed when serum albumin and globulin are heated together; these have been detected by electrophoresis [Van der Scheer, Wyckoff & Clarke, 1941], as well as by changes in the ease of sedimentation during centrifugation, and by changes in precipitability with salts [Kleczkowski, 1941*a*].

Antibodies and protein-antigens can combine to form complexes with other proteins simultaneously undergoing denaturation by heat. Van der Scheer *et al.* [1941] and Krejci, de Spain Smith & Dietz [1941] showed that when antisera are heated, antibodies are found in products with changed electrophoretic mobility, and Kleczkowski [1941*b*] and Bawden & Kleczkowski [1941, 1942] have described changes in the serological behaviour of both antibodies and protein-antigens after heating with unspecific serum proteins. These changes are most striking when 'O'-type antigens, giving granular precipitation with antisera, or their antibodies, are heated in the presence of serum albumin. The complexes formed by such antigens are no longer precipitated by antibodies, although they still combine with them to inhibit the precipitation of any unheated antigens added subsequently. Similarly, the complexes formed by antibodies still combine with the antigens but are unable to precipitate them.

In this paper the effects of salts on the formation of such complexes are described and compared with their effects on the separation of floccules.

MATERIAL AND METHODS

The proteins used for studying the changes in precipitability produced by the formation of complexes were the 'soluble euglobulin' and albumin fractions from rabbit serum. The proteins used as antigens were tomato bushy-stunt virus, and the soluble whole globulin and albumin fractions from human serum. These were all prepared by the methods previously described [Kleczkowski, 1941 *a*, *b*; Bawden & Kleczkowski, 1941].

The antisera used were against tomato bushy-stunt virus, against the whole globulin of human serum and against human serum albumin. They