72. THE ESTIMATION OF RIBOFLAVIN

PART 1. A NEW BIOLOGICAL METHOD

By M. M. EL SADR, T. F. MACRAE and C. ELIZABETH WORK

From the Division of Nutrition, Lister Institute, London

PART 2. THE ESTIMATION OF RIBOFLAVIN IN MILK: COMPARISON OF FLUORIMETRIC AND BIOLOGICAL TESTS

By K. M. HENRY, J. HOUSTON and S. K. KON

From the National Institute for Research in Dairying, University of Reading

PART 3. STATISTICAL ANALYSIS OF THE DATA

By J. O. IRWIN

Of the Medical Research Council's Statistical Staff

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PART 1

The importance of riboflavin in the nutrition of the rat has been recognized for some years. Rats given a diet deficient in this factor cease to grow [Edgar *et al.* 1937] and later develop severe pathological symptoms [Day *et al.* 1938; Bessey & Wolbach, 1939; El Sadr, 1940].

It is comparatively recently that riboflavin has been proved to be an essential nutrient of other mammals. Sebrell & Onstott [1938] and Street & Cowgill [1939] showed that dogs, when given a diet deficient in riboflavin, developed symptoms which rapidly led to collapse and death; these symptoms were prevented by addition of riboflavin to the diet and certain sick animals were cured by administration of this compound. Margolis *et al.* [1939] demonstrated that a black-tongue-producing diet supplemented by nicotinic acid would not maintain dogs in good health unless further supplements of aneurin and riboflavin were given. Hughes [1939] has indicated that riboflavin is required for the well-being of the pig.

Sebrell & Butler [1938] fed a restricted diet, used previously by Goldberger & Tanner [1925], to a group of women and observed the development of lesions at the corners of the mouth; these symptoms were rapidly cured by administration of riboflavin but not of nicotinic acid. Oden *et al.* [1939] found a similar condition occurring naturally in Georgia; again remission of the symptoms was effected by administration of riboflavin. It is possible that some pellagra-producing diets are deficient in riboflavin as well as in nicotinic acid and aneurin, since nicotinic acid alone or with aneurin has not always been found to cure pellagra [Schmidt & Sydenstricker, 1938; Ghalioungui & Hanna, 1938]. According to

Vilter *et al.* [1939] patients who had been cured of pellagra by administration of nicotinic acid and aneurin, and were maintained on the pellagra-inducing diet and the above supplements, later developed certain nervous and digestive symptoms which were cured by administration of riboflavin.

Since the dietary value of riboflavin is established, the estimation of this vitamin in foodstuffs is a matter of importance; many methods have already been described, some biological and others physico-chemical. The latter methods (see Karrer [1939]) at present cannot be considered always to be reliable, but have the advantage of rapidity. The usual procedure is to extract the riboflavin either with aqueous alcohol or acetone, purify the extract by various methods and finally to estimate, fluorimetrically or colorimetrically, the riboflavin in the extract. In some cases the riboflavin is converted into lumiflavin which is estimated colorimetrically. Errors may arise from incomplete extraction of riboflavin from the foodstuffs, losses during the purification of the extract, interference by coloured or fluorescent impurities and, when conversion into lumiflavin is employed, losses due to incompleteness of such conversion. In certain cases the physico-chemical methods may yield satisfactory results but many careful experiments of comparison with satisfactory biological methods must be carried out before their adoption becomes justifiable.

In the biological methods for estimation of riboflavin which have been described, the growth of rats is usually taken as a criterion of biological activity. The provision of a basal diet free from riboflavin but containing all other essential nutrients has hitherto not been attained, and most of the diets which have been used are deficient in at least one factor of the vitamin B_2 complex besides riboflavin. Any growth response obtained by the addition of a foodstuff to these diets may be due to the supplementary effect of riboflavin or of the other nutrients in which the particular basal diet is deficient, or, more probably, to the combined effects of riboflavin and the other factors. Such methods of estimation must tend to give too high values for the riboflavin content of foodstuffs.

