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The Effect of Sulphonamides and of Cooking or Grinding the Diet on the Excretion of Vitamins of the B Complex by the Refected Rat

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Refection in rats was first recognized by Fridericia (1926), who described the condition as 'a transmissible change in the intestinal content, enabling rats to grow and thrive without B-vitamin in the food'. The existence of refection has been confirmed by later workers (cf. review by Kon, 1945), but the full mechanism of establishing and maintaining the refected state has not yet been satisfactorily explained.

It is now known that the intestinal flora of rats, and indeed of most animals, synthesizes all the well-characterized vitamins of the B complex. The contribution of this microbial synthesis to the vitamin requirements of rats on normal diets is relatively small, but refected rats are evidently able to derive the whole of their B vitamins from the products of such synthesis. Thus either the total amount of vitamins synthesized must be greater in the refected than in the normal rat or the efficiency of utilization must be higher or, more probably, both. Kon, Kon & Mattick (1938) suggested that the presence of undigested starch in the caecum of the refected rat, along with the requisite starch-splitting enzymes led to a vigorous fermentation and a resulting acid pH that created conditions favourable for both vitamin synthesis and absorption.

During recent years the effect of sulphonamides on the synthetic activities of intestinal micro-organisms has been extensively studied, and much valuable information

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has been acquired about the synthesis of vitamins and other substances in the gut and their availability to the host.

Experiments on the effect of sulphonamides on the growth of refected rats and on the excretion by them of thiamine and riboflavin have already been briefly reported by Coates, Henry, Kon, Kon, Mawson, Stanier & Thompson (1946), who found that sulphonamides depressed the growth rate of the rats and their faecal excretion of both vitamins. Untreated refected rats excreted no measurable amounts of thiamine in the urine, whereas their urinary excretion of riboflavin was comparable with that of stock rats. Administration of sulphonamides caused a marked depression in this excretion. It was concluded from these experiments that thiamine is probably the limiting factor in refection, and that the bacterial flora of the refected rat synthesizes riboflavin in excess of requirements. In further work a more detailed study was made of the excretion by the refected rat of several members of the vitamin B complex and of the effect thereon of sulphonamides and of cooking or grinding the diet.

EXPERIMENTAL

General

The experiments were done with two batches of rats. The first series of collections was made from the first of these batches in 1947, and the second series of collections from the second batch in 1949. The urine, faeces and caecal contents of the groups of rats were analysed for seven vitamins of the B complex: thiamine, riboflavin, nicotinic acid (and in urine N-methylnicotinamide), pantothenic acid, biotin, folic acid and vitamin B_6. After the second series of collections the livers of the rats were also analysed for these vitamins.

Diets

The first batch of rats received one of the following four diets: (1) The refective diet of Coates *et al.* (1946). (2) The refective diet steamed with an equal weight of water to gelatinize the starch. (3) The refective diet with 0·5% sulphathiazole. (4) The refective diet with 0·5% succinylsulphathiazole.

Rats in the second batch received one of these diets, or (5) the refective diet, but with milled starch replacing raw potato starch. Grinding starch in a ball-mill for several days breaks the starch-grain capsule (Lampitt, Fuller & Goldenberg, 1947) and makes the starch readily digestible (Levy & Jacquot, 1948). Thus it was expected that the ability of starch to support refection would be destroyed on milling in the same way as on gelatinizing the starch by cooking.

Management of rats

Male and female rats from the stock colony were placed at weaning on the refective diet (1). At the end of 7–8 weeks, when refection was well established as judged by steady growth and healthy appearance, the animals were divided into equal groups and given the experimental diets.

The rats in the first batch were divided into four groups and given diets (1), (2), (3) or (4). At the end of 1 week the different groups were placed in metabolism cages

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of the Hopkins type (Ackroyd & Hopkins, 1916) and 24 h samples of urine and faeces were collected. After the collection the rats were given diet (1) for 1 week and then for another week their respective experimental diets; a second 24 h collection of urine and faeces was then made. The rats were again given diet (1) for 1 week and their experimental diets for another week, when a third 24 h collection of urine and faeces was made. The rats were then killed and their caecal contents were removed for analysis.

For comparison, urine and faeces were also collected from a control group of rats from the same litterings as the first batch but fed on the stock-colony diet (Folley, Ikin, Kon & Watson, 1938) from weaning. Three collections were made at the same times as those from the refected rats.

The rats in the second batch were divided into six groups, two groups receiving diet (1) and the remaining groups diets (2), (3), (4) or (5). At the end of 1 week one of the groups receiving diet (1) was killed and the livers and caecal contents were removed for analysis; the other groups were placed in the metabolism cages and a 24 h collection of urine and faeces was made. The rats were then given diet (1) for 1 week and their experimental diet for a further week, when a second 24 h collection of urine and faeces followed. The rats were then killed and livers and caecal contents were removed for analysis.

