4. The shell gland has three-quarters of its riboflavin present as dinucleotide, the rest being equally divided between the free form and the mononucleotide.

5. It is suggested that the magnum is the tissue involved in the day-to-day mechanism affecting the amount of riboflavin in the egg.

The author thanks Dr A. W. Greenwood, the Director of this Centre, for his advice and criticism, and Miss D. R. Mitchell for assistance both in the care of the birds and in the analyses.

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The Use of Chicks for the Biological Assay of Members of the Vitamin B Complex

2. Tests on Natural Materials and Comparison with Microbiological and Other Assays

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In a previous paper Coates, Kon & Shepheard (1950) suggested methods for the biological assay of nicotinic acid, riboflavin, pyridoxin, pantothenic acid and folic acid with chicks. The accuracy of the methods was determined for preparations of the pure vitamins. The present paper reports their application to assaying the same vitamins in various natural materials, and the results are compared with those found by microbiological or other means.

EXPERIMENTAL

Substances tested

While the technique of the chick assays for the B-vitamins was still in the experimental stage, the materials chosen for study were those, such as milk and yeast products, already well studied by other methods. However, the chick tests were primarily designed for comparison with results obtained by microbiological techniques,

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which, mainly because of their speed and simplicity of operation, are increasingly being used for routine determinations of the vitamins. Since they are applied in particular at this laboratory to experiments on the synthesis of vitamins by the micro-organisms of the alimentary tract, it was thought advisable to examine certain gut-content materials by the chick method.

The following twelve substances were tested for one or more of the vitamins listed; the first three were issued by the Vitamin B Subcommittee of the Accessory Food Factors Committee (Medical Research Council) for tests which were part of a co-operative research on methods organized by that Subcommittee:

(1) Flour of 85 % extraction, prepared under the supervision of the staff of the Research Association of British Flour Millers, through the courtesy of Dr T. Moran, Director.

(2) Dehydrated minced beef obtained through the co-operation of Dr F. Kidd, F.R.S., then Superintendent of the Low Temperature Research Station, Cambridge.

(3) Dried food yeast; a roller-dried preparation of *Torula utilis* supplied by Dr A. C. Thaysen from the Chemical Research Laboratories, Teddington.

(4) Dried brewer's yeast.

(5) A yeast extract.

(6) Dried beer solids.

(7) Dried skim milk; a commercial sample of good quality.

(8) Sow's milk; a pooled, freeze-dried sample of milk from several sows, prepared as described by Davis, MacVicar, Ross, Whitehair, Heidebrecht, Braude, Coates, Henry, Kon, Thompson & Wilby (1950).

(9) Dried spinach-beet; a mixture of spinach-beet leaves freshly picked, freed from stalk, immediately dried in a current of warm air at about 45° and milled before use.

(10) Dried rat faeces, collected from normal stock-colony rats and dried at about 70°, then milled finely.

(11) Dried whole rumen contents, kindly supplied by our colleague Dr J. W. G. Porter, collected from a fistulated cow over several weeks, spread on stainless steel trays, dried in a current of warm air at about 45°, then milled and the whole sample remixed mechanically before use.

(12) Fish solubles, kindly supplied by Dr J. A. Lovern of the Torry Research Station, Aberdeen; a concentrate of a watery extract obtained in the course of manufacture of herring oil by the 'cook-and-press' method.

Standards

For the work with chicks commercially pure samples of the vitamins were used throughout, since the quantities involved were large. For microbiological tests the Medical Research Council Provisional Standards were used when available; synthetic pteroylglutamic acid (Lederle Laboratories Inc.) was used as folic-acid standard in both methods. The two sets of standards were compared microbiologically and corrections were made when necessary, the results being expressed in terms of microbiological standards.