Many of the rat growth methods of estimation of riboflavin are based on the method of Bourquin & Sherman [1931]. The diet employed by these authors contained, as source of B-vitamins other than riboflavin, an 80 % alcoholic extract of wheat evaporated on to corn starch. Booher [1932], Bessey & Wolbach [1939] and Sherman & Langord [1938] used this diet. György *et al.* [1934] observed increased growth rates when the diet was supplemented either with a fuller's earth filtrate of yeast extract or a Peters's preparation of vitamin B_4 [Kinnersley *et al.* 1933]. György [1935] reported a considerable variation in the content of B_2-vitamins of different samples of wheat. Booher *et al.* [1934] and Copping [1936] showed that, for a given dose of riboflavin, the growth rate could be increased by raising the level of the wheat extract in the diet. Carlsson & Sherman [1938] pointed out the possibility of a deficiency of some members of the vitamin B_2 complex occurring in the basal diet, and suggested that food products such as liver, milk and muscle may contain unknown growth-promoting factors which are absent from the basal diet. It appears therefore that the Bourquin-Sherman diet is not entirely satisfactory for the estimation of riboflavin, as it does not contain adequate amounts of all the members of the vitamin B_2 complex other than riboflavin. This is not surprising as the diet was originally intended to provide vitamin B_1 free from what was then known as vitamin B_2 or vitamin G. The Bourquin and Sherman "unit" of riboflavin potency was defined as that amount which, when given daily, maintained an average growth rate in rats of 3 g. weekly. This "unit" was later calculated to be equivalent to 2–2·5 μg. of riboflavin [Bessey, 1938]. The use of a standard growth rate as a reference unit is to

be criticized on theoretical grounds and in any case a weight increase of 3 g. weekly is too low, since at this level the margin of error is large.

Day *et al.* [1937] employed a diet in which an 80% alcoholic extract of rice polishings provided the riboflavin-free supplement, and claimed that their method measured riboflavin when the growth rate was less than 10 g. weekly. Ansbacher *et al.* [1936] and Cook *et al.* [1937] used as sources of additional B-vitamins concentrates prepared from rice polishings, and Supplee *et al.* [1939] used these concentrates after they had been autoclaved. Lindholm [1938], in the construction of a standard response curve to be used for the estimation of riboflavin in foodstuffs, provided the other members of the vitamin B_2 complex as an aqueous extract of liver treated with fuller's earth at *p*H 4; fuller's earth is known to remove vitamin B_6 (adermin) as well as riboflavin from solution. Lunde *et al.* [1939] fed the standard "Peters's eluate" preparation of vitamin B_1 which is known to contain some vitamin B_6, but which cannot supply adequate amounts of all B_2-vitamins. Euler *et al.* [1934] used as source of additional B_2-vitamins an extract of yeast which had been treated with frankonite.

It is improbable that any one of the above diets contained adequate amounts of all B_2-vitamins other than riboflavin and many probably contained appreciable amounts of riboflavin due to the incorporation of inadequately purified casein. Supplee *et al.* [1936] effected progress in the purification of the basal diet by showing that when casein is washed with salt solution at its isoelectric point, riboflavin is demonstrable in the washings even when the casein had been washed previously with alcohol and acetic acid.

Other biological methods for the estimation of riboflavin have been described. Jukes [1937] used, as criterion of riboflavin activity, the growth rate of chicks fed on a grain diet supplemented by a concentrate prepared from rice polishings. Snell & Strong [1939] employed the growth-promoting action of riboflavin for *Lactobacillus casei*. The latter method is quick and by its use concentrations of riboflavin as low as 0·05 μg. per 10 ml. of medium can be estimated, but it involves the preliminary extraction of riboflavin from the foodstuffs; this is known to be difficult to carry out quantitatively in certain cases.

In this laboratory it is considered that riboflavin may best be estimated by using the growth of young rats as the criterion of biological activity. In earlier experiments [Edgar *et al.* 1937], a growth response curve to graded doses of riboflavin was constructed with the use of a diet which contained yeast fuller's earth filtrate as source of B_2-vitamins other than riboflavin. It was soon realized that this diet was deficient in vitamin B_6 and probably in other factors, as well as in riboflavin, and was therefore not suitable for the estimation of riboflavin in foodstuffs.