The alternation between the refective diet (1) and the experimental diets was adopted because previous experience (Coates *et al.* 1946) had shown that refected rats did not survive for long on the experimental diets (2), (3) or (4). As can be seen from Table 1, it proved possible with the first batch of rats to collect urine and faeces on three separate occasions, with the loss of only one rat from the group given the cooked refective diet and of three rats from the group given the refective diet with succinylsulphathiazole. Two collections were made from the second batch of rats, with the loss of two rats on the cooked refective diet and of one rat from each of the groups on the refective diet with succinylsulphathiazole and on the refective diet containing milled starch.

Some difficulty was experienced in preventing the rats on diets (1), (3), (4) and (5) from scattering their food into the collection vessels, whether the diets were given mixed with water or dry. The stock-colony diet was not scattered, and the cooked refective diet dried into hard lumps that were easily separated from the faeces. During the third collection period of the first series of collections, food, but not water, was withheld from all the rats; though this prevented contamination of the faeces by the diet it caused some reduction in the weight of the faeces collected. Stainless steel hoods with narrow side openings were then used to reduce food scattering for all of the second batch of rats.

Collection of samples

Urine

The urine samples were collected under toluene in brown-glass bottles. After measurement of its volume the urine was filtered into brown-glass containers, and stored at -25° until required.

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Table 1. *Information about the materials obtained during the collection periods*, numbers of rats in each group and weights of the rats*

	Collection no.	Diet: Stock	Refective	Cooked refective	Refective with starch milled	Refective with sulphathiazole	Refective with succinyl-sulphathiazole
Series 1:							
No. of rats in group†	1	6	9	10	—	10	10
	2	6	9	9	—	10	8
	3	6	9	9	—	10	7
Mean weight of rats (g)	1	286	154	128	—	142	140
	2	297	179	129	—	157	143
	3	309	200	134	—	143	140
Weight of faeces (g/rat/24 h)	1	4·3	3·8	0·32	—	1	1·1
	2	3·6	1·4	0·37	—	1·1	1·1
	3	2·0§	1·1§	0·19§	—	0·43§	0·46§
Volume of urine (ml./rat/24 h)	1	9·8	4·8	4·0	—	4·2	2·9
	2	9·6	2·0	4·2	—	2·6	1·8
	3	5·7	7·0	5·2	—	3·8	3·7
Weight of caecal contents (g/rat)		4·8	4·6	1·0	—	8·6	12·5
Series 2:							
No. of rats in group‡	1	—	5	5	5	5	5
	2	—	5	3	4	5	4
Mean weight of rats (g)	1	—	121	103	104	114	113
	2	—	136	97	107	93	102
Weight of faeces (g/rat/24 h)	1	—	1·3	0·12	0·27	0·34	0·10
	2	—	1·7	0·43	0·32	1·0	0·37
Volume of urine (ml./rat/24 h)	1	—	2·9	3·9	1·5	1·0	1.6
	2	—	3·8	4·4	3·8	4·8	3·8
Weight of caecal contents (g/rat):							
Before collection		—	4·5	—	—	—	—
After collection		—	4·3	2·3	2·5	1·7	1·6
Weight of liver (g/rat):							
Before collection		—	6·2	—	—	—	—
After collection		—	8·7	5·6	5·6	4·8	5·6

* For details of arrangements see p. 68.
† Each group initially contained equal numbers of males and females.
‡ All females.
§ Food withheld during this collection period.

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Faeces

The faeces were collected on filter-paper moistened with toluene in beakers covered with black paper. They were picked off twice during the 24 h period and brushed free from any adherent particles of diet, weighed, and then mixed, with a spatula, in brown-glass bottles; these were stored at $-25°$ until required.

Caecal contents

The caecal contents were weighed and were then well mixed in brown-glass bottles before storage at $-25°$.

Livers

The livers were weighed, homogenized in a Waring blendor and stored in brown-glass bottles at $-25°$.

Measurement of vitamins

Chemical methods

Thiamine. The thiamine content of the urine was measured by the method of Mawson & Thompson (1948).

The other materials required a preliminary extraction: 5 g (or less) of the sample were suspended in 40 ml. of 0·1 N-H_2SO_4 and heated on the steam bath for 1 h; after cooling the pH was adjusted to 4·5 by the addition of 2·5 M-sodium acetate. The mixture was then centrifuged and the precipitate washed twice with water, the supernatant fluid and washings being then made up with water to 60 ml.; 20 ml. were set aside for the estimation of riboflavin (see below) and the remaining 40 ml. were diluted to 75 ml. and divided into three 25 ml. portions. One portion was used for the estimation of free thiamine; a known amount of thiamine standard was added to the second; the third was incubated for 2 h at 37° with 100 mg Takadiastase (Parke, Davis & Co.) for measurement of total thiamine. The thiamine present in the three extracts was measured fluorimetrically after zeolite purification (cf. Mawson & Thompson, 1948).

Riboflavin. The riboflavin content of the urine samples was measured fluorimetrically, directly on the urines, as described by Slater & Morell (1946). The other materials were examined by the same fluorimetric method to determine the riboflavin in the 20 ml. portions of extract set aside during the thiamine estimations.

Nicotinic acid. Suitable portions of urine and faeces from the first series of collections were analysed by the method of James, Norris & Wokes (1947).

