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AN INVESTIGATION OF FACTORS GOVERNING POTATO-STARCH REFECTION IN RATS

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(With Plate I and 9 Figures in the Text)

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INTRODUCTION

REFECTION in rats was first recognized in 1926 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". Fridericia *et al.* (1927) later described the condition in detail. The existence of refection has since been confirmed by Roscoe (1927), Kon & Watchorn (1928), Taylor & Thant (1929), Kon (1931), and Parsons *et al.* (1933). Mendel & Vickery (1929) were unable to produce refection in their laboratory, but as very few animals were used in their experiments the results cannot be considered too seriously. Bliss (1936), working on potato-starch refection, confirmed the findings of Kon & Watchorn (1928) and of Kon (1931).

No accepted or acceptable explanation of the phenomenon has yet been put forward. It has been suggested by the various authors and particularly by Fridericia *et al.* (1927) that bacterial synthesis of vitamin B in the intestine of the rat may be the explanation of the condition. Schieblich & Rodenkirchen (1929) have described a "vibrio-like" organism which they claim to be responsible for refection, but this finding has so far not been confirmed. In a recent dissertation on refection Nathan (1935) believes that the explanation is coupled with the nature of the starch in the diet rather than with an infection with a specific type of organism.

The refection originally described by Fridericia *et al.* (1926, 1927) was observed in rats fed on a vitamin B-free diet in which the carbohydrate used was rice starch. On this diet only a few of the rats became refected, but as was shown by Kon & Watchorn (1928), and since confirmed by Kon (1931), Parsons *et al.* (1933) and by Bliss (1936), when potato starch is the chief source of carbohydrate in the diet refection amongst the rats is almost the rule.

The present study is concerned mainly with the problem of potato-starch refection on diets containing a large proportion of potato starch. The work was carried out over a period of 3 years starting in 1932, and was embodied in a thesis for the Ph.D. degree, University of Reading, by P. M. Kon in May 1935.

Throughout this paper reference is made to "vitamin B", and by this term is meant the vitamin B complex.

EXPERIMENTAL METHODS

The rats were kept in experimental cages of galvanized iron, 9 × 9 × 9 in. with wire fronts hinging at the top and opening outwards, and movable wire screen bottoms above movable trays containing either sterilized sawdust or absorbent paper. The mesh of the wire screens varied with the experiment, the normal being a mesh of a quarter of an inch. In some cases screens half or two-thirds of an inch mesh were used. The cages were combined in batteries of forty-eight.

EXPERIMENTS ON REFECTION

I. General

(*a*) *Potato-starch refection*

Refection in this laboratory was first obtained in 1931. At first there was the usual difficulty in establishing the condition. Once, however, the condition had become well established there was no further trouble in refecting rats on a potato-starch diet. The following is the diet (D. 20) on which refection has been obtained in this laboratory:

	%
Casein grain curd*	23
Potato starch (from Harrington Bros.) ...	40
Sugar (castor)	17
Palm-kernel oil	15
Salts (Hopkins)	5

* Prepared in the laboratory by the method of Clark *et al.* (1920).

When the main study was started in the autumn of 1932, two rats (299 and 300) had been on the refective diet (D. 20) for 1 year, and were used for the foundation of a refected colony. The growth curves for these two rats are shown in Text-fig. 1. They grew steadily and well from the time they were placed on diet 20 until some 15 months later, when the decline which then took place may well have been expected in rats of that age. At post-mortem all organs of both rats appeared normal macroscopically, although there was a fairly marked degree of emaciation.

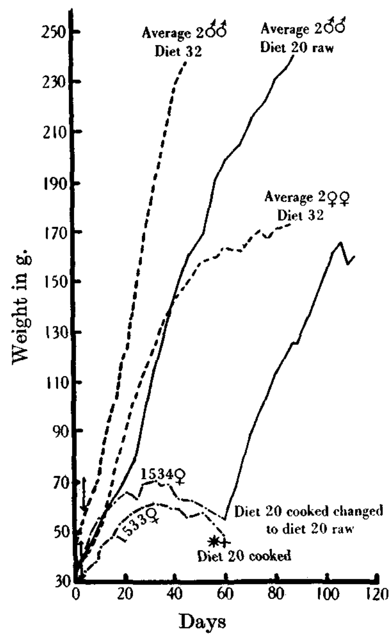
Text-fig. 1. Growth curves of rats 299 and 300 on the refective diet 20.
↑ Changed to stock diet.

Effect of cooking and of addition to the diet of yeast, on the refective power of the diet

An experiment comparing the effect of cooking and of adding yeast to the refective diet was carried out. The cooked diet (D. 20) was prepared daily by adding distilled water and heating until the starch was gelatinized. Littermate rats were fed the cooked diet, the raw diet, and the raw diet + 5 per cent brewer's yeast (diet 32), and the results are shown in Text-fig. 2. Rat 1533 on diet 20 (cooked) declined and died, and rat 1534 was declining steadily until it was changed to diet 20 (raw), when a marked recovery took place and the animal began to grow rapidly. There was little difference between the growth of well-refected rats on diet 20 and those on diet 20 + 5 per cent brewer's yeast (diet 32).

Time factor in susceptibility to refection

It was found in the early stages of the investigation that rats became refected more easily if placed in a cage with an already well-refected rat, although this was not necessary when the condition had become well established in the laboratory. Failures to refect animals on the potato-starch diet occurred earlier in this investigation but were almost unknown later when we found that rats just weaned could be placed on diet 20 with almost complete assurance that they would become refected. This observation suggests an infection theory, the supposition being that, 4 years after refection was first established in this laboratory, the refective agent was so strong and the infection so heavy that refection on a suitable diet was almost a certainty. The rats not only grew well on the refective diet, but presented a healthy appearance and looked perfectly normal.



Text-fig. 2. Effect of cooking and of addition of yeast on the refective power of diet 20.

↑↕ Diets start. + Death. ∗ Beri-beri symptoms.

Colour, size and refective power of faecal pellets in potato-starch refection

When refection was first established here the animals passed the large pale faeces described by Fridericia *et al.* (1927) as typical of refection, although in potato-starch refection the faeces are never quite so pale as in rice-starch refection. When the condition became well established the faecal pellets of the refected rats, although larger than normal and containing raw starch, were of a darker colour than previously. Periods of passing dark pellets were alternated with periods of passing light pellets, although there seemed to be no correlation between the growth of the animals and the colour of their faeces.

Fridericia *et al.* (1927) have stated that the feeding of faeces of refected rats to non-refected rats on a diet containing a large percentage of rice starch will establish in the latter a definite state of refection. Such a statement, in the case of potato-starch refection, cannot be made, because refection on a diet containing large quantities of raw potato starch is so nearly the rule that when faeces are fed it is impossible to say whether refection is due to this or has arisen spontaneously.