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Chick assays

The procedure followed was essentially as described previously (Coates, Kon & Shepheard, 1950). The inclusion of the test materials in the diet presented difficulties. Most of the materials were bulky and of low potency, so that it was sometimes necessary to replace as much as 20 % of the diet by the test sample to get a measurable response. The basic components of the diet were then adjusted to preserve the nutritive value of the basal diet as far as possible unchanged. Where the sample, such as dried faeces or flour, consisted mainly of fibrous or carbohydrate material, it was added at the expense of the dextrin. If it contained an appreciable amount of protein, as in dried milk, some of the casein was omitted from the basal diet. Certain materials for which a chick assay was required were of too low potency to give a measurable response even when forming 20 % of the diet. Such samples were fortified before testing by the addition of a known amount of the vitamin to be assayed. The limits of error of such an assay were inevitably large, and it could hence be used only for detecting marked divergences from the results of the corresponding microbiological test. This technique was used in the assay of riboflavin in flour and of riboflavin, pantothenic acid and folic acid in rumen contents.

In early assays the diet was given in powdery form, but it was given granulated as soon as the advantages of that method of feeding were realized (Coates, Kon & Shepheard, 1950). The amount of water used in granulation was varied to suit the material tested, but fairly satisfactory granules were obtained in all instances.

Whenever possible four doses of the test material were assayed against four doses of standard, with about fifteen birds in a group, although occasionally the amount of test material available was insufficient for so many. Frequently results for one or more of the groups could not be included because their response was in the maximum or minimum range or because in the groups with low doses many birds had died before the end of the test.

Microbiological assays

Technique

Assay methods. The methods used were according to published procedures or modifications of them described below.

Recovery tests. In a number of instances, shown in the tables of results, extracts were prepared, from samples of the material studied, with and without addition of a known amount of the vitamin under test. From the measured differences in the potencies of the two extracts the percentage recovery of the added vitamin was calculated.

Internal and external standards. The potency of each test extract was compared with that of a dilute solution of the relevant vitamin (external standard). Also in a number of instances, before carrying out the final dilution step in the preparation of an extract, two equal portions were taken and to one of them was added a known amount of the vitamin. Both portions were then equally diluted with water to provide the solutions for assay. A comparison of the potencies of these two solutions made it possible to calculate the potency of the extract in terms of an internal standard. In

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some assays the solutions containing internal standard were tested at fewer dose levels than those of the external standard, and results with such internal standards may hence have been less precise than those with external standards, but in all the examples reported there was broad agreement between results obtained by the two different methods.

Vitamins unstable to light. Assays for riboflavin, vitamin B_6 and folic acid were carried out by red light or in a darkened room.

Incubation. For most of the assays carried out with *Lactobacillus casei*, *Lb. arabinosus*, *Streptococcus faecalis* and *Leuconostoc mesenteroides*, the baskets of tubes, covered with towels, were incubated in an enclosed water-bath fitted with a pump to circulate the water continuously during the period of incubation. In some of the earlier assays an air incubator was used. The usual period of incubation was 60–72 h, whether responses were to be measured turbidimetrically or by titration.

General

The design of the microbiological assays was in general such that no straightforward statistical assessment of the errors of the results was possible. In some instances results were obtained by reference to both internal and external standards or by the application of a variety of extraction procedures, and for certain assays of riboflavin and folic acid by the use of two different assay organisms. In some assays we have been unable to apply a simple linear transformation to the standard-response curve. All available results are therefore presented, supported in many instances by recovery values or statements of the extent of agreement between values obtained by reference to internal with those by external standards. The problem of statistical interpretation of the results has been studied by Mr E. C. Fieller of the Mathematics Division of the National Physical Laboratory, Teddington, and his findings will be published separately. In general, there was reasonable agreement between the results quoted here, which were obtained by the usual process of 'reading-off' and those obtained by Mr Fieller's more detailed calculations. The limits of error for his results proved in most cases to be within 90–110 % of either calculated figure.

Riboflavin

Substances tested. Dried skim milk, 85 % extraction flour, dried sow's milk and dried rumen contents were assayed.

Methods of assay. The method of the Analytical Methods Committee (1946), with the medium designated (Y), was applied to dried skim milk. Five strains of *Lb. casei* were used; three were obtained by propagation from isolated colonies of *Lb. casei* A.T.C.C. 7469; the other two, L.G.N. and Y.T.C. 1, had growth characteristics different from one another and from the other three cultures.