N-*Methylnicotinamide*. The N-methylnicotinamide contents of the urines collected in the first series of collections were measured by the method of Huff & Perlzweig (1947).

Microbiological methods

General. Progress in the development of microbiological methods during the course of these experiments resulted in certain modifications of the procedures used in the assay of samples from the second series of collections; these are noted under the results for the individual vitamins.

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The refective diet was analysed by the methods used for the analysis of the samples of faeces from the first series of collections.

Since two of the diets given to the rats contained sulphonamides, *p*-aminobenzoic acid was added to all assay tubes to a final concentration of 10 μg/ml. The results of some microbiological assays, the media being supplemented with *p*-aminobenzoic acid in this way, of samples of rat excreta and caecal contents to which known amounts of sulphathiazole and succinylsulphathiazole had been added are given in Table 2. These results show the validity of the assay techniques for samples containing sulphonamides at concentrations similar to those expected in the samples from rats given sulphonamides in their diets.

Table 2. *Results of the microbiological assays* of samples of urine, faeces or caecal contents, alone and after addition of sulphonamides*

Sample	Sulphonamide addition (%)		Vitamin content (μg/g) Without sulphonamide	With sulphonamide
			Riboflavin	
Urine	Sulphathiazole	0·1	0·27	0·27
Faeces		1·0	17·0	17·0
Caecal contents	Succinylsulphathiazole	0·5	20·0	20·0
			Nicotinic acid	
Urine	Sulphathiazole	0·1	7·2	7·3
Faeces		0·6	120	130
Caecal contents	Succinylsulphathiazole	0·5	26·0	27·0
			Pantothenic acid	
Urine	Sulphathiazole	0·1	26·0	26·0
Faeces		1·0	60·0	60·0
Caecal contents	Succinylsulphathiazole	0·5	15·0	15·0
			Vitamin B_6	
Caecal contents	Sulphathiazole	0·5	2·1	2·1
Caecal contents	Succinylsulphathiazole	0·5	1·6	1·5
			Biotin	
Urine	Sulphathiazole	0·1	0·055	0·055
Faeces		0·8	0·64	0·72
Caecal contents	Succinylsulphathiazole	0·5	0·11	0·13
			Folic acid (assayed with *Streptococcus faecalis*)	
Caecal contents	Sulphathiazole	0·5	0·23	0·21
Caecal contents	Succinylsulphathiazole	0·5	0·073	0·058
			Folic acid (assayed with *Lactobacillus casei*)	
Urine	Sulphathiazole	0·1	0·013	0·013
Faeces		1·0	0·20	0·20
Caecal contents	Succinylsulphathiazole	0·5	0·049	0·050

* For details of assay procedures, see p. 73.

Different extraction procedures were investigated in detail and those used are noted under the results reported for the several vitamins. Urine samples, unless otherwise stated, were not extracted, preliminary tests having shown this to be unnecessary; they were diluted with water to give a suitable concentration for assay.

Added vitamins were always almost completely recovered by the methods described. The validity of several of the microbiological tests has been verified by comparison

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with the results of assays with chicks (Coates, Ford, Harrison, Kon, Shepheard & Wilby, 1952).

Riboflavin. The extraction procedure and, in the first series of collections, the assay medium, were those of the Analytical Methods Committee (1946). For the second series of collections the medium was that of Roberts & Snell (1946). *Lactobacillus casei* ATCC 7469 was the test organism.

Nicotinic acid. All samples (including urine) were extracted by autoclaving with 20 parts of N-HCl at 15lb. pressure for 15 min. The extracts of samples from the first series of collections were assayed with *Lb. arabinosus* 17–5 and the medium of the Analytical Methods Committee (1946), modified by the addition of 0·2 g L-asparagine and 0·2 g L-glutamic acid to 500 ml. basal medium (Coryell, Harris, Miller, Williams & Macy, 1945); the same organism and the medium of Roberts & Snell (1946) were used for the samples from the second series of collections.

Biotin. The samples were extracted with 5 parts of 6N-H_2SO_4. The assays were done with the same organism as for nicotinic acid and with the same medium except that nicotinic acid was included and biotin omitted.

Vitamin B_6. Samples from the first series of collections were extracted with 5 parts of 2N-H_2SO_4, and those from the second series with 100 parts of 0·055N-H_2SO_4 (Rabinowitz & Snell, 1947); the latter method gave somewhat higher results.

The assay was that described by Atkin, Schultz, Williams & Frey (1943), with *Saccharomyces carlsbergensis* 4228, except that ammonium phosphate and nicotinic acid were included in the medium, as recommended by Hopkins & Pennington (1947). The total volume of liquid in a tube was 5 ml.; the tubes were mechanically shaken during the 18 h of incubation at 30°.

Folic acid. The samples from the first series of collections were incubated with one-fiftieth of their weight of papain (British Drug Houses Ltd.) and of Takadiastase (Parke, Davis & Co.) at pH 4·6 and 37° (Luckey, Briggs, Moore, Elvehjem & Hart, 1945); after incubation the mixtures were autoclaved for a moment at 15 lb. pressure before filtering and adjustment of the pH to 6·8. The resulting extracts were assayed with *Streptococcus faecalis* or *Lb. casei* ATCC 7469, or both, according to the method of Teply & Elvehjem (1945).