In further experiments, designed to yield a method for the biological estimation of riboflavin, various supplements from yeast and liver were investigated as sources of B_2-factors other than riboflavin. Treatment of aqueous extracts of whole liver with norite charcoal was found to remove the riboflavin quantitatively, and the resulting filtrate contained adequate amounts of all other B_2-vitamins recognized to be essential for the rat. With this supplement, a growth response curve to graded doses of riboflavin has been constructed. The application of this method to the estimation of riboflavin in foodstuffs is now being carried out in this laboratory and the results obtained will be published later. We submitted details of our method with the liver charcoal filtrate to Dr S. K. Kon and his collaborators, who have not only repeated our experiments and constructed a growth response curve similar to our own, but have also applied the method to the estimation of riboflavin in certain milk products and have compared the

resulting values with those obtained by fluorimetric means. Part 2 of this paper gives the results obtained. Dr J. O. Irwin has analysed the results obtained in both laboratories; his report is contained in Part 3.

Methods and results

The animal technique employed was essentially that described previously [Edgar *et al.* 1938]. Young rats, whose mothers had received the stock breeding diet except during the last week of lactation when no yeast was fed, were weaned at 21 days; they weighed 40–50 g. They received the vitamin-deficient ration employed in this laboratory, consisting of casein 100,[1] rice starch 300, cottonseed oil 60, lard 15, salt mixture (McCollum's No. 185) 25 and water 500; the diet was cooked by steaming for 3 hr. Each rat received daily supplements of 0·08–0·1 ml. cod liver oil and 10–15 μg. aneurin chloride hydrochloride. Male rats only were used.

The animals, after gaining slightly in weight for about 7–10 days, reached a constant weight and were then given the test dose of riboflavin together with the supplements described below. The animals from various litters were divided as equally as possible amongst the groups receiving the different doses of riboflavin. The growth rate was observed for a period of 4 weeks.

A. *Growth response of rats to graded doses of riboflavin when additional B_2-vitamins are supplied as a crude yeast fuller's earth filtrate fraction and yeast fuller's earth eluate fraction (Table 1, Group A; Fig. 1)*

When the first curve of growth response to graded doses of riboflavin was made in this laboratory [Edgar *et al.* 1937] the rats received yeast fuller's earth filtrate as source of B_2-vitamins other than riboflavin. It was then realized [Edgar & Macrae, 1937] that these animals were deficient in what was then termed "yeast eluate factor", now known to be identical with vitamin B_6 [El Sadr *et al.* 1939, 2]. A further response curve therefore was made from results of tests in which each animal received the B_2-vitamins other than riboflavin as daily doses of (1) crude yeast fuller's earth filtrate = 1 g. dry yeast [Edgar *et al.* 1937] and (2) yeast fuller's earth eluate = 2 g. dry yeast [Edgar & Macrae, 1937]. The presence in the diet of the yeast eluate fraction caused a considerable increase in the growth rates at the various levels of riboflavin dosage (Table 1, Group A; Fig. 1), the maximum growth response being 30·4 g. weekly compared with 18 g. weekly observed when no eluate fraction was given [Edgar *et al.* 1937]. In these experiments the casein was not purified by washing with salt solution.

B. *Growth response of rats to graded doses of riboflavin when additional B_2-vitamins are supplied as a purified yeast fuller's earth filtrate fraction purified by extraction with amyl alcohol and yeast fuller's earth eluate fraction (Table 1, Group B; Fig. 1)*

The yeast fuller's earth filtrate was purified by the method described by Edgar *et al.* [1938]; each rat received amounts equivalent to 2 g. dry yeast daily of both the filtrate and eluate fractions. The casein in the basal diet was purified

[1] Casein used was "Glaxo ashless extracted". In later experiments this was washed by the following method: 2·5 kg. were stirred for 30 min. with 30 l. tap water containing 600 g. NaCl and 30 ml. glacial acetic acid. After an hour the supernatant liquor was poured off. The washing was repeated 6 times. The casein was then pressed as dry as possible on Büchner funnels, suspended in 96% alcohol, filtered and finally dried on open trays in a current of air.