It is certain, however, that the administration of fresh faeces from rats well refected on diet 20 has some slight effect in prolonging the life of rats "run out" on a vitamin B-deficient diet and then transferred to diet 20. Though it does not necessarily establish in all of them the state of refection, the prognosis for such rats is more hopeful. The explanation of this may well be that the vitamin B present in the faeces saves the life of the rat and gives it a chance to survive long enough to become refected.

Effect of size of mesh of screen and of number of rats in a cage on the establishment of refection

In the authors' experience once refection is well established a change to coarser mesh or confinement to individual cages does not seem to affect the rate of growth of younger rats or the general well being of adult rats. But either of these factors exerts a definite influence in the initial stages of refection. As a rule a rat is more likely to fail to become refected if kept alone on a $\frac{3}{4}$ in. screen than on the customary $\frac{1}{4}$ in. mesh. The size of the mesh does not seem to play a definite role when several rats are kept together in one cage. Similarly, a group of rats started together in one cage generally become refected more readily than rats started singly. Even towards the end of this investigation the authors failed to refect two young rats placed in individual cages. These animals became refected after the administration of a few pellets of "refected" faeces and thereafter grew rapidly and steadily, although still confined to individual cages.

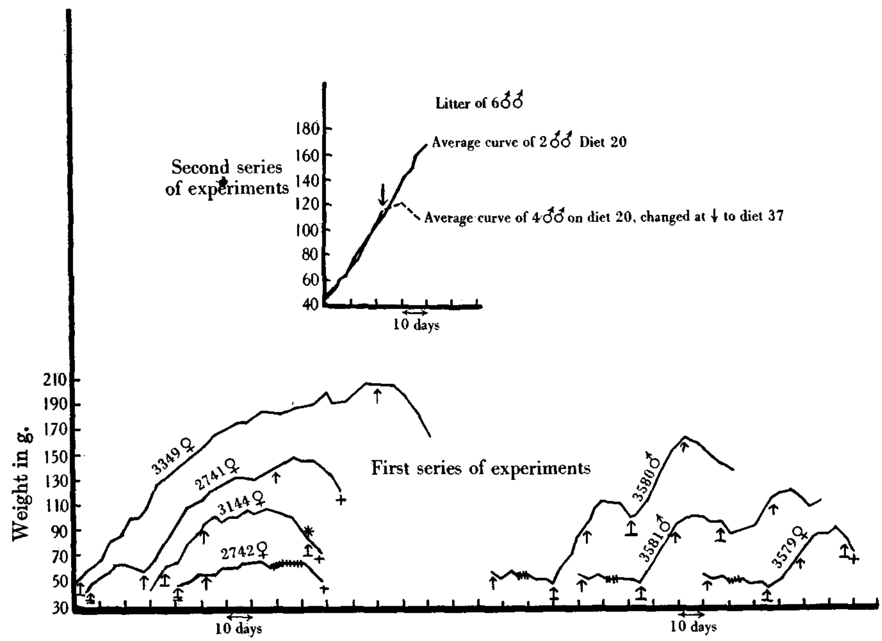
It seems that the ingestion of faeces (either their own, those of cage mates or faeces specially administered) materially helps rats in getting over the difficult first stages of refection.

In the authors' opinion once refection is well established there is no necessity for the last two mentioned sources of faeces. Even when rats are kept singly on very wide-mesh screens, auto-coprophagy cannot be entirely prevented. It is therefore impossible to say whether or not this practice is an essential condition of sustained refection.

(b) *Attempts to produce refection by feeding tannia starch*

Clark & Waters (1933) have described the occurrence of nephritis in rats fed on raw aroid tubers, such as the tannia tuber. They also observed that animals so fed produced large pale faeces containing a high percentage of starch. This fact, coupled with the constant finding at post-mortem of very large caeca in these rats, aroused the authors' interest, suggesting, as it did, the possibility of a state of the intestinal tract similar to that observed in potato-starch refection. A very enlarged caecum containing quantities of undigested starch is invariably found in rats refected on a potato-starch diet. Moreover, tannia starch in common with potato and arrowroot starches (two refective starches) can be extracted from the tuber by simple elution. Dr Clark very kindly discussed the problem with the authors and arranged to supply them with fresh tannia tubers.

The starch was extracted from the tubers by elution, dried and pulverized. An analysis, by Dr W. L. Davies, showed that the starch contained only 0·033 per cent N corresponding to 0·22 crude protein. A diet (D. 37) was made of exactly the same composition as diet 20 but containing the tannia starch in place of potato starch. Rats were given this diet, and the resulting growth curves are given in the lower half of Text-fig. 3.



Text-fig. 3. Attempts at producing refection on a diet containing tannia starch.

↑ Diet 37. ↑· Diet 20. ↥ Diet 19. +++++ Faeces fed.
\+ Death. * Beri-beri symptoms.

All attempts to refect rats on this diet failed. Even when rats were in a state of well-established potato-starch refection, a change to the tannia-starch diet resulted in marked loss of weight, and when the experiment was continued, in death. The feeding of faeces from well-refected rats to rats on diet 37 produced only a temporary stimulation of growth, and when the feeding of faeces was stopped, growth ceased again.

As the first series of experiments with tannia starch were carried out at a

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comparatively early stage in the investigation it was thought advisable to repeat them when potato-starch refection was firmly established. The results of this second experiment shown in the upper half of Text-fig. 3 confirm the results of the first series. From these results it is clear that refection cannot readily be obtained on a diet containing tannia starch as a source of carbohydrate.

II. The vitamin B content of the faeces and caecal contents of refected and of vitamin B-deficient rats

For some time it has been recognized that an animal subsisting on a synthetic diet will frequently benefit from consuming its excreta (Osborne & Mendel, 1911). Numerous workers have also demonstrated the presence of vitamin B in the excreta of birds and mammals (Cooper, 1914; Muckenfuss, 1918; Gaglio, 1919; van der Walle, 1922; Portier & Randoin, 1920), and this discovery led to discredit being thrown upon the routine methods of estimating vitamin B, as it was thought that coprophagy amongst the rats might vitiate the results (Steenbock *et al.* 1923*a*; Steenbock *et al.* 1923*b*; Dutcher & Francis, 1924; Salmon, 1925; Smith *et al.* 1925; Heller *et al.* 1925; Booher & Kaneko, 1932; Guerrant & Dutcher, 1932, 1934).

Fridericia *et al.* (1927) stated that the large pale faeces of refected rats contained considerable amounts of vitamin B, while those of non-refected rats on a diet deficient in this factor contained very little. No quantitative comparisons were carried out.

Roscoe (1927) stated that, for a rat to continue in the refected state it must consume some of its own faeces, but found that the white faeces of refected rats contained little if any of the antineuritic vitamin B. Guerrant & Dutcher (1932, 1934) showed that the type of carbohydrate used in the basal ration has an effect on the vitamin B and G (B_1 and B_2) potency of the faeces.