The remaining substances were assayed with *Lb. casei* A.T.C.C. 7469 by the method of Roberts & Snell (1946) and also with *Leuc. mesenteroides* A.T.C.C. 10100 by the method of Kornberg, Langdon & Cheldelin (1948), modified by supplementing each 100 ml. of the double-strength basal medium with the following: DL-tryptophan, 20 mg; adenine, guanine, uracil and xanthine, 2 mg each; nicotinic acid, pyridoxin

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hydrochloride, calcium DL-pantothenate and aneurin hydrochloride, 20 μg each; *p*-aminobenzoic acid, 100 μg; biotin, 0·1 μg; folic acid, 0·4 μg. Use of the supplement slightly increased the sensitivity of the assay and resulted in a more nearly linear standard-response curve. The effect of omitting individual constituents of the supplement was not investigated.

Treatment of samples. All the samples were prepared for assay by extraction with 0·1 N-hydrochloric acid, as described by the Analytical Methods Committee (1946). From the 85 % extraction flour, extracts were prepared also by digesting the sample for 4 h with takadiastase (Parke, Davis & Co.) at pH 4·6 and the acid-hydrolysis procedure referred to above. For dried sow's milk comparisons were made between extracts treated and not treated with ether and between results obtained by direct addition of aqueous dilutions of the dried sow's milk to the assay tubes and those obtained from extracts prepared as described above.

Nicotinic acid

Substances tested. Dried rat faeces, dried brewer's yeast, yeast extract, dried beer solids and dried rumen contents were tested.

Method of assay. Lb. arabinosus 17-5 was used. For the first four substances the method was that described by the Analytical Methods Committee (1946), except that the medium was supplemented with asparagine and glutamic acid, 40 mg of each per ml. double-strength basal medium; the addition increased the responses. For the remaining substances the medium of Roberts & Snell (1946) was used, modified by omission of nicotinic acid and inclusion of riboflavin.

Vitamin B_6

Substances tested. Dried food yeast, dried beef, 85 % extraction flour and dried rumen contents were tested.

Method of assay. Saccharomyces carlsbergensis 4228 was used as described by Atkin, Schultz, Williams & Frey (1943), with the following modifications. The medium was supplemented with nicotinic acid and ammonium phosphate, as recommended by Hopkins & Pennington (1947); the final liquid content of assay tubes was 5 ml.; the tubes were autoclaved at 10 lb. pressure for 10 min, cooled in ice before inoculation and brought to 30° in a water-bath before transfer to a shaker in an air incubator.

Treatment of substances tested. Extracts were as a rule prepared by autoclaving the substances for 5 h at 20 lb. pressure with 0·055 N-sulphuric acid, as recommended by Rabinowitz & Snell (1947). For the 85 % extraction flour, 0·44 N-acid was used for the extraction, as recommended by Atkin *et al.* (1943).

Folic acid

Substances tested. Dried food yeast, dried spinach-beet leaves and dried rumen contents were tested.

Method of assay. The assays were usually made with *Lb. casei* A.T.C.C. 7469 in the medium of Roberts & Snell (1946), modified by omission of folic acid and inclusion

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of riboflavin. In some instances extracts were assayed also with *Strep. faecalis* Rogers in the medium of Teply & Elvehjem (1945).

In a recent publication, Koft, Sevag & Steers (1950) remark on the difficulty they encountered in preserving folic-acid solutions. We have used a stock solution with 1000 μg folic acid (Lederle Laboratories Inc.)/ml. in 25 % ethyl alcohol containing 1 % sodium bicarbonate. The stock solutions were prepared at intervals of several months and with each assay two standard solutions were included, one being diluted from the most recent stock solution and the other from an older one. In tests with *Lb. casei* or *Strep. faecalis*, we have obtained no evidence of loss of folic acid from the stock solutions after storage for 3 months at 2° or after incubation for 10 days at 30°. A stock solution containing 200 μg folic acid/ml. in M/15 phosphate buffer at pH 7·3 was unchanged by incubation for 10 days at 30° in the presence of 1 % of the preservative of Hutner & Bjerknes (1948); in the absence of preservative there was bacterial growth and a 40 % loss of folic-acid activity.

Treatment of substances tested. The routine method adopted was that of Sreenivasan, Harper & Elvehjem (1949), and involved digestion of the substance with an aqueous extract of pig kidney at pH 4·6 and then digestion with an extract of chick pancreas at pH 7·0. We departed from the published method by not autolysing our extract of chick pancreas and by carrying out the digestion of rumen contents and dried spinach-beet leaves under the preservative of Hutner & Bjerknes (1948) instead of under toluene.