Table 1

Growth response of young male rats to increasing doses of riboflavin. Group A animals received ashless extracted casein diet with crude yeast filtrate fraction (=1 g. dry yeast daily) and yeast eluate fraction (=2 g. dry yeast daily) as sources of B_2-vitamins. Group B animals received washed casein diet with yeast filtrate fraction purified by amyl alcohol extraction (=2 g. dry yeast daily) and yeast eluate fraction (=2 g. dry yeast daily) as source of B_2-vitamins.

	Group A		Group B	
Daily dose of riboflavin μg.	No. of rats	Av. weekly wt. increase during 4 wk. period (g.)	No. of rats	Av. weekly wt. increase during 4 wk. period (g.)
0	4	7·5	2	3·5
3	5	10·6	2	7·2
6	5	13·9	2	10·0
12	7	19·3	2	14·1
25	5	20·6	—	—
37	2	27·6	—	—
50	9	30·4	6	20·4

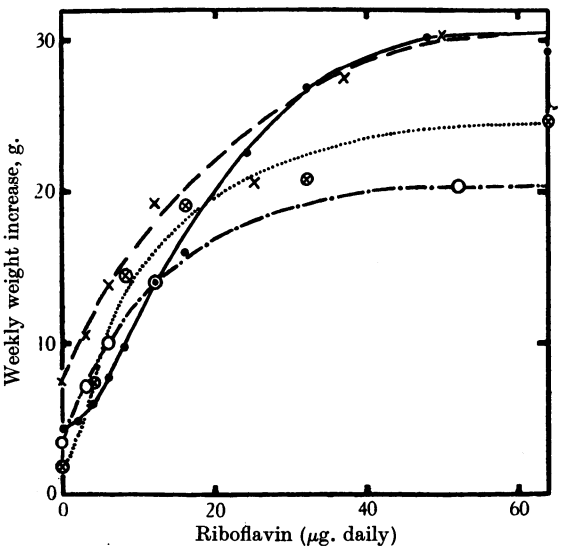

Fig. 1. Response of rats to increasing doses of riboflavin. Data of El Sadr *et al.* — — (Exp. points ×) rats received crude yeast filtrate fraction and yeast eluate fraction. ·—·— (Exp. points o) rats received purified yeast filtrate fraction and yeast eluate fraction. ——— (Exp. points •) rats received liver charcoal filtrate. Data of Kon & Henry. ..... (Exp. points ⊗) rats received liver charcoal filtrate. The curves have been fitted by eye to display the observed points more clearly.

by washing with salt solution. The growth rates observed at the various dose levels of riboflavin (Table 1, Group B; Fig. 1) were considerably less than the corresponding values obtained using the less pure fractions. The growth rate of 3·5 g. weekly of rats receiving no added riboflavin may be compared with the previous value of 7·5 g. weekly (Table 1, Group A) and suggests that the further purification of the diet had resulted in the removal of appreciable amounts of riboflavin. The maximum growth rate of 20·4 g. weekly, observed when optimal amounts of riboflavin were administered, indicated, however, that the diet was deficient in at least one member of the vitamin B_2 complex additional to riboflavin; this must have been lost in the purification processes. This possibility has been substantiated by further work [El Sadr *et al.* 1939, 1].

C. *Growth response of rats to graded doses of riboflavin when additional B_2-vitamins are supplied as a liver extract treated with norite charcoal (Table 2; Fig. 1)*

The diet finally adopted for the biological estimation of riboflavin contained casein which had been purified by washing with salt solution, while additional B_2-vitamins were supplied by a liver charcoal filtrate.

Preparation of liver charcoal filtrate. 1 l. of liver extract, prepared by extraction of 6 kg. of whole liver with 50 % aqueous acetone and subsequent removal of the acetone *in vacuo*, was adjusted to *p*H 5 with HCl and 36 g. norite charcoal (Messrs Hopkin & Williams) were added. After stirring for 30 min. the charcoal was allowed to sediment for 2 hr. and was then removed by filtration. The treatment was repeated with an equal amount of norite charcoal, and the final filtrate was fed to the rats without further treatment. Each rat received 1 ml. (=6 g. fresh liver) daily.