Having regard to these varying reports on the vitamin B content of faeces and the effect of coprophagy on rats subsisting on a vitamin B-deficient diet, it was thought that some light might be thrown upon the problem of refection by comparing the vitamin B content of the faeces and caecal contents of refected rats with those of rats kept on a vitamin B-deficient diet.

(*a*) *The vitamin B content of the faeces of refected and of vitamin B-deficient rats*

Faeces were collected from refected rats and rats declining on a vitamin B-deficient diet. These rats were kept in the experimental cages described earlier on screens of ¼ in. mesh over trays covered with absorbent paper from which the faeces were collected daily, roughly cleaned of adhering particles of food and dried in air. When dried the faeces were finally cleaned and stored in jars in a desiccator. Some of the "refected" faeces were stored for about 3 months as they were easily and rapidly collected, but much more difficulty was encountered in collecting sufficient quantities of faeces from rats declining on a vitamin B-deficient diet. These rats, as a rule, eat very little of a diet poor in roughage and pass only a small quantity of faeces.

Table I shows the difference in faecal output between the two groups of rats.

Table I

	No. of rats	Period over which faeces were collected days	Total dry weight of faeces g.	Approx. av. dry weight of faeces per rat per day g.
Refected rats, batch 1	8	41	174	0·53
Refected rats, batch 2	6	18	50	0·46
Vitamin B-deficient rats	50	13½*	96	0·14

* With vitamin B-deficient rats the period of collection varied from rat to rat. An average figure is given.

The average dry weight of faeces excreted daily by refected rats is about three to four times that excreted by rats declining on a vitamin B-deficient diet (Table I).

When sufficient faeces had been collected they were ground in an almond mill and kept in a desiccator ready for feeding.

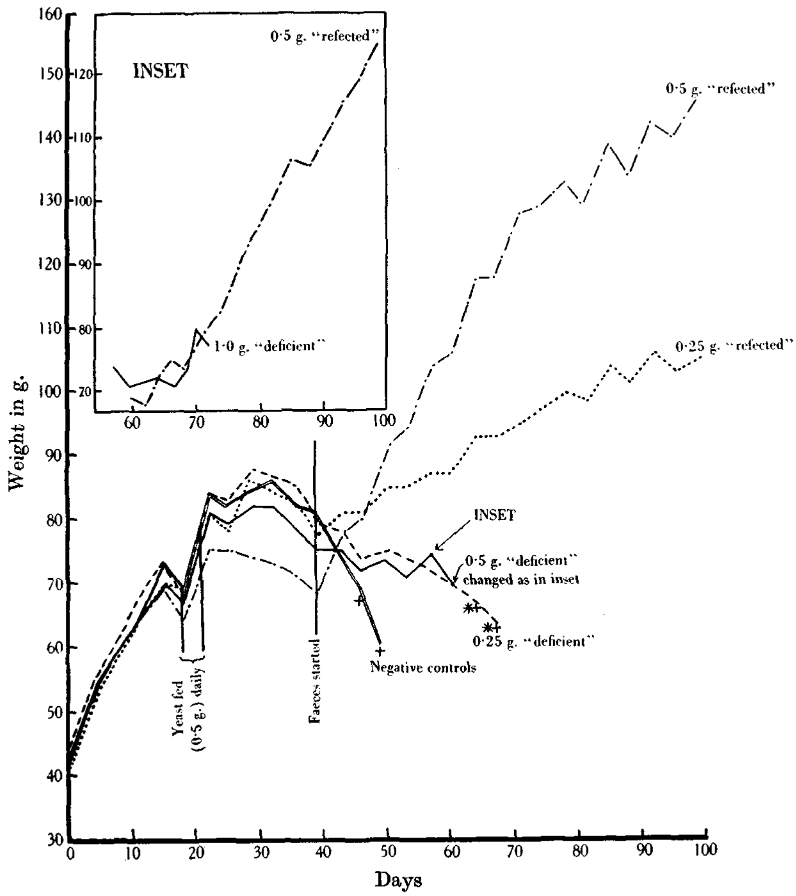
The groups which were to receive faeces consisted of four litters of five rats each, and were arranged in five groups of four (two bucks, two does), littermates being evenly distributed throughout the groups. Each group of four rats was kept in one cage. Two days after weaning all the groups were placed on the vitamin B-deficient diet of this laboratory (diet 19[1]) plus two drops of cod-liver oil daily. When they had been little over a fortnight on this diet and were declining in weight, their power of response to a vitamin B stimulus was tried out and all groups were given 0·5 g. of yeast per rat daily for 3 days. The rats responded by an immediate increase in weight, and this was maintained for about 10 days, after which all groups started to lose weight again. After 5 weeks on the diet, when all the groups were either stationary or losing weight, the feeding of faeces was started. The faeces were fed at the following levels:

Group 1 was fed 0·25 g. of "refected" faeces per rat per day.
,, 2 ,, 0·5 g. ,, ,, ,, ,,
,, 3 ,, 0·25 g. of "deficient" ,, ,,
,, 4 ,, 0·5 g. ,, ,, ,, ,,
,, 5 was the negative control group.

The animals were still kept together in groups of four in a cage, and received diet 19 and cod-liver oil. In the afternoon of each day, with the exception of Sunday, the allotments of faeces for each rat were weighed out into palette dishes and mixed with a little distilled water and sugar. The faeces were fed individually, each rat being placed under a plant pot with its

[1] Diet 19: casein (repeatedly washed with acidulated water at the isoelectric point) 24 per cent; dextrinized potato starch 67 per cent; ground-nut oil 5 per cent; salt (Steenbock 40) 4 per cent.

dish of faeces and left there until the faeces were consumed. From the start of the experiment the rats took the faeces readily, and there was no difficulty in getting them to clean up their dishes completely. The feeding of faeces was continued for almost 8 weeks, and the results of the experiment are shown in Text-fig. 4. In the graph each curve is an average for four rats (two bucks and two does). By the tenth day after the start of the feeding two of the negative control rats were dead and the last two died a day or two later.



Text-fig. 4. The vitamin B content of faeces of refected and of vitamin B-deficient rats. Each curve is an average for four rats (two bucks and two does).

\+ Death. * Beri-beri symptoms.

The rats receiving 0·25 g. of "deficient" faeces showed a steady decline in weight but lived for 28 days after the start of the experiment. All four died showing symptoms of beri-beri. As these rats lived longer than the negative controls, it seems that some slight benefit was derived from the faeces. The rats receiving 0·5 g. of "deficient" faeces declined slightly and then maintained their weight for about 3 weeks. At the end of this time one of the rats (inset in Text-fig. 4) was given 1 g. of "deficient" faeces daily. This produced a temporary slight acceleration of growth, which, however, ceased again after about 10 days. The other three rats of this group, after having been kept for 7 days longer on 0·5 g. "deficient" faeces, were given 0·5 g. "refected" faeces daily. The results given in the figure (inset) were striking The rats immediately resumed growth and continued steadily to increase in weight for 32 days, at the end of which they were killed.