Pantothenic acid

Substances tested. Dried food yeast, condensed fish solubles, skim milk powder and dried rumen contents were tested.

Method of assay. Yeast, fish solubles and dried rumen contents were assayed with *Lb. arabinosus* 17-5 in the medium of Roberts & Snell (1946), modified by the omission of pantothenic acid and inclusion of riboflavin. For the assay of skim milk powder the medium of Barton-Wright (1946) was used.

Treatment of substances tested. Extracts of yeast, fish solubles and dried rumen contents were prepared by the method of Nielands & Strong (1948), which was published after the tests had been made on skim milk powder. The milk was extracted by the variety of procedures set out in Table 1.

RESULTS

Chick tests

The results are set out in Table 2.

Microbiological tests

A summary of the mean results obtained is set out in Table 2. More detailed results are presented under the appropriate vitamin headings. The result given in Table 2 was generally the mean of all results available, but in some instances certain results were omitted, because they were not considered sufficiently precise. In all tables the results from which the means quoted in Table 2 were derived are indicated.

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Riboflavin. For skim milk powder the results obtained with the five cultures of *Lb. casei* by external and internal standards were similar to one another. They are given in Table 3. For the other samples assayed (dried sow's milk, dried rumen contents and 85 % extraction flour) the results are given in Table 4.

Table 1. *Pantothenic-acid content of four natural materials, assayed microbiologically*

Substance tested	Method of extraction	Pantothenic-acid* content (μg/g)	Recovery (%)
Dried food yeast	Digestion at pH 8 with extracts of calf intestine and of chick liver	160	106
		156	73
		166	117
		148	85
	Mean	158	95
Fish solubles	As above	51	—
		79	116
		57	—
		60	—
		44	—
		47	—
	Mean	56	—
Dried rumen contents	As above	14	—
Skim milk powder	Dissolved in water	28	—
	As above, then autoclaved at 15 lb. for 15 min	30	—
		27	—
	Dissolved in water and made to pH 4·6. Heated to 100° and filtered. Digested with mylase P (British Drug Houses, Ltd.) (after Ives & Strong, 1946)	23	100
	Mean	27†	—

* Expressed as calcium pantothenate.
† Reference to internal standards gave comparable results, with mean value 24 μg/g.

Nicotinic acid. Results for the five substances assayed are set out in Table 5.

Vitamin B_6. Results for the four substances assayed are set out in Table 6.

Folic acid. Results for the three substances assayed are set out in Tables 7 and 8.

Pantothenic acid. Results for the four substances assayed are set out in Table 1.

Rat tests

In several instances results are available for rat tests carried out on some of the substances examined in the present study. The tests were done for a different purpose by Dr K. M. Henry, but have so far not been published. They are set out in Table 9 and quoted in Table 2.

Physical and chemical tests

Results are also available for physical and chemical tests done in this laboratory on certain substances. They are quoted in Table 2.

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Table 2. *Content of certain B vitamins in natural materials determined with chicks, microbiologically, by physical or chemical methods and by rat assay*