With this basal diet the growth rates varied consistently with the dose of riboflavin as shown in Table 2 and Fig. 1. The maximum growth rate of 30·2 g. weekly was similar to that observed when crude yeast fuller's earth filtrate and

Table 2. *Data of El Sadr* et al.

Growth response to increasing doses of riboflavin of male rats receiving purified casein in the diet and whole liver extract treated with norite charcoal =6 g. fresh liver daily.

Daily dose μg.	No. of animals	Mean observed increase in wt. per week (over 4 weeks) g.	Calculated values		
			Equation (1)	Equation (2)	Equation (3)
0	8	4·34	—	—	—
2	6	4·92	0·52	5·50	—
4	6	6·04	6·49	4·83	—
6	6	7·88	9·99	7·31	6·59
8	5	9·80	12·47	9·94	9·88
12	8	14·12	15·96	14·44	14·52
16	8	16·03	18·44	17·89	17·81
24	7	22·75	21·94	22·65	22·46
32	8	26·75	24·42	25·68	25·75
48	10	30·22	27·91	28·98	—
64	10	29·28	30·39	30·35	—

yeast fuller's earth eluate fractions were given as sources of additional B_2-vitamins (Table 1, Group A); however, the lower growth rate of 4·3 g. weekly given by the basal diet containing the liver supplement without added riboflavin suggested that this diet was less contaminated with riboflavin.

The liver charcoal filtrate described above contains adequate amounts of all the recognized members of the vitamin B_2 complex required by the rat except riboflavin. It is known that the factor we have named liver filtrate factor is adsorbed to some extent by norite charcoal [Macrae *et al.* 1939] but adequate amounts appear to be present in the filtrate as no increased growth rate was observed after addition of purified concentrates of this factor. It has long been recognized that norite charcoal is not a good absorbent for vitamin B_6. The high growth rates observed when optimal amounts of riboflavin were administered prove that the less well characterized members of the vitamin B_2 complex were present in the charcoal filtrates. It is true that somewhat better growth rates were obtained with untreated extract of whole liver as source of all B_2-vitamins than with the liver charcoal filtrate and optimal amounts of riboflavin. The difference, however, is so slight that it is considered improbable that

the presence of B-vitamins other than riboflavin in foodstuffs tested would appreciably affect the growth rate of rats receiving the above liver charcoal filtrate in their basal diet. We therefore consider that this basal diet and procedure may be satisfactorily used for the estimation of riboflavin in all foodstuffs. The basal diet has the advantage of being easily prepared.

Statistical examination shows that the usual method of biological estimation, involving the administration of both unknown and standard at two dose levels may be employed.

Norite charcoal has been found to remove riboflavin from the other B_2-vitamins of yeast extracts. In the above method of riboflavin estimation, therefore, yeast charcoal filtrate may be substituted for liver charcoal filtrate.

SUMMARY

Treatment of extracts of liver or yeast with norite charcoal yielded a filtrate which, while deficient in riboflavin, contained all other known constituents of the vitamin B_2 complex. Young rats when given a diet complete in other respects and containing liver charcoal filtrate as source of B_2-vitamins showed graded growth response to graded doses of riboflavin. A method is therefore suggested whereby the riboflavin contained in foodstuffs may be determined by employing one of these filtrates as the riboflavin-free supplement to the diet.

We express our thanks to Dr H. Chick for her advice and criticism. The whole liver extract used in the above experiments was supplied by Messrs Glaxo Laboratories Ltd., and the aneurin and riboflavin by Messrs Roche Products Ltd. We are most grateful to these firms for their continued help.

PART 2

A fluorimetric assay of the riboflavin content of milk has been in use for some time in this laboratory [Henry *et al.* 1939], but without parallel biological tests it had not so far been possible to judge how reliable it was.

Dr Macrae very kindly kept us informed of the developments of the biological method described in Part 1 of this paper and we were able to carry out two series of tests on milk in time for a joint publication.