Group 3, receiving 0·25 g. "refected" faeces, grew fairly well but sub-normally throughout the experiment, making an average gain of 28 g. over 60 days. Group 4, however, fed on 0·5 g. "refected" faeces made good growth, showing an average gain of 78 g. over the experimental period of 60 days. The peculiar fluctuations in this and the previous curve are noteworthy. The explanation probably lies in the fact that the rats were not given faeces on Sundays, and responded to this temporary absence of vitamin B from their food by a loss of weight at the Monday weighing.

The rats receiving 0·25 g. of "refected" faeces grew much better even than the rat which was given 1 g. of "deficient" faeces. From these results it appears that, dry weight for dry weight, the faeces of refected rats are more than four times as potent, as a source of vitamin B, as those of rats fed vitamin B-deficient diet.

(*b*) *The vitamin B content of the caecal contents of refected and of vitamin B-deficient rats*

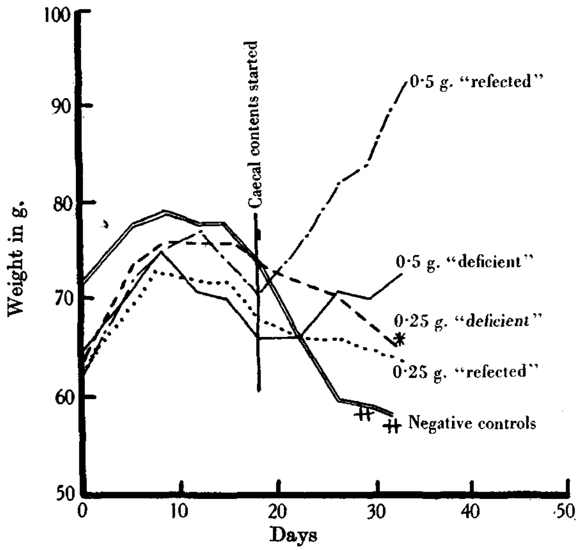
For this experiment the caecal contents of refected rats and rats on the vitamin B-deficient diet were collected and stored over a period of about 1 year. This lengthy period was due to the fact that large numbers of rats on the vitamin B-deficient diet had to be killed in order to obtain the necessary amount of material. The rats were killed some six or seven at a time; the caeca were taken out, and the contents evacuated into an evaporating dish, mixed up well with a little distilled water and evaporated to dryness on a water-bath. As the caecal contents of refected rats are slightly acid, and those of vitamin B-deficient rats alkaline, the latter were acidified with a little dilute acetic acid before evaporating to prevent any possibility of destruction of vitamin B taking place in one case and not in the other. (The important relationship between the state of refection and the hydrogen-ion concentration of the caecal contents will be described later.) When the material was dry, it was collected in a bottle, stored in a desiccator and, before feeding, was ground in an almond mill.

From thirty-nine refected rats, 79 g. of dried material were obtained giving an average yield of approximately 2 g. per rat, while from 175 rats on the vitamin B-deficient diet there were 69 g. of dried material giving only about 0·4 g. per rat. Thus the caecum of a refected rat contained about five times as much dry material as the caecum of a rat declining on the vitamin B-deficient diet.

As in the previous experiment, five groups of four rats each were used, littermates being distributed throughout the groups. The groups were fed at the following levels of caecal contents:

Group 1. 0·25 g. "deficient" caecal contents
„ 2. 0·5 g. „ „ „
„ 3. 0·25 g. "refected" „ „
„ 4. 0·5 g. „ „ „
„ 5. Negative controls.

The rats were allowed to decline on diet 19 and were then fed caecal contents daily (except Sundays) in the way described for the feeding of faeces (p. 8). The results of this experiment are shown in Text-fig. 5. Unfortunately



Text-fig 5. The vitamin B content of the caecal contents of refected and of vitamin B-deficient rats.

+ Death. * Beri-beri symptoms.

all the rats did not start on the same date, although they had all to finish at the same time, when the supply of material collected from vitamin B-deficient rats was exhausted. This means that while some rats were not on the experiment quite as long as others, the average period was about 15 days. All the negative control rats were dead 14 days from the start of the experiment. The rats on 0·25 g. "deficient" caecal contents lost weight (7 g. on the average), and at the end of the experiment two of them showed symptoms of beri-beri. Rats fed 0·5 g. of "deficient" caecal contents grew slightly, making an average gain in weight from the beginning of the feeding of about 8 g. The rats fed 0·25 g. "refected" caecal contents declined slightly and then maintained their weight, showing an average loss of about 3 g. during the experimental period. The rats fed 0·5 g. "refected" caecal contents made good growth, showing an average gain of about 25 g. From this it will be seen that 0·5 g. of "refected" caecal content is definitely more potent than 0·5 g. of "deficient" contents, but this quantity is slightly more potent than 0·25 g. of "refected" caecal contents.

Relatively speaking, the "refected" caecal contents are less potent than refected faeces when compared with corresponding material from vitamin B-deficient rats. This difference between relative potencies of "deficient" and "refected" caecal contents and faeces may be explained by the fact that the caecal contents of vitamin B-deficient rats are more concentrated when compared with the caecal contents of refected rats, than the faeces of vitamin B-deficient rats compared with the faeces of refected rats. It will be remembered that the dry weight of faeces obtained from refected rats was about three to four times that from non-refected rats, whereas the dry weight of caecal contents of refected rats was five to six times that from vitamin B-deficient rats. The caecal contents of refected rats contain a large proportion of undigested starch as well as bacterial bodies and other residues, but as the material passes farther down the intestinal tract the numbers of bacterial bodies will increase and some of the starch will be broken down. Thus in the *faeces of a refected rat, a larger percentage of bacterial bodies (presumably containing vitamin B) and a smaller percentage of starch will be present than* in the caecum. This will not be so in the case of rats on the vitamin B-deficient diet in which the starch is cooked and so easily digested, that there is very little starch to provide bulk in the caecum. In these animals, dry weight for dry weight, caecal contents and faeces should be more equally concentrated.

III. Bacteriological investigation of the faeces and intestinal contents of rats receiving different diets

Previous workers on refection claimed that bacterial synthesis of vitamin B in the intestine of the refected rat may be the reason why these animals are able to grow and thrive on a diet deficient in this factor. Fridericia *et al.* (1927) found the bacterial flora of the caecal contents of refected rats to be different from that of other rats, and describe "Gram-negative rods curved as vibrio" and large Gram-positive cocci to be present in large numbers. But feeding of five isolated strains of cocci and nine strains of bacilli gave no definite result. Kon & Watchorn (1928) observed that Gram-positive organisms were more numerous than Gram-negative organisms in the faecal flora of their refected rats. Schieblich & Rodenkirchen (1929) describe, in all cases, a predominance of "vibrio-like" organisms in faecal smears from their refected rats. All attempts to culture the vibrio were abortive, but nevertheless these workers consider that this organism was responsible for refection.