		Content from				
		Chick assay		Micro-biological assay.	Other available sources	
Vitamin	Substance assayed	Value (μg/g)	True fiducial limits at $P=0.95$ (μg/g)	Value (μg/g)	Method	Value (μg/g)
Riboflavin	Dried skim milk 1	19·5	18·1–21·1	18·1	Fluorimetric*	18·3
	Dried skim milk 2	21·0	19·0–23·5	Not tested	Fluorimetric*	20·1
	Dried sow's milk	7·0	5·8–8·6	6·3	Fluorimetric*	6·4
					Rat assay†	8·2
	Dried rumen contents	15·6	8·8–24·8	11·1	Fluorimetric‡	11·0
	Flour, 85 % extraction	0·50	0·16–0·83	0·7	Fluorimetric‡	0·63
Nicotinic acid	Dried rat faeces	243	155–373	191	Chemical§	181
	Dried brewer's yeast	687	470–1068	700	—	—
	Yeast extract	429	269–760	916	Chemical§	871
	Dried beer solids 1	54	27–94	163	Chemical§	160
	Dried beer solids 2	52	35–71	148	—	—
	Dried rumen contents	62	47–81	57	—	—
Vitamin B_6 (expressed as pyridoxin hydrochloride)	Dried food yeast	38	33–46	30	Chemical§	33
					Rat assay†	39‖
	Dried beef	3·3	1·7–5·1	2·9	Chemical¶	3·4
					Rat assay†	3·6‖
	Flour, 85 % extraction	2·1	1·6–2·6	1·4	Chemical¶	4·0
					Rat assay†	2·5‖
	Dried rumen contents	3·7	2·6–4·9	5·1	—	—
Folic acid	Dried food yeast	19·6	16·3–23·8	7·2	—	—
	Dried spinach-beet leaves	10·5	7·3–14·8	15·9	—	—
	Dried rumen contents	0·74	0·39–1·14	1·2	—	—
Pantothenic acid (expressed as calcium pantothenate)	Dried food yeast	150	132–172	158	—	—
	Condensed fish solubles	67	55–84	56	—	—
	Skim milk powder	41	28–55	27	—	—
	Dried rumen contents	17·6	7·9–28·4	14	—	—

* See Davis *et al.* (1950).
§ Method of James, Norris & Wokes (1947).
† See Table 9.
‖ Combined result of two assays.
‡ Unpublished method of S. Y. Thompson.
¶ Method of Brown, Bina & Thomas (1945).

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Table 3. *Riboflavin content of skim milk powder, assayed microbiologically*

Designation of *Lb. casei* culture	Riboflavin content by External standard (μg/g)	Riboflavin content by Internal standard (μg/g)
A.T.C.C. 7469 isolate 1	18·8	18·0
A.T.C.C. 7469 isolate 2	18·5	19·0
A.T.C.C. 7469 isolate 3	19·8	17·4
L.G.N.	16·7	16·2
Y.T.C. 1	17·7	18·8
Mean	18·1	

Table 4. *Riboflavin content of dried sow's milk, dried rumen contents and 85 % extraction flour, assayed microbiologically*

Substance tested	Method of preparing extract	Test organism*	Riboflavin content (μg/g)	Recovery (%)
Dried sow's milk	Dissolved in water	Lb. c.	6·3	86
		L. mes.	6·8	96
	As Analytical Methods Committee (1946)	Lb. c.	7·0	125
		L. mes.	5·4	84
	As Analytical Methods Committee (1946), but extracts treated with ether	Lb. c.	7·5	106
		L. mes.	4·8	96
		Mean	6·3	99
Dried rumen contents	As Analytical Methods Committee (1946)	Lb. c.	12·3	74
		Lb. c.	13·5	—
		L. mes.	8·7	75
		L. mes.	9·7	—
		Mean	11·1	—
Flour, 85 % extraction	As Analytical Methods Committee (1946), but extracts treated with ether	Lb. c.	0·67	100
		Lb. c.	0·72	—
	Digested with takadiastase, then as Analytical Methods Committee (1946)	Lb. c.	0·67	—
		Lb. c.	0·72	—
		L. mes.	0·68	—
		L. mes.	0·70	—
		Mean	0·69	—

* Lb. c.: *Lb. casei* A.T.C.C. 7469; L. mes.: *Leuc. mesenteroides* A.T.C.C. 10100.

Table 5. *Nicotinic-acid content of five natural materials, assayed microbiologically*

Substance tested	Nicotinic-acid content by External standard (μg/g)	Nicotinic-acid content by Internal standard (μg/g)		Recovery (%)
Dried rat faeces	191	163*		—
Dried brewer's yeast	700	671*		—
Yeast extract	916	768*		—
Dried beer solids 1	163	—		—
Dried beer solids 2	145	158	Mean 148	90
	149	138		87
Dried rumen contents	56	—	Mean 57	80
	57	—		80

* These results are less precise than those obtained by reference to external standards (see p. 77) and the latter are therefore quoted in Table 2.