EXPERIMENTAL

1st experiment

A. *Biological tests on rats.* 1. Preparation of animals. Mothers with young were changed on the 16th day of lactation from the stock colony diet [Folley *et al.* 1938] to a similar diet from which yeast, liver and milk had been removed. Males only were used for these tests. They were weaned at 21 days, weighing 30–50 g., placed in individual cages and given the riboflavin-deficient diet as described in Part 1. They received daily a solution containing 10 μg. of aneurin chloride hydrochloride and 2 drops of cod liver oil. There was hardly any growth on this diet and after 7–10 days, when the animals were either stationary in weight or losing slightly, they were divided into groups of 8, the allowance of aneurin was increased to 15 μg. daily and each rat was given in addition 0·5 ml. (= 6 g. of fresh liver) of the liver charcoal filtrate prepared as described in Part 1, and concentrated in a current of air at 40°.

One control group received no further additions, five other groups received respectively 4, 8, 16, 32 and 64 μg. daily of pure synthetic riboflavin ("Roche") which we found by fluorimetric tests to be equal to the highest commercially available grade of the natural substance. Two more groups received respectively 0·9 and 1·8 g. daily of spray-dried full cream milk to supply riboflavin at presumptive levels of approximately 10 and 20 μg.

2. Riboflavin standard. A stock solution containing 100 μg./ml. was made up in 50 % methyl alcohol. From this the necessary quantity was removed daily, evaporated *in vacuo* almost to dryness and made up in water at *p*H 2 so that the solution contained 40 μg./ml. The strength of this solution was checked against a fluorimetric standard each day before feeding. The solutions were kept in dark bottles.

3. Milk. The dried milk was dissolved each day in water so that 0·9 g. was contained in 5 ml. Fluorimetric tests were done at weekly intervals. Double doses of all supplements were fed on Saturdays to avoid Sunday dosing. The test lasted 4 weeks.

B. *Fluorimetric tests.* The milks were prepared for assay by the method of Emmerie [1938] and measurements were carried out in the outfit described by Henry *et al.* [1939].

C. *Results.* The mean gains in weight of the experimental animals are shown in Table 3 and Fig. 1. The riboflavin content of milk calculated from these data and the results of fluorimetric tests are given in Table 5; the value of 15·2 μg. per g. was obtained by calculating a pooled slope for test and standard [Irwin, 1937]. When the smaller and larger doses were interpreted separately with reference to the standard curve, results of 12·2 μg. per g. and 19·1 μg. per g. were obtained. These do not differ significantly, but there is a discrepancy between the higher value and the result of the fluorimetric test.

Table 3. *Data of Henry* et al. (*1st experiment*)

Daily dose of standard (μg.)	0	4	8	16	32	64
No. of animals	8	8	8	8	8	8
Observed mean increase in wt. per week (over 4 weeks) (g.)	1·84	7·31	14·59	19·09	20·94	24·94
Daily dose of spray-dried milk (g.)	0·9	1·8				
No. of animals	8	8				
Observed mean increase in wt. per week (over 4 weeks) (g.)	15·12	21·97				

2nd experiment

A. *Biological tests on rats.* 1. Preparation of animals. The preliminary procedure was the same as in the first experiment. The rats weighed 34–48 g. at weaning. Again, males only were used in groups of eight. Riboflavin was fed at levels of 4, 8, 16 and 32 μg. daily.

2. Riboflavin standard. An aqueous solution at *p*H 2 containing 40 μg. in 1 ml. was freshly made every 3 days. It was checked daily by fluorimetric tests.

3. Milk. The same sample of full cream spray-dried milk was used, 0·5 and 1·0 g. were fed corresponding to presumptive levels of intake of 5 and 10 μg. In addition an evaporated milk was also tested. Of this 2 and 4 g. (0·58 and 1·16 g. on the dry basis) were given daily diluted with water to 5 and 10 ml. respectively. The presumptive levels of feeding were in this case approximately 6 and 12 μg. of riboflavin.

B. *Results.* The results are given in Tables 4 and 5. With one exception the control animals gained little weight. One animal however gained 6 g. in the first fortnight of the experiment and 46 g. in the second. There was no obvious

Table 4. *Data of Henry* et al. (*2nd experiment*)

Daily dose of standard (μg.)	0	4	8	16	32
No. of animals	7	7	8	8	8
Observed mean increase in wt. per week (over 4 weeks) (g.)	0·48	9·14	14·91	19·06	22·31
	(*a*)			(*b*)	
Daily dose of:					
(*a*) spray-dried milk	0·5	1·0			
(*b*) evaporated milk (g.)				2·0	4·0
No. of animals	8	8		8	8
Observed mean increase in wt. per week (over 4 weeks) (g.)	9·78	15·88		10·84	16·28

Table 5. *Results calculated from the data of Henry* et al.