Numerous workers have shown that certain bacteria are capable of synthesizing vitamin B (Pacini & Russell (1918), Damon (1923, 1924), Scheunert & Schieblich (1923, 1927 and 1927*a*), Schieblich (1929, 1930), Heller *et al.*

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(1925)), while Bechdel *et al.* (1926) showed that calves could grow to maturity on a diet devoid of vitamin B and Bechdel *et al.* (1927, 1928) isolated an organism of the genus *Flavobacterium* from the rumen of a cow on a vitamin B-free diet. They consider this organism which they stated constituted 90 per cent of the rumen microflora to be responsible for the supply of vitamin B to the cow. In view, however, of the extensive and complex flora of the intestinal tract of ruminants, this explanation seems to be too optimistic in its simplicity. It nevertheless points again to the dominant part played by bacteria as a source of vitamin B for ruminants and to their probable role in the same capacity in the refected rat.

In an attempt to find a bacteriological explanation for the phenomenon of potato-starch refection the authors carried out numerous microscopical and cultural examinations of the caecal contents and faeces of normal, refected and vitamin B-deficient rats.

(*a*) *Examination of faeces*

A daily examination over a period of 3 months was made of the faeces of refected rats fed diet 20 raw, rats fed diet 20 + 5 per cent yeast and rats fed diet 20 cooked (these latter rats declined and exhibited symptoms of vitamin B deficiency). Direct microscopical examination and cultural examination were made in all cases. Amongst media used were the following: nutrient agar, nutrient agar + 10 per cent serum, MacConkey's agar, yeast dextrose agar, dextrose agar and beer-wort agar. No essential difference was found between the types of organisms present in the faeces of the three groups of rats. In no group did one particular organism appear which was not present in the faeces of the rats of the other groups. But one significant fact which emerges from the direct study was that the faeces of the refected rats showed consistently a predominantly Gram-positive flora when compared with the faeces of the rats of the other two groups. Another fact to be noted was that in rats declining on a vitamin B-free diet faecal smears showed aberrant forms. The organisms appeared small and stunted, poorly staining and irregular when compared with the clear-cut well-stained organisms in the faecal smears of refected rats. This is well illustrated in Pl. I, figs. 1 and 2, which show photomicrographs of faecal smears of a refected rat and of a rat declining on a vitamin B-free diet. Cultural examinations showed no difference in the types of organisms obtained from the faeces of the different groups.

(*b*) *Attempts to reduce the intestinal flora of rats*

As no positive information had been obtained from the examination of the faeces of refected and non-refected rats, it was thought that if it were possible to reduce the number of organisms in the intestines of a refected rat some information as to the role played by the intestinal flora might be obtained. It seemed that a likely way to effect this reduction would be to feed some germicidal substance, and it was thought that a solution of sodium hypochlorite (strength about 10,000 parts of available chlorine per million) might be suitable for this purpose. A preliminary trial was made with an adult doe kept on the stock diet. The disinfectant was given in the drinking water, 0·35 ml. in 15 ml. of water (the average daily intake). The mixture was offered in an automatic drinker daily and was taken quite readily and without ill effect over a period of 10 weeks. Faeces from this rat were plated and bacterial counts made before the administration of the sodium hypochlorite and at intervals throughout the 10 weeks' experimental period. Table II gives the results of these counts.

Table II. *Plate counts on nutrient agar of faeces of a rat given a solution of sodium hypochlorite daily*

Days on treatment	Organisms per g. of faeces
0	86,600,000
3	74,200,000
10	15,000,000
13	16,800,000
24	10,200,000
28	20,800,000
46	8,400,000
61	5,200,000

From these figures it will be seen that the bacteria of the intestine of the rat were greatly reduced by the daily administration of sodium hypochlorite. An examination of the bacterial flora at the end of the experiment showed that the surviving organisms were predominantly spore formers of the *B. mesentericus* and *B. vulgatus* type, large Gram-positive cocci and coliforms.

Experiment with well-refected rat

An adult buck weighing 228 g. which had been on the refective diet for 3 months was given 0·4 ml. daily of the sodium hypochlorite solution in the drinking water for 6 weeks in addition to the diet. A plate count of faecal bacteria was made before and at intervals during the experimental period. Total counts and coliform counts were made. The results are given in Table III.

Table III. *Plate counts of faeces of a refected rat given daily a solution of sodium hypochlorite*

Days on treatment	Count on MacConkey's agar per g. of faeces	Total count on nutrient agar per g. of faeces
0	*24,000,000*	*56,300,000*
7	Not done	10,100,000
14	*5,400,000*	*13,000,000*
23	14,800,000	17,200,000
36	*9,800,000*	*9,000,000*

From this table it will be seen that the bacteria in the faeces of the refected rat have been greatly reduced in number, but that the coliforms have not been reduced to the same extent as the other bacteria. Throughout the experiment the rat remained perfectly healthy on the refective diet and increased in weight from 228 to 260 g.

(c) *Examination of the contents of the stomach, caecum and colon*

As the bacterial synthesis of vitamin B, suggested by other workers as the explanation of refection, is believed to take place somewhere in the intestine, it was thought that a bacteriological examination of the contents of different portions of the intestine should be made for comparison with the results secured from examinations of the faeces. For this purpose, rats growing well on the refective diet alone and on the refective diet plus yeast, and rats declining on a vitamin B-free diet were killed and their intestinal contents examined. Particular attention was paid to the caecum which Fridericia *et al.* (1927) considered to be especially concerned in refection, while the present authors have shown that in refected rats, its contents are a good source of vitamin B.

Direct examination

Animals from the three groups mentioned above were killed, stomachic, caecal and colonic contents were removed under sterile conditions, and direct microscopical examinations were made. Very few bacteria were found in the stomach, and examinations of the stomach contents were discontinued. The results obtained for caecal and colonic contents were similar to those from direct faecal examination in that no difference was observed between the types of bacteria present in the intestinal contents of the rats of the three different groups. It was, however, very noticeable that smears from the caecal and colonic contents of vitamin B-deficient rats showed poorly staining organisms and many aberrant forms. The bacteria appeared to be growing in an unsuitable medium and swollen distorted organisms formed a large part of a not very heavy flora. In contrast smears from well-refected rats showed clearly stained, well-defined organisms and a heavy flora. Pl. I, figs. 3 and 4, show photomicrographs of smears from the caecal contents of refected and vitamin B-deficient rats showing the different pictures obtained.