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Table 6. *Vitamin B_6 content of four natural materials, assayed microbiologically**

Substance tested		Vitamin B_6 content by External standard (μg/g)	Internal standard (μg/g)	Recovery (%)
Dried food yeast		30	—	—
		30	—	95
	Mean	30	—	—
Dried beef		2·9	—	—
		2·8	—	102
	Mean	2·9	—	—
Flour, 85 % extraction		1·2†	—	—
		1·5	—	91
	Mean	1·4	—	—
Dried rumen contents		5·2	— (Mean 5·1)	142
		5·3	—	112
		4·8	4·4	104
		5·2	5·6	94
	Mean			113

* Each substance was assayed twice, with an interval of 2 years' storage at −20° between assays.
† Extracted with 0·055 N-sulphuric acid.

Table 7. *Folic-acid content of dried food yeast, assayed microbiologically after different extraction procedures*

Method of extraction	Folic-acid content by *Lb. casei* (μg/g)	*Strep. faecalis* (μg/g)	Recovery (%)
Digestion with pig-kidney extract followed by dilution with water and filtration (Bird, Bressler, Brown, Campbell & Emmett, 1945)	3·7	3·0	—
	3·3	3·0	—
	3·3	—	—
Digestion with pig-kidney extract, filtration omitted	4·3	—	—
	4·5	—	—
Digestion with chick-pancreas extract, pH 5	6·3	5·8	—
	6·8	—	—
As above, but with larger amounts of chick-pancreas extract	6·7	—	86
Digestion with chick-pancreas extract, pH 8	6·0	—	97
Digestion with pig-kidney extract, pH 4·6, then with chick-pancreas extract, pH 7 (Sreenivasan *et al.* 1949)	6·9	—	86
	7·5	—	80

Each result is for a separately weighed portion of yeast. The highest results, 6·9 and 7·5 (mean 7·2), obtained after digestion with pig-kidney and chick-pancreas extracts, were those taken for comparison with the results of the chick assay in Table 2.

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Table 8. *Folic-acid content of dried spinach-beet leaves and dried rumen contents, assayed microbiologically*

Substance tested	Method of extraction	Test organism	Folic-acid content (μg/g)	Recovery (%)
Dried spinach-beet leaves	Digestion with pig-kidney extract, pH 4·6, then with chick-pancreas preparation, pH 7·0	*Lb. casei*	16·9	128
			16·5	65
		Strep. faecalis	15·6	86
			14·6	77
		Mean	15·9	89
Dried rumen contents	As above	*Lb. casei*	1·1	—
			1·2	75
		Mean	1·2	

Table 9. *Content of riboflavin and vitamin B_6 in natural materials determined by tests with rats*

Vitamin	Method	Substance assayed	No. of groups: Standard	No. of groups: Test	No. of animals/group	Value (μg/g)	True fiducial limits at $P=0·95$ (μg/g)
Riboflavin	Henry, Houston & Kon (1940)	Dried sow's milk	3	2	10	8·2	7·3–9·3
Vitamin B_6	Copping (1943)	Dried food yeast	3	2	4♂	41	30–61
			3	2	4♀	38	29–52
		Dried beef	3	2	4♂	3·3	2·1–4·4
			3	2	4♀	3·9	2·7–5·1
		Flour, 85 % extraction	3	3	4♂	2·0	1·3–2·5
			2	2	4♀	3·0	2·3–4·3

DISCUSSION

The biological assay by the chick method of members of the vitamin B complex in natural materials proved possible, although, as might be expected, the error was somewhat large, especially for samples of low potency. As the test sample was incorporated directly into the chicks' diet without any preliminary extraction or other preparatory treatment, the results are a measure of the physiological availability of the vitamins rather than their total amount.

For the microbiological tests the reproducibility by any one set technique was generally good, and the inherent error was hence comparatively small. However, a microbiological test, though reproducible, may fail to measure the activity of a vitamin as established by direct feeding test with higher animals, not only because it assays the activity for a different organism but also because the test is generally applied not to the substance itself but to an extract prepared from it. In none of the microbiological tests reported here was there any marked discrepancy between results obtained with external and with internal standards, indicating that the vitamins had the same growth-promoting potency in presence or absence of the other substances present in test extracts. Agreement between results with both types of standard therefore provides some evidence for the validity of the results. Recovery tests also, where applied, generally showed little or no loss and indicated that the procedures

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used were capable of estimating the different vitamins, at any rate in so far as they were available in the test extracts to the appropriate micro-organisms.