Folic acid was liberated from the samples of faeces and caecal contents in the second series of collections by incubation with a pig-kidney suspension at pH 4·6 (Bird, Robbins, Vandenbelt & Pfiffner, 1946). Preliminary trials had shown that further treatment with the chick-pancreas suspension, as practised by Sreenivasan, Harper & Elvehjem (1948), did not increase the liberation of folic acid. The liver samples were autolysed in 0·1 M-phosphate buffer (Olson, Fager, Burris & Elvehjem, 1948). The extracts were assayed with *Lb. casei* ATCC 7469 and the medium of Roberts & Snell (1946).

Pantothenic acid. The samples from the first series were incubated with papain and Takadiastase, as for folic acid. The extracts were assayed with *Lb. arabinosus* 17–5 and the medium of the Analytical Methods Committee (1946).

The samples from the second series were incubated with a phosphatase preparation

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from calf intestinal mucosa and a chick-liver suspension to liberate bound pantothenic acid (Neilands & Strong, 1948). The extracts were assayed with *Lb. arabinosus* 17–5 and the medium of Roberts & Snell (1946).

RESULTS

Diet. The vitamin content of the refective diet, as determined by analysis, is shown in Table 3.

Table 3. *Vitamin content of the refective diet*

(Thiamine measured fluorimetrically, the other vitamins microbiologically)

Vitamin	Content (μg/g diet)
Thiamine	0·008
Riboflavin	0·06
Nicotinic acid	0·82
Pantothenic acid	0·10
Vitamin B_6	0·01
Biotin	0·007
Folic acid	0·0002

Urine and faeces. The vitamin contents of the samples of urine and faeces from the three collections in the first and the two collections in the second series are listed in Table 4. The results are expressed on a daily basis for a unit weight of rat, to facilitate comparison of findings in the two series of collections and in groups of rats on different diets. The daily excretion of vitamins can be derived from the data in Tables 1 and 4.

Table 4 shows that the agreement between chemical and microbiological methods for the assay of nicotinic acid was good; for riboflavin in urine the agreement was not so satisfactory, the microbiological assay usually giving higher results, but the two methods showed fair agreement for the other samples. Satisfactory agreement was obtained in determinations of the folic-acid content of urine and faeces when both *Strep. faecalis* and *Lb. casei* were used.

It will be seen from Table 4 that the daily excretion of each vitamin by the groups of rats on all the diets, except the stock diet, varied widely. Some of this variation in the first series of collections was probably due to the withholding of food during the third period of collection, resulting in small amounts of faeces and relatively large amounts of urine (see Table 1). But even when food was given the variation in the excretion of vitamins was great.

The rats receiving the stock diet excreted the vitamins much more uniformly, and it is difficult to explain why the refective diet, or modifications of it, should be associated with such wide variability, unless it is connected with fluctuations in bacterial synthesis.

The effects of adding sulphonamides to the refective diet, of cooking the diet and of milling the potato starch can most readily be seen from Table 5. In this table the mean values for the vitamin contents of the urine and of the faeces from the different groups of rats for the five collections (from Table 4) are expressed as percentages of the vitamin contents of the urine and faeces of the rats fed on the refective diet.

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The fine milling of potato starch breaks the capsules of its grains and renders the starch soluble and digestible (Levy & Jacquot, 1948) in the same way that cooking does. The results for the cooked refective diet and for the diet containing milled starch should, therefore, be similar. In fact with both diets the rats stopped growing and excreted markedly less vitamins, especially folic acid, in the faeces (Table 5). The urinary excretion of riboflavin was increased, that of nicotinic acid remained substantially unchanged, but the other vitamins were somewhat reduced in amount. Some of these effects may possibly have been due to the mobilization of body stores; the urinary excretion of riboflavin in man is said to increase in the absence of thiamine and in starvation (Mickelsen, Doeden & Keys, 1945).

On a diet with either sulphonamide the faecal excretions of vitamins was considerably reduced. The relatively insoluble drug, succinylsulphathiazole, caused, as expected, larger depressions than the more soluble sulphathiazole, but the extent of the difference was not as great as anticipated. The urinary excretions were also depressed, rather more than by the cooked refective diet, and again succinylsulphathiazole produced the greater effect. It will be noted that both urinary and faecal excretion of nicotinic acid was depressed by sulphonamides, which also reduced the urinary excretion of N-methylnicotinamide by more than half. It is probable, therefore, that the refected rat derives more nicotinic acid from microbial synthesis than by the conversion of tryptophan to nicotinic acid, of which the rat is capable (Krehl, Teply, Sarma & Elvehjem, 1945), since according to Hundley (1949) this process is independent of the intestinal microbial flora.

Caecal contents. Table 6 shows the vitamin content, in μg/100 g rat, of the caecal contents of the rats from the two batches. There were considerable variations for the individual vitamins of caecal contents removed on different occasions, but the general pattern of results was similar to that found for faeces.