Cultural examination

Cultural examinations of the intestinal contents of refected and non-refected rats were made, using media reported on p. 13. Coliforms, aerobic spore formers and Gram-positive cocci were obtained, but there appeared to be very little difference in their relative distributions in the two groups. Numerous attempts were made on special media to culture vibrios from the intestinal contents of refected rats but without success. Fridericia *et al.* (1927) have shown that the faeces of rats refected on a rice-starch diet retained the refective agent after heating at 80° C. for 10 min., but not after heating at 100° C. for the same time. It was, therefore, thought that examination of the flora of the caecal contents after heating might yield interesting results. As a result of this treatment (heating emulsions of caecal and colonic contents to 80° C. for 10 min.), organisms of the *B. mesentericus* and *B. vulgatus* type were obtained, but from both refected and non-refected rats.

Scheunert & Schieblich (1927, 1927*a*), have shown that *B. vulgatus* can synthesize vitamin B, but that the amount varies with the *p*H of the medium in which the organism is grown, and that vitamin B is only produced in slightly acid, neutral or very slightly alkaline medium, and not at all in a medium over *p*H 7·6. In view of the marked difference in the *p*H of the caecal contents of refected and vitamin B-deficient rats,[1] these observations acquire, in the opinion of the authors, a special significance which will be dealt with in the general discussion.

An anaerobic study of the caecal and colonic contents of both groups of rats was also carried out, but without significant results.

Attempts to confirm the findings of Schieblich & Rodenkirchen (1929) with regard to vibrios have proved negative. Vibrios have been seen in faecal and caecal smears from refected rats, but have also been obtained from similar smears from rats dying of vitamin B deficiency. This was also the experience of one of us (S. K. K.) in Warsaw in 1929 and of another (A. T. R. M.) in this Institute in 1931.

IV. Relation between the hydrogen-ion concentration of the caecal contents of rats and the phenomenon of potato-starch refection

During the present study, frequent determinations of the *p*H of the caecal contents of rats receiving refective and non-refective diets have been carried out. For this purpose, rats were killed by coal gas, their caeca removed without delay, and the hydrogen-ion concentration of the caecal contents measured at once by means of the quinhydrone electrode. In some cases where the animal died on a deficient diet, the *p*H was estimated immediately after death.

According to experimental treatment the rats used may be divided into four main groups. The results are therefore tabulated under four headings in Table IV.

The results are quite clear-cut. Without a single exception, the contents of the caeca of rats growing well on the refective diet were acid. In an equally conclusive way, an alkaline *p*H characterizes caecal contents of rats which failed to become refected on diet 20, as well as rats receiving the standard vitamin B-deficient diet. Rats on the tannia-starch diet occupied an intermediate position. The contents of their caeca, though of a higher *p*H than those of refected animals on diet 20, are still on the acid side.

In the authors' experience, therefore, an acid reaction in the caecum is an essential condition of the phenomenon of potato-starch refection.

All rats which failed to become refected on diet 20 showed an alkaline *p*H. But one or two rats which were not refected on the tannia-starch diet retained an acid *p*H of the caecal contents, thus showing that, in addition to an acid *p*H of the caecum, other factors are necessary to bring about the condition of refection.

[1] This is presented more fully in section IV.

Table IV. *The* pH *of the caecal contents of rats studied under different conditions*

Rat group	Rat no.	Diet	Remarks	pH of caecal contents
1	1534	Refective, 20	Growing well, obviously refected	5·40
	2267	,,	,, ,,	5·09
	2268	,,	,, ,,	5·09
	2747	,,	,, ,,	4·83
	2748	,,	,, ,,	5·03
	3997	,,	,, ,,	5·59
	3998	,,	,, ,,	6·14
	4005	,,	,, ,,	5·69
	4017	,,	,, ,,	5·54
	4032	,,	,, ,,	5·52
	4266	,,	,, ,,	5·47
	4268	,,	,, ,,	5·83
			Average	5·43
2	2743	Refective, 20	Declining or moribund, obviously not refected	8·84
	2745	,,	,, ,, ,,	7·00
	3127	,,	,, ,, ,,	7·52
	3129	,,	,, ,, ,,	7·27
	3314	,,	,, ,, ,,	7·61
	3315	,,	,, ,, ,,	7·87
			Average	7·59
3	4302	Vitamin B-free, 19	Declining or moribund	7·47
	4319	,,	,, ,,	7·43
	4362	,,	,, ,,	7·30
	4364	,,	,, ,,	7·51
	4764	,,	,, ,,	6·98
			Average	7·34
4	3349	Tannia starch, 37	Declining	6·18
	3580	,,	,,	5·60
	3581	,,	,,	5·75
	4776	,,	,,	6·98
	4777	,,	,,	7·45
			Average	6·39

In addition to its acid reaction, another striking feature of the caecum of a refected rat is its size, which is very much greater than that of the caecum of a rat kept on a normal stock diet or than that of the caecum of a rat declining on a vitamin B-free diet.

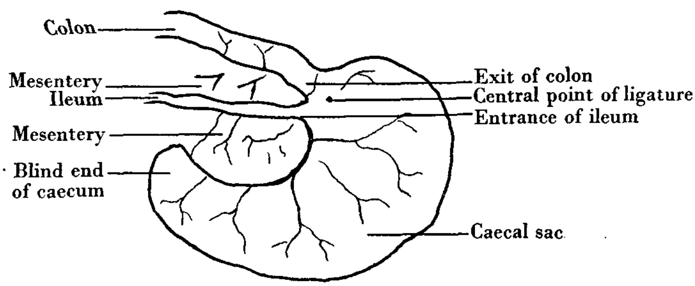
These two conditions of the caecum are always present in the successfully refected rat, and for this reason the authors have been led to investigate whether refection is possible in the absence of the caecum. Such experiments are described in the following section.

V. The surgical removal of the caeca of rats and the effect of the operation on the growth of rats on normal and refective diets

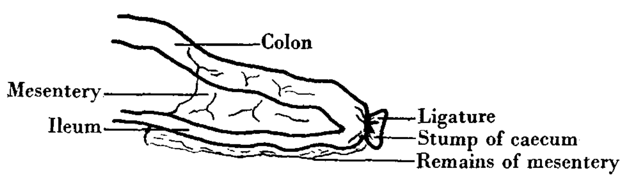
At the time when the following experiments were planned and carried out, the only description known to the authors of the technique of a surgical removal of the caecum in lower animals or birds was that given by Durant (1930). Consequently a technique had to be developed.

(a) Operative procedure and effect of operation on rats on normal diets

Three adult rats kept on the normal stock diet were operated upon as a preliminary to the experiment proper, and to ascertain whether a rat could live without its caecum. An operating table for rats described by Shohl (1931) was used and found to be very successful. An A.C.E. mixture was used for anaesthesia. The animal was starved for 24 hours before operation. In all cases the caecum was ligatured and removed at a point just below the entrance of the ileum and exit of the colon, care being taken to prevent occlusion of the gut by the ligature. (Text-fig. 6 gives a diagrammatic drawing of the operation.)