	Biological test			
		Limits of error (%)		Fluorimetric
Experimental material	Result μg./g.	(P=0·95)	(P=0·99)	test result μg./g.
(*a*) Spray-dried milk	15·2	75–133	69–145	10·0
(*b*) Spray-dried milk	9·3	76–131	70–142	10·3
(*c*) Evaporated milk	2·6	78–128	72–139	2·9

explanation of this behaviour but its abnormal increase in weight has been omitted from the mean. One animal, receiving 4 μg. of riboflavin, died of purulent pneumonia on the 23rd day of the experiment.

The results show that when milk is fed at lower levels, yielding weight gains of 10–15 g. a week, biological findings are in good agreement with fluorimetric tests.

Discussion

It is of interest to compare results obtained in the present study with other reports of biological and especially of simultaneous biological and chemical assays of riboflavin in milk. Kramer *et al.* [1938] applied the Bourquin-Sherman [1931] method to milk and obtained a value of 2 μg. per g. for winter herd milk. Whitnah *et al.* [1938] assayed milk from the same farm fluorimetrically and obtained, before cows went out to pasture, values varying according to breed from 1·17 to 1·73 μg. per g. In a later paper Kramer *et al.* [1939] reported an average difference of only 10 % between the results of biological (Bourquin-Sherman method) and fluorimetric riboflavin estimation on milk. Snell & Strong [1939] obtained by biological tests, presumably by the Bourquin-Sherman [1931] method, a value of 17 μg. per g. for skim milk powder and an identical value by their microbiological test with *Lactobacillus casei*. Hodson & Norris [1939] report values of 17–22 μg. per g. for skim milk powder by fluorimetry and very similar figures using the microbiological test of Snell & Strong [1939]. Finally, Lunde *et al.* [1939] quoted a biological value for fresh milk of 2·4 μg. per g. and 2·7 μg. by fluorimetry. The agreement in all these tests is excellent but it should be noted that the biological tests consisted mostly in feeding only one level of the standard and one of the unknown.

Summary

1. The biological method of assay of riboflavin described by El Sadr *et al.* (Part 1) has been compared with fluorimetric tests for full cream spray-dried milk and for evaporated milk.

2. Good agreement was found when the milks were fed at levels supplying up to 10 μg. daily. For a higher level the agreement was not so satisfactory.

PART 3

(1) *Data of El Sadr et al. (Tables 2 and 6; Fig. 2)*

Table 2 shows the observed mean increase in weight per week over a period of 4 weeks for the negative controls and each of the 10 doses. The best fitting straight line relating response to the logarithm of the dose is

$$y = -5{\cdot}458 + 19{\cdot}849x \qquad \text{.....(1)}$$

where y = response, and x = log dose.

Table 6. *Slopes and their standard errors; variance ratios for the linearity test*

	Slope	Standard error	Variance ratio	5% point of variance ratio
El Sadr *et al.*:				
Standard, all points	19·85	0·76	10·4	2·1
Central, 6 points	26·36	1·40	2·5	2·6
Henry *et al.*:				
Exp. 1 (*a*) Standard	13·82	1·10	2·7	2·9
(*b*) Spray-dried milk	22·73	3·87	—	—
(*a*) and (*b*) together	14·24	1·09	3·2	2·6
Exp. 2 (*a*) Standard	14·41	1·59	0·7	3·4
(*b*) Spray-dried milk	20·24	3·73	—	—
(*c*) Evaporated milk	18·06	2·55	—	—
(*a*) and (*b*) together	14·97	1·38	1·1	2·8
(*a*) and (*c*) together	14·76	1·29	0·9	2·8