Broadly speaking, there was in our experience good agreement between the microbiological and chick tests, although there were some marked discrepancies, of which three outstanding examples were the determinations of nicotinic acid in dried beer solids and yeast extract and of folic acid in dried yeast.

The results obtained by chick assay for nicotinic acid in beer solids was on two separate occasions only about one-third of that found microbiologically and by chemical test. For yeast extract, the chick value was less than half that found by these two methods. Several explanations may be suggested to account for the differences. First, samples were prepared for microbiological and chemical test by autoclaving with N-hydrochloric acid, and this rather drastic treatment may have released some of the vitamin from a form not readily available to animals. Secondly, compounds may have been present that were as active for bacteria as nicotinic acid and were chemically measured as such, but useless to the chick, though this possibility is rather remote, not only because of the good agreement between microbiological tests on beer solids with internal and external standards (Table 5) but also because of the good agreement between the chemical measurement and microbiological assay. The third possibility is that some inhibitor or antivitamin may have been present in the beer solids and yeast extract and have interfered with proper utilization of the nicotinic acid by the chicks.

Whatever the reason for the discrepancy, it is clear that very much less nicotinic acid was biologically available to the chicks than was indicated by the microbiological and chemical techniques.

The interpretation of these comparisons is in any event complicated by the biological interrelationship of nicotinic acid and tryptophan. It is known that chicks can utilize tryptophan in place of nicotinic acid (Krehl, Sarma, Teply & Elvehjem, 1946; Rosen, Huff & Perlzweig, 1946; Briggs, Groschke & Lillie, 1946); in our experience with the diet used in the present studies 40 mg of additional tryptophan gave a growth response equivalent to that usually produced by about 2 mg nicotinic acid.

When chicks were used to assay dried yeast for folic acid the result obtained was several times higher than any found microbiologically. It is possible that the enzyme treatment in vitro, used before the microbiological test, was less effective in the breakdown of conjugated forms of folic acid than was the complicated system of enzymes in the digestive tract of the chick. Possibly also there may exist in yeast precursors or metabolites of the pteroylglutamates of greater activity to chicks than to the microorganisms used. It is unlikely that the high result found by the chick method was due to a lack in the basal diet of some nutrient present in yeast, for satisfactory agreement between the two techniques was obtained in the assays of pyridoxin, pantothenic acid and nicotinic acid in yeast.

Though other workers (Bird *et al.* 1945; Burkholder, McVeigh & Wilson, 1945) obtained good agreement between microbiological and chick tests for folic acid on yeast, Tables 2 and 7 show that we failed to do so, whether we applied the enzyme treatments used by these authors separately or together.

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Discrepancies such as those just described amply justify caution in interpretation of microbiological results and emphasize that the reactions of micro-organisms to any nutrient as present in natural materials are not necessarily those of higher animals. A microbiological assay may, however, have considerable advantages over a non-biological technique, as is shown by the measurement of riboflavin in sow's milk (see Davis *et al.* 1950). Good agreement was reached between the microbiological and chick techniques, whereas a chemical method well-tried for cow's milk gave much lower results; further investigation revealed the presence in the sow's milk of riboflavin in a form not detectable by the chemical procedure used.

Apart from these examples of marked discrepancy between microbiological and chick tests, there were several smaller differences that may have been due to imperfections in technique. Thus, the agreement between the tests for pantothenic acid by the two techniques studied was reasonably good for yeast, fish solubles and rumen contents, but for milk the chick test gave a higher value. Pantothenic acid is similar to folic acid in often occurring naturally in combined forms, from which it must be liberated by enzyme action before it becomes available to the micro-organisms used by us. It is possible that milk supplied the chicks with an essential factor absent from the basal diet or that the enzyme treatment before microbiological assay may have been less efficient than that of the chick's digestive system.