The caecums of rats given the refective diet contained appreciable quantities of all the vitamins measured. In many instances these were greater than those in the caecums of rats on the stock diet, indicating considerably more synthetic activity of the microbial flora in the refected rats. It should be noted that part of the vitamins in the caecums of stock-colony animals was probably from unabsorbed food residues, whereas in the refected rats all the vitamins in the caecum must have been synthesized, since their diet was almost vitamin-free (see Table 3).

The cooking of the refective diet, or the milling of the starch included in it, caused a general lowering of the vitamin content of the caecal contents, and sulphonamides, particularly succinylsulphathiazole, caused an even greater reduction. With all these diets the reduction in the microbial synthesis of folic acid was the most marked.

Livers. The vitamin content of the livers of the rats used in the second series of collections, and of a group of rats fed on the refective diet and killed before the collection period are shown in Table 7. The results are expressed in μg/100 g rat. It is clear that the giving of the experimental diets for periods of 1 week did not cause any significant change in the content of any of the vitamins measured, except for some decrease in pantothenic acid. Unfortunately, no samples of liver were available from rats that had been on the experimental diets for longer periods.

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Table 4. *Vitamin content (μg/100 g rat/day) of samples from five collections of urine and faeces from groups of rats on different diets*

(The numbers of rats in each group and the amounts of excreta collected are given in Table 1)

			Diet											
			Stock		Refective		Cooked refective		Refective with starch milled		Refective with sulphathiazole		Refective with succinyl-sulphathiazole	
Vitamin	Series	Collection	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces
Thiamine														
Free	1	1	—	2·1	—	1·9	—	0·86	—	—	—	0·56	—	0·36
		2	—	5·5	—	1·6	—	1·7	—	—	—	1·1	—	2·8
		3	—	1·8	—	1·2	—	0·55	—	—	—	—	—	—
	2	1	—	—	—	0·32	—	—	—	—	—	—	—	—
		2	—	—	—	0·54	—	—	—	—	—	—	—	—
Total	1	1	7·5	4·9	0·0	15·0	0·0	3·0	—	—	0·0	2·0	0·0	1·4
		2	9·3	5·8	0·0	3·0	3·7	3·7	—	—	0·0	3·0	0·0	3·5
		3	9·1	2·4	0·0	3·6	0·0	0·89	—	—	0·0	—	0·0	—
	2	1	—	—	—	4·6	—	—	—	—	—	—	—	—
		2	—	—	—	4·0	—	—	—	—	—	—	—	—
Mean (total)			8·6	4·4	0·0	6·0	0·0	2·5	—	—	0·0	2·5	0·0	2·4
Riboflavin:														
Determined chemically	1	1	4·7	6·9	0·87	25·0	0·78	4·4	—	—	0·29	2·9	0·0	2·2
		2	3·0	8·7	2·4	14·0	2·4	7·6	—	—	1·5	4·4	1·1	4·5
		3	2·7	5·7	7·1	11·0	4·3	1·5	—	—	4·1	—	2·3	—
Determined microbiologically	1	1	9·2	8·6	1·2	27·0	1·2	5·8	—	—	1·3	5·1	—	4·8
		2	12·0	10·0	7·2	25·0	5·7	8·6	—	—	2·1	6·5	1·1	7·9
		3	7·3	6·9	21·0	16·0	13·0	6·2	—	—	11·0	2·6	2·3	6·8
	2	1	—	—	8·2	32·0	24·0	3·6	6·3	5·9	5·9	3·9	8·3	3·4
		2	—	—	7·1	23·0	25·0	14·0	15·0	11·0	16·0	18·0	7·7	5·4
Mean			6·5	6·3	6·9	23·0	9·5	6·4	11·0	8·45	5·1	6·2	3·3	5·0
Nicotinic acid:														
Determined chemically	1	1	35·0	93·0	31·0	230·0	20·0	24·0	—	—	25·0	26·0	—	36·0
		2	14·0	110·0	8·2	80·0	18·0	23·0	—	—	9·0	25·0	6·9	36·0
		3	7·6	32·0	13·0	44·0	10·0	12·0	—	—	9·1	8·0	6·6	16·0
Determined microbiologically	1	1	37·0	87·0	23·0	210·0	14·0	21·0	—	—	16·0	27·0	—	33·0
		2	12·0	84·0	7·4	92·0	13·0	32·0	—	—	7·1	24·0	5·3	38·0
		3	—	—	—	—	—	—	—	—	—	—	—	—
	2	1	—	—	15·0	120·0	16·0	13·0	6·4	18·0	5·5	16·0	10·0	10·0
		2	—	—	13·0	130·0	19·0	56·0	15·0	42·0	13·0	84·0	12·0	27·0
Mean			21·0	81·0	16·0	130·0	16·0	26·0	11·0	30·0	12·0	30·0	7·8	26·0