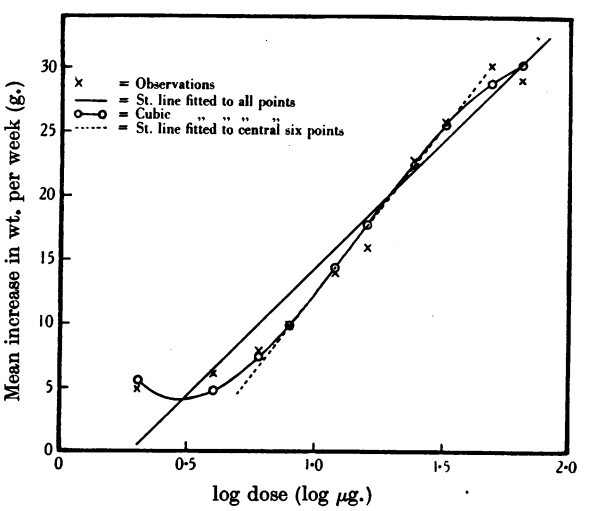

Fig. 2. Data of El Sadr *et al.* Relation between growth and log dose.

The usual test for linearity of regression [Fisher, 1936], however, shows a significant departure from linearity (the variance ratio is 10·4 with a 0·1 % point of only 3·8;

$$z = 1{\cdot}17, \quad 0{\cdot}1\,\%\ \text{pt.} = 0{\cdot}67).$$

It is clear that the straight line is too low for very low and rather high doses and too high elsewhere. The negative controls show that the response to low doses is largely non-specific while the response to the two highest doses suggests a slackening rate of increase. The data are satisfactorily graduated by the cubic curve

$$y = 14{\cdot}951 - 49{\cdot}239x + 64{\cdot}785x^2 - 18{\cdot}161x^3, \qquad \ldots\ldots(2)$$

the deviations from which are only such as might arise by chance (variance ratio $= 1{\cdot}1$, 5 % pt. $= 2{\cdot}2$, $z = 0{\cdot}05$, 5 % pt. $= 0{\cdot}40$).

In practice however it would be simpler and more satisfactory to use doses between the ranges 6 and 32 μg. The best fitting straight line to the responses within the range, that is to the central 6 doses, is

$$y = -13{\cdot}923 + 26{\cdot}357x. \qquad \ldots\ldots(3)$$

This gives a satisfactory fit, showing no significant departure from linearity (variance ratio $= 2{\cdot}5$, 5 % pt. $= 2{\cdot}6$; $z = 0{\cdot}46$, 5 % pt. $= 0{\cdot}48$).

The values yielded by equations (1), (2) and (3) are given in Table 2, while Table 6 gives the slopes of the two straight lines together with their standard errors (see also Fig. 2).

(2) *Data of Henry* et al. (*Tables 3, 4, 5 and 6*)

The data were obtained from two experiments. In the first, doses of 0·9 and 1·8 g. of spray-dried milk were compared with 5 doses of the standard. In the second, doses of 0·5 and 1·0 g. spray-dried milk and of 2·0 and 4·0 g. evaporated milk were compared with 4 doses of standard. The average responses are shown in Tables 3 and 4.

In each experiment the slopes of the straight lines relating response to log dose were calculated separately for the standard and for each milk. The pooled slopes were also calculated for standard and spray-dried milk and standard and evaporated milk, respectively. Linearity of regression was tested in each case and no significant departures from it were found. Table 6 gives the slopes and their standard errors together with the variance ratios and their corresponding 5 % points for the linearity test. In the first experiment the slope for spray-dried milk is significantly higher than for the standard, the difference being 2·2 times its standard error, in the second experiment the difference is in the same direction but only 1·2 times its standard error and does not reach the significance level. None of the slopes in the second experiment differ significantly. The results of the three assays are given in Table 5.

(3) *Comparison of results*

The slope of the standard curve obtained from the data of El Sadr *et al.* is significantly steeper than the slopes for standard from the data of Henry *et al.* even if all the doses are included in the calculation of the former. This overall slope does not differ significantly from the slopes for milk.

The fact that significantly different slopes for the standard curve may occur in different laboratories emphasizes the necessity of using at least 2 doses of standard and 2 doses of the unknown preparation in routine testing.

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