Caecum of rat before operation.



Colon, ilium and caecal stump after operation.

Text-fig. 6. Operation for removal of caecum in the rat. Diagrammatic drawing of caecal region before and after operation.

The abdominal wound was sutured in two layers with silk. For 48 hours at least after the operation the rats were kept on a milk diet only. Of the three rats operated in the preliminary experiment the first one died from peritonitis after 3 days because of the slipping of the ligature and leakage of the intestinal contents. The other two rats survived the operation. At the end of 8 weeks during which it had been in perfect health, one rat was killed. At autopsy a fairly marked dilatation of the first part of the colon was noticed. The second rat was killed 12 days after operation to see the extent of healing. It was seen that the ends of the caecal stump had healed together, and it was almost impossible to find the ligature.

As these results showed that a rat on a normal diet can dispense with a large part of its caecum, the following experiments were carried out to observe the effect of absence of caecum on the state of refection.

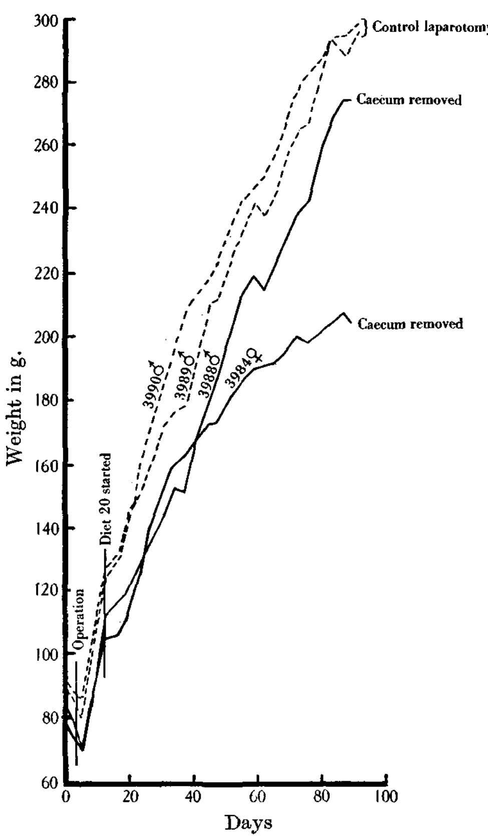
(b) Refection of rats after removal of caecum

Exp. 1. Eight rats on the stock diet, weighing between 70 and 86 g., were divided into two groups 2♂♂ and 2♀♀ in each group and were treated as follows:

Group 1. Had their caeca removed by operation as described above.

Group 2. In these rats an abdominal incision was made under anaesthesia and the intestines handled as for the complete operation but the caecum was not removed. The wound was then sutured and the animals allowed to recover. This will be referred to as the "control laparotomy".

One rat died under anaesthesia, but the operation was successful in the seven remaining cases and the animals recovered. There were two fatalities in group 1 later. One rat died of pneumonia 3 days after operation and another of peritonitis 7 days after operation. On the tenth day four of the remaining rats, one buck and one doe from group 1 and 2 bucks from group 2, were put on to diet 20 and remained on this diet for 10 weeks, at the end of which time they were killed and post-mortems made. Pl. I, figs. 5 to 8 show photographs of the caeca and a small part of the large intestine of these rats. It will be seen that there is a very marked difference between the sizes of the caeca of the two groups of rats. The small pea-like swelling (the remains of the caecum) in the operated rats consisted very largely of fibrous tissue, and contained very little lumen. There was, however, some compensatory hypertrophy of the proximal end of the colon and distal end of the ileum. An examination of the accompanying growth curves (Text-fig. 7) shows very little difference in growth between the two groups of animals. It should be noted that there is a doe in group 1.



Text-fig. 7. Effect of removal of caecum on growth of rats on the refective diet 20. First experiment.

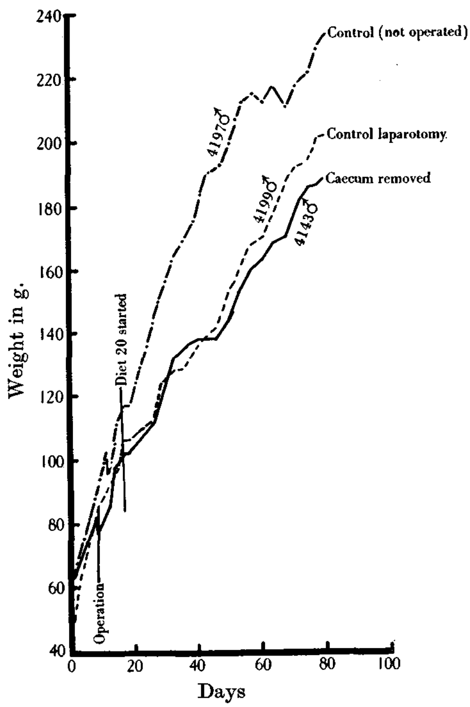
Exp. 2. Twelve rats, similarly on the stock diet and weighing between 60 and 80 g., were divided into three groups and dealt with as follows:

(1) Two bucks and two does had their caeca removed.

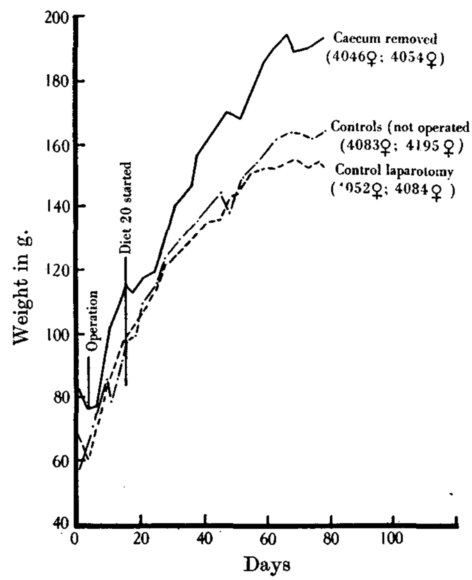
(2) Two bucks and two does acted as operated controls (control laparotomy).

(3) Two bucks and two does were starved for 1 day only and then placed on diet 20 with groups 1 and 2, and acted as normal controls.

Twelve days after operation all three groups of rats were placed on diet 20. The animals were placed in four cages, each cage containing one operated rat, one control operated rat and one normal control rat. During the experimental period of 9 weeks, there were two deaths, one in group 2, the rat dying of an abdominal abscess after 3 days, and the other in group 3, a rat which died



Text-fig. 8. Effect of removal of caecum on growth of rats on the refective diet 20. Second experiment. Bucks.



Text-fig. 9. Effect of removal of caecum on growth of rats on the refective diet 20. Second experiment. Does.

$2\frac{1}{2}$ weeks after the start of the experiment from a twisted bowel and an enormously distended caecum. At the end of the experiment, a rat from group 1 was found to be suffering from pneumonia, and its growth curve has consequently not been included.