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N-Methylnicotinamide	1	1	330.0	—	470.0	—	270.0	—	—	—	240.0	—	—	—
		2	290.0	—	280.0	—	280.0	—	—	—	130.0	—	77.0	—
		3	240.0	—	520.0	—	280.0	—	—	—	220.0	—	190.0	—
Mean			290.0	—	420.0	—	280.0	—	—	—	200.0	—	130.0	—
Pantothenic acid	1	1	53.0	36.0	19.0	190.0	31.0	15.0	—	—	11.0	14.0	18.0	14.0
		2	43.0	32.0	54.0	115.0	3.9	25.0	—	—	4.0	16.0	2.3	20.0
		3	32.0	22.0	135.0	78.0	36.0	15.0	—	—	28.0	7.1	3.9	12.0
	2	1	—	—	75.0	160.0	87.0	9.0	28.0	22.0	28.0	14.0	37.0	9.1
		2	—	—	96.0	160.0	90.0	70.0	70.0	40.0	72.0	90.0	13.0	27.0
Mean			43.0	30.0	76.0	140.0	50.0	27.0	49.0	31.0	36.0	39.0	15.0	18.0
Vitamin B_6	1	1	6.1	11.0	0.89	22.0	0.64	0.50	—	—	1.2	9.4	—	3.1
		2	8.1	10.0	1.1	11.0	1.8	4.8	—	—	1.3	2.2	1.1	2.8
		3	—	—	—	—	—	—	—	—	—	—	—	—
	2	1	—	—	7.6	13.0	5.4	1.5	2.2	2.0	2.9	2.0	4.3	1.6
		2	—	—	11.0	12.0	18.0	5.2	8.2	2.5	14.4	5.7	8.5	5.0
Mean			7.1	10.5	5.1	14.0	6.4	3.0	5.2	2.3	4.85	4.8	4.6	3.1
Biotin	1	1	0.18	1.5	0.12	1.7	0.22	0.41	—	—	0.068	0.064	0.044	0.10
		2	0.10	1.5	0.22	0.90	0.14	0.30	—	—	0.057	0.36	0.043	0.43
		3	0.10	0.68	0.75	0.87	0.22	0.22	—	—	0.13	0.11	0.18	0.19
	2	1	—	—	0.14	1.0	0.29	0.14	0.083	0.23	0.024	0.36	0.050	0.030
		2	—	—	0.12	0.94	0.17	0.36	0.11	0.84	0.12	0.41	0.087	0.15
Mean			0.13	1.2	0.27	1.1	0.21	0.28	0.096	0.53	0.082	0.26	0.081	0.38
Folic acid:														
Determined with *Streptoccocus faecalis*	1	1	0.20	1.5	0.055	7.8	0.033	0.31	—	—	0.037	0.115	0.031	0.095
		2	0.22	1.3	0.012	2.3	0.026	0.18	—	—	0.013	0.088	0.012	0.070
		3	0.20	0.62	0.055	1.8	0.020	0.13	—	—	0.012	0.027	0.0095	0.054
Determined with *Lactobacillus casei*	1	1	—	—	—	—	—	—	—	—	—	—	—	—
		2	0.17	1.3	0.0067	2.4	0.014	0.18	—	—	0.0078	0.086	0.0048	0.10
		3	0.17	0.59	0.051	1.8	0.013	0.13	—	—	0.010	0.036	0.0092	0.033
	2	1	—	—	0.031	7.2	0.028	0.49	0.013	0.59	0.029	0.93	0.0081	0.13
		2	—	—	0.033	6.1	0.029	0.95	0.020	0.52	0.024	0.35	0.0094	0.063
Mean			0.19	1.1	0.033	4.2	0.023	0.34	0.017	0.56	0.019	0.23	0.012	0.089

A dash indicates that no estimation was made.

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Table 5. *Mean values for the vitamin content of the urine and faeces of groups of rats on different diets expressed as percentages of the vitamin content of the urine and faeces of rats fed on the refective diet*

	Vitamin or metabolite																	
	Thiamine																	
	Free		Total		Riboflavin		Nicotinic acid		N-Methyl-nicotin-amide	Pantothenic acid		Vitamin B_6		Biotin		Folic acid		
Diet	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Urine	Faeces	Urine	Faeces	Urine	Faeces	Urine	Faeces
Refective	—	100	—	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Cooked refective	—	69	—	42	140	28	100	20	67	66	19	125	21	78	25	70	8
Refective containing milled starch	—	—	—	—	160	37	69	23	—	64	22	100	16	35	48	50	13
Refective with sulphathiazole	—	81	—	42	74	27	75	23	48	47	28	95	34	30	24	58	1
Refective with succinylsulphathiazole	—	110	—	40	48	22	49	15	31	20	13	90	22	30	35	36	2
Stock	—	190	—	73	94	100	130	62	69	57	21	140	75	48	110	580	26

A dash indicates that no estimation was made.