The microbiological assay of vitamin B_6 was done with *Saccharomyces carlsbergensis*, reported by Snell & Rannefeld (1945) and Miller & Baumann (1945) to respond about equally to the three forms of the vitamin, pyridoxal, pyridoxamine and pyridoxin. According to Luckey, Briggs, Elvehjem & Hart (1945) the activity for chicks of pyridoxal or pyridoxamine is less than that of pyridoxin. Hence if these compounds when present in natural materials behave in the same way as in the free form, one might expect the chick tests to yield on the whole lower results than microbiological tests. This was so in our experience with rumen contents, but not with yeast, meat and flour. The chick findings agreed, however, very well with those we obtained on rats and, except for flour, with admittedly rather unreliable chemical measurements. It is possible that the extraction before the microbiological test was not complete or that the basal diet for chicks and for rats was lacking in some factor other than vitamin B_6. Of the known factors, vitamin B_{12} was not added, but examination of the chicks used in this laboratory has shown that they carry reserves of vitamin B_{12} (Coates, Harrison & Kon, 1950). Evidence is accumulating (Stokstad, Jukes, Pierce, Page & Franklin, 1949) that the so-called 'animal protein factor' may well be of a complex nature. Whether the supply of 'animal protein factor' to the chicks used in these assays is optimum, and how best it may be supplemented if it is not, can be decided only when more is known of its identity and distribution.

Until the importance of the various factors just quoted is thoroughly investigated, they may give rise to error in any biological assay carried out by these methods. However, if the possibilities of error are borne in mind when interpreting results, assays by the chick method can contribute reliable information about the biological availability of vitamins of the B complex and can also serve as checks on the applicability of microbiological and chemical methods to estimating the requirements of higher animals.

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SUMMARY

1. The methods of assaying vitamins of the B_2 complex with chicks, described by Coates, Kon & Shepheard (1950) for pure substances, have been applied to twelve natural materials, and the results have been compared with those of microbiological tests and of some chemical and rat assays as well.

2. Dried milks, rumen contents, faeces, yeast products, flour, meat, fish solubles and spinach-beet leaves were assayed for one or more of the following vitamins: riboflavin, nicotinic acid, vitamin B_6, folic acid and pantothenic acid.

3. On the whole the agreement was satisfactory, the notable exceptions being nicotinic acid in yeast extract and dried beer solids, for which the chick assay gave much lower results, and folic acid in yeast, where the reverse was true.

4. The tests show that the microbiological methods used can be applied to a wide range of natural materials. They also emphasize the need for a biological check before microbiological results are interpreted in terms of animal requirements.

We are greatly indebted to our colleagues, Dr S. Y. Thompson and Dr J. W. G. Porter, for the physical and chemical tests quoted in this paper, and we should especially like to thank Dr K. M. Henry for permission to quote the results of biological tests with rats.

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The Importance to Sheep of Frequent Feeding

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It has long been the practice of good stockmen to feed their animals 'a little and often'. This paper describes how this belief has been tested experimentally.

EXPERIMENTAL

Twenty-two Cheviot ewe hoggs were paired and divided into two equal groups which were comparable in age, body-weight and previous history. The sheep were penned singly, and to each was fed a daily ration consisting of 1 lb. chopped hay mixed with 1½ lb. of the following concentrate mixture: yellow maize meal 57, crushed oats 15, wheat bran 14, linseed-cake meal 7, and white fish meal 7%.

In addition to these rations each animal was offered an *ad lib.* supply of water and had access at all times to both plain salt and mineral licks. This quantity of food was maintained throughout the 18 weeks of the experiment as it was considered that to increase it with increasing body-weight would serve only to complicate the experiment.

During the first part of the experiment, which lasted for a period of 9 weeks, group A was fed eight times daily at approximately hourly intervals between the hours of 9.30 a.m. and 5.15 p.m. On each occasion one-eighth of the total ration was given, i.e. 3 oz. concentrates and 2 oz. chopped hay. Group B received its daily ration as one large feed at 9.15 a.m. For the second 9-week period the treatments were reversed, group B being given the frequent treatment and group A fed once daily. The animals were weighed twice weekly throughout the experiment. Food residues were weighed, no account being taken of their composition, and the total quantity of food eaten daily was recorded. Animals on a single feed rarely left a food residue, but, on the other hand, those fed frequently commonly did this as is shown in Table 2.

RESULTS

The criteria used in the interpretation of the results are those of body-weight gains and efficiencies of food utilization. The latter refer to net efficiencies and are calculated by dividing the starch equivalent (lb.) consumed over and above maintenance requirements during each 9-week period by the body-weight gain (lb.) during that time. The results of the experiment are shown in terms of body-weight gains in