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Table 6. *Vitamin content (μg/100 g rat) of caecal contents from rats fed different diets*

(The number of rats in each group and the weight of caecal contents are given in Table 1)

Vitamin	Series	When killed	Diet: Stock	Refective	Cooked refective	Refective containing milled starch	Refective with sulphathiazole	Refective with succinylsulphathiazole
Thiamine								
Free	1	After collection 3	3·3	7·3	1·0	—	2·7	10·5
	2	Before collection 1	—	0·56	—	—	—	—
		After collection 2	—	0·38	3·6	8·0	0·8	1·7
Total	1	After collection 3	3·8	13·0	0·81	—	9·9	1·8
	2	Before collection 1	—	3·5	—	—	—	—
		After collection 2	—	4·2	5·0	8·5	11·1	2·2
Riboflavin:								
Determined chemically	1	After collection 3	4·9	21·0	6·2	—	16·0	12·5
	2	Before collection 1	—	20·0	—	—	—	—
		After collection 2	—	22·0	17·0	11·0	4·7	5·5
Determined microbiologically	1	After collection 3	9·6	23·0	8·8	—	27·0	50·5
	2	Before collection 1	—	35·0	—	—	—	—
		After collection 2	—	30·0	17·0	21·0	15·0	9·7
Nicotinic acid	1	After collection 3	69·0	99·0	48·0	—	140·0	230·0
	2	Before collection 1	—	160·0	—	—	—	—
		After collection 2	—	130·0	80·0	91·0	49·0	39·0
Pantothenic acid	1	After collection 3	47·5	46·5	44·0	—	108·0	133·0
	2	Before collection 1	—	160·0	—	—	—	—
		After collection 2	—	140·0	89·0	90·0	62·0	40·0
Vitamin B_6	1	After collection 3	5·0	19·5	2·7	—	13·0	14·0
	2	Before collection 1	—	13·0	—	—	—	—
		After collection 2	—	12·0	5·4	3·9	3·1	2·9
Biotin	1	After collection 3	0·68	1·6	0·36	—	0·95	0·97
	2	Before collection 1	—	1·3	—	—	—	—
		After collection 2	—	0·8	0·33	0·47	0·18	0·16
Folic acid:								
Determined with *Streptococcus faecalis*	1	After collection 3	7·45	5·1	0·68	—	1·4	0·65
Determined with *Lactobacillus casei*	1	After collection 3	6·4	4·4	0·45	—	1·2	0·44
	2	Before collection 1	—	6·5	—	—	—	—
		After collection 2	—	6·6	1·4	2·0	0·77	0·22

A dash indicates that no estimation was made.

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In Table 8 the vitamin content of the livers of the rats on the refective diet is expressed in µg/g wet tissue, for comparison with the data of Mitchell & Isbell (1942) for normal stock-colony rats. The only vitamins present in lower concentration in the refected rats were vitamin B_6 and folic acid.

Table 7. *Vitamin content (µg/100 g rat) of the livers of the rats used in the second series of collections*

(The weights of the livers are given in Table 1)

	Refective diet					
	Raw					
Vitamin	Rats killed before collection	Rats killed after collection	Cooked	Containing milled starch	With sulphathiazole	With succinylsulphathiazole
Thiamine, total	6·9	3·6	1·8	2·2	1·8	2·3
Riboflavin:						
Determined chemically	110·0	—	110·0	—	79·0	83·0
Determined microbiologically	140·0	130·0	155·0	110·0	110·0	120·0
Pantothenic acid	590·0	685·0	420·0	340·0	310·0	350·0
Nicotinic acid	910·0	770·0	810·0	650·0	550·0	700·0
Vitamin B_6	34·0	29·0	41·0	22·0	29·0	32·0
Biotin	5·1	4·7	5·1	3·6	3·7	4·1
Folic acid	6·9	4·7	6·8	3·4	4·8	6·3

A dash indicates that no estimation was made.

Table 8. *Vitamin content of the livers (µg/g wet tissue) of rats receiving the refective diet (or modifications of it), and of a group of normal rats receiving the stock diet*

	Liver content	
Vitamin	Refected rats	Normal rats*
Thiamine	5·0	6·0
Riboflavin	22·0	25·0
Nicotinic acid	130·0	170·0
Pantothenic acid	73·0	75·0
Vitamin B_6	6·0	21·0
Biotin	0·7	0·6
Folic acid	1·0	6·0

* Data from Mitchell & Isbell (1942).

DISCUSSION

These results demonstrate clearly that the refected rat can synthesize B vitamins and absorb enough of them for growth and can also excrete in the urine detectable quantities of all the vitamins measured except thiamine. Further, the faeces and caecal contents of rats receiving the refective diet contained more of most vitamins than those of rats receiving the stock diet, showing that more vitamin synthesis occurs in the caecum of the refected rat than in that of the stock rat. The urinary vitamin excretion of the stock rats was higher than that of the refected rats in most instances—in accord with the view that the stock rat derives most of its vitamins from the diet.

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The available evidence (cf. Kon, 1945) suggests that the establishment and maintenance of refection is dependent on a flourishing caecal flora. Some measure of the starch-splitting activity of this flora can be obtained from the pH of the caecal contents of rats receiving the various diets. Thus the caecal contents of rats receiving the refective diet had a pH of 5–6·6, those of rats receiving the refective diet with sulphonamide a pH of 5·5–6·5, and those of rats receiving the cooked or milled starch a pH of 7–7·5.

The rats receiving sulphonamides, as well as those receiving cooked or milled starch stop growing (Table 1) and their excretion of vitamins is reduced (Tables 4 and 5). The mechanisms by which these changes are brought about are different.

The acid reaction of the caecums of rats receiving sulphonamides suggests that starch breakdown is proceeding. This has been confirmed by microscopical examination (Baker, Nasr, Morrice & Bruce, 1951). As the synthesis and excretion of vitamins are reduced by sulphonamides, it would appear that the organisms responsible for starch breakdown are different from those responsible for vitamin synthesis. Preliminary bacteriological examinations of the caecal contents and faeces of rats receiving the refective diet with sulphonamides (Mann, unpublished) show that, as expected, the coliform organisms are depressed. This indicates that coliforms may be among the principal vitamin synthesizers in the caecums of the refected rats.

Cooking or grinding starch breaks the starch-grain capsule and allows the digestive enzymes in the stomach and intestines to attack the carbohydrate. The starch is broken down and the products absorbed before they reach the caecum. The small amount of undigested food residue reaching the caecum of rats fed on these diets provides a very limited substrate for bacterial action. Thus the pH is not lowered, vitamin synthesis is restricted and conditions are unfavourable for the absorption of any products of the synthesis.

These experiments do not provide a full explanation of the phenomenon of refection—in particular why it sometimes happens that a batch of rats given the refective diet from weaning fails to become refected, though their circumstances are, as far as is known, identical with those under which preceding and successive batches refect readily. The experiments indicate, however, that the establishment and maintenance of refection depend not only on the presence of undigested starch and starch-splitting organisms in the caecum, but also on the presence of additional organisms, which are susceptible to sulphonamides, for the symbiotic synthesis of vitamins.

The attainment of the state of refection appears to require a delicate balance between the organisms present in the caecum of the rat, and a full explanation of the phenomenon does not seem possible without a knowledge of the types, numbers and biochemical potentialities of the micro-organisms present both in the rat that has become refected and in the apparently identical rat that declines and dies on the same diet. It is also possible that the length of time the food remains in the caecum of rats on the refective diet may vary, those rats with a slower rate of passage having, therefore, a greater chance of becoming refected than those in which the food passes quickly through the caecum.

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SUMMARY

1. Five collections of urine and faeces were made, each for a 24 h period, from groups of rats given the refective diet from weaning and then one of the following experimental diets for 1 week before the collection period: the refective diet, the cooked refective diet, the refective diet with sulphathiazole, the refective diet with succinylsulphathiazole and the refective diet made with milled starch. For purposes of comparison three collections of urine and faeces were made from a group of rats of the same age as the refected rats and fed on the stock-colony diet.

2. The excreta, the caecal contents and the livers of the rats were analysed for seven vitamins of the B complex: thiamine, riboflavin, nicotinic acid (and in urine N-methylnicotinamide), pantothenic acid, biotin, folic acid and vitamin B_6. Chemical or microbiological methods were used for these estimations.

3. Rats fed on the refective diet, containing only traces of vitamins, excreted in their urine substantial, though very variable, quantities of all the vitamins measured except thiamine. The faeces and caecal contents of these rats contained appreciable amounts of all the vitamins.

4. Rats fed on the refective diet with sulphonamides excreted less vitamins in both urine and faeces, and the vitamin content of the caecal material was reduced, particularly that of folic acid.

5. More riboflavin was excreted in the urine of rats fed on the cooked refective diet, or the refective diet with milled starch, than in that of rats on the unmodified raw refective diet, probably because body stores were being used; there was no change in the urinary excretion of nicotinic acid and some reduction in the excretion of the other vitamins. The vitamin contents of the faeces and caecal contents of these rats were markedly lowered.

6. The significance of these findings in relation to the establishment of refection is discussed.

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The Heat of Combustion of the Tissues of Cattle in Relation to their Chemical Composition

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The estimation of energy retention by cattle from the results of simultaneous determinations of balances of carbon and of nitrogen is based on the assumption that fat and protein of constant chemical composition and calorific value are the only materials retained. The values employed for this purpose are usually those given by Armsby (1917):

Nitrogen retention × 6·25 = protein retention; protein retention × 0·5254 = carbon retention in protein; non-protein carbon retention × 1·31 = fat stored; protein stored (g) × 5·7 = calories stored as protein (Cal.); fat stored (g) × 9·5 = calories stored as fat (Cal.).

The factors for protein are derived from Köhler's (1900-1) studies on the elementary composition of fat-free muscles of cattle. These results with those of other investigators are summarized in Table 1. Köhler's (1900–1) results with horse muscles, which are not included, gave lower values for nitrogen and for calories

Table 1. *Carbon and nitrogen content and the calorific value of fat- and ash-free mammalian muscles*

		Composition on an ash-free basis		
Investigator*	Date	Carbon (%)	Nitrogen (%)	Cal./kg
Rubner	(1885)	53·40	16·30	5656·9
Stohmann & Langbein	(1891)	52·02	16·36	5640·9
Argutinsky	(1894)	52·33	16·15	—
Köhler	(1900–1)	52·69	16·57	5700·8

* Quoted in References, p. 90.

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