It will be seen from the growth curves in Text-figs. 8 and 9 that this experiment confirms the results of the first experiment, and shows very little difference over an experimental period of 9 weeks, in growth rate on diet 20 between animals with a normal caecum and those with a much reduced caecum. The growth of the operated does from group 1 is slightly better than that of the control-operated does and the normal control does, but in the case of the bucks growth is best in the normal control buck, with very little difference between operated and control-operated bucks.

At the end of 9 weeks' experimental period the animals were killed, and at autopsy the operated animals were found to show very much the same picture as found in the operated animals of Exp. 1 (Pl. I, figs. 9 to 18).

These experiments indicate that the size of the caecum is not a controlling factor in the state of refection, as it appears that rats with much reduced caeca are able to grow and thrive on a vitamin B-deficient diet containing raw potato starch, almost as well as rats with normal caeca. A compensatory hypertrophy of the last part of the small intestine and the first part of the large intestine was observed in a number of the operated rats (Pl. I, figs. 16 and 17), and it may well be that some of the functions of the caecum are taken over by the hypertrophied intestine.

Note (see p. 17). Since this work was completed three papers (Griffith, 1935; Guerrant *et al.* 1935; and Innes & Nicolaysen, 1937) have appeared describing the removal of the caecum of the rat.

DISCUSSION

The results of this work show clearly that potato-starch refection cannot be attributed to a single property of the refective diet or to an infection with a specific organism.

No spectacular difference was found in the bacteriological picture of the caecal contents or of the faeces when rats thriving on a refective diet were compared in this respect with rats declining on a vitamin B-deficient diet or on the refective diet after its refective properties had been destroyed by cooking. Unless one adopts the unlikely explanation that refection is due to some virus capable of synthesizing large quantities of vitamin B or to the presence of small numbers of an organism even more exceptionally endowed, one is forced to conclude that a purely bacteriological explanation of the phenomenon of potato-starch refection would not be in keeping with the experimental results.

In our opinion the explanation is more complex, and to facilitate appreciation of the relevant observations on refection made in the course of the present investigation, these are given in a concise form in the table on p. 22.

Taking all these points into consideration, we suggest the following explanation of the phenomenon of potato-starch refection in the rat:

(*a*) The presence of undigested starch and of requisite starch-splitting organisms in the caecum of the rat leads to a vigorous fermentation and results in an acid *p*H.

(*b*) The acid *p*H coupled with the presence of ample amounts of a nutrient medium (starch) allows of more normal and more vigorous growth of the caecal flora than the alkaline caecum of a vitamin B-deficient rat.

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Factor or finding	Bearing on, or relation to, the state of refection
Diet	Must contain raw potato starch
Coprophagy:	
Administration of faeces Small meshed screens Presence of companions in the cage	Helpful, but not absolutely essential in the initial and early stages of refection—immaterial once refection is well established in a rat. Faeces from well refected rats do not necessarily always produce refection when fed
Caecal contents or faeces:	
Their vitamin B content	Definitely much higher than in rats on the standard vitamin B-deficient diet
Presence of undigested starch	*Essential to refection, but may be present in non-refected rats*
Bacterial flora	Not essentially different in kind from that of non-refected rats, but of more vigorous growth
Effect of intestinal disinfectant	Appreciably reduces total bacterial count of faeces. Leaves mostly coliform organisms and aerobic spore formers. Has no effect of the course of refection
*p*H of caecal contents	Acid reaction always present in refected rats
Caecum, size and function	Very enlarged in refected animals, but functions can apparently be taken over by hypertrophy of the adjacent intestine
Fermentation in caecum	Vigorous in refected rats, not so noticeable in rats declining on refective diet, although starch present

A reference was made in the section on bacteriology to the work of Scheunert & Schieblich (1927*a*) who showed that the vitamin B synthesizing powers of *B. vulgatus* were affected by the *p*H of the medium in which the organism was grown and largely disappeared when the *p*H became definitely alkaline. A similar behaviour on the part of organisms normally present in the intestinal tract of the rat would lead to a greatly increased production of vitamin B in the caecum of a refected rat, while the same organism would not produce vitamin B so readily in the unfavourable alkaline medium in the caecum of a non-refected rat. Our experimental findings speak very definitely in favour of such an explanation. Although we were unable to find any marked difference in the types of organism present in the caecal contents and faeces of refected and non-refected rats, it was clearly observed that organisms in faecal and caecal smears from refected rats were much more normal and more prolific than those found in vitamin B-deficient rats or in rats declining on the refective diet. Further, we found that caecal contents and faeces of refected rats are, per unit of dry weight, much richer in vitamin B than those of rats subsisting on a vitamin B-deficient diet.

(*c*) We believe also that the acid *p*H of the caecum benefits the refected rat in yet another respect in that it creates conditions favourable for the *absorption of the vitamin B formed.*

Such favourable conditions certainly exist in the ruminant, which is known to be able to dispense with an exogenous source of vitamin B, because no doubt it can utilize the vitamin B formed by bacterial synthesis in the rumen. As the vitamin B so formed travels with the fermented cud through the acid abomasum or "true stomach", the analogy with the refected rat is apparent, though in the rat, fermentation, synthesis of vitamin B and absorption probably all take place in the acid caecum. It appears from this investigation that when the caecum is removed, these functions may be assumed by the hypertrophied parts of the adjacent intestine. That vitamin B is either absorbed or rendered assimilable by passage through an acid part of the intestinal tract is also shown by the fact that coprophagy can lengthen the life of a rat on a vitamin B-free diet; although small amounts of vitamin B (presumably produced by bacterial synthesis) are present in the caecum of a rat on a vitamin B-free diet, the animal is unable to use the factor, which passes out in the faeces. When, however, the faeces are re-fed, the vitamin B they contain becomes available, probably because, instead of being in contact only with the alkaline gut, the faeces have to pass through the acid stomach.

The observations made during this investigation suggest that in the rat, refected on a potato-starch diet, processes analogous to those normally obtaining in the bovine take place and the rat, like the bovine, is rendered independent of an exogenous supply of vitamin B.

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EXPLANATION OF PLATE I

Figs. 1 and 2. Photomicrographs of faecal smears of a refected rat (1) and of a rat declining on a vitamin B-deficient diet (2).

Figs. 3 and 4. Photomicrographs of caecal smears of a refected rat (3) and of a rat declining on a vitamin B-deficient diet (4).

Figs. 5–8. Caecal and colonic parts of intestines of rats refected after control laparotomy (5 and 6) and after removal of caecum (7 and 8).

Figs. 9–18. Caecal and colonic parts of intestines of a rat on a normal stock diet (9), of normally refected rats (10, 11 and 12), of rats refected after control laparotomy (13, 14 and 15) and of rats refected after removal of caecum (16, 17 and 18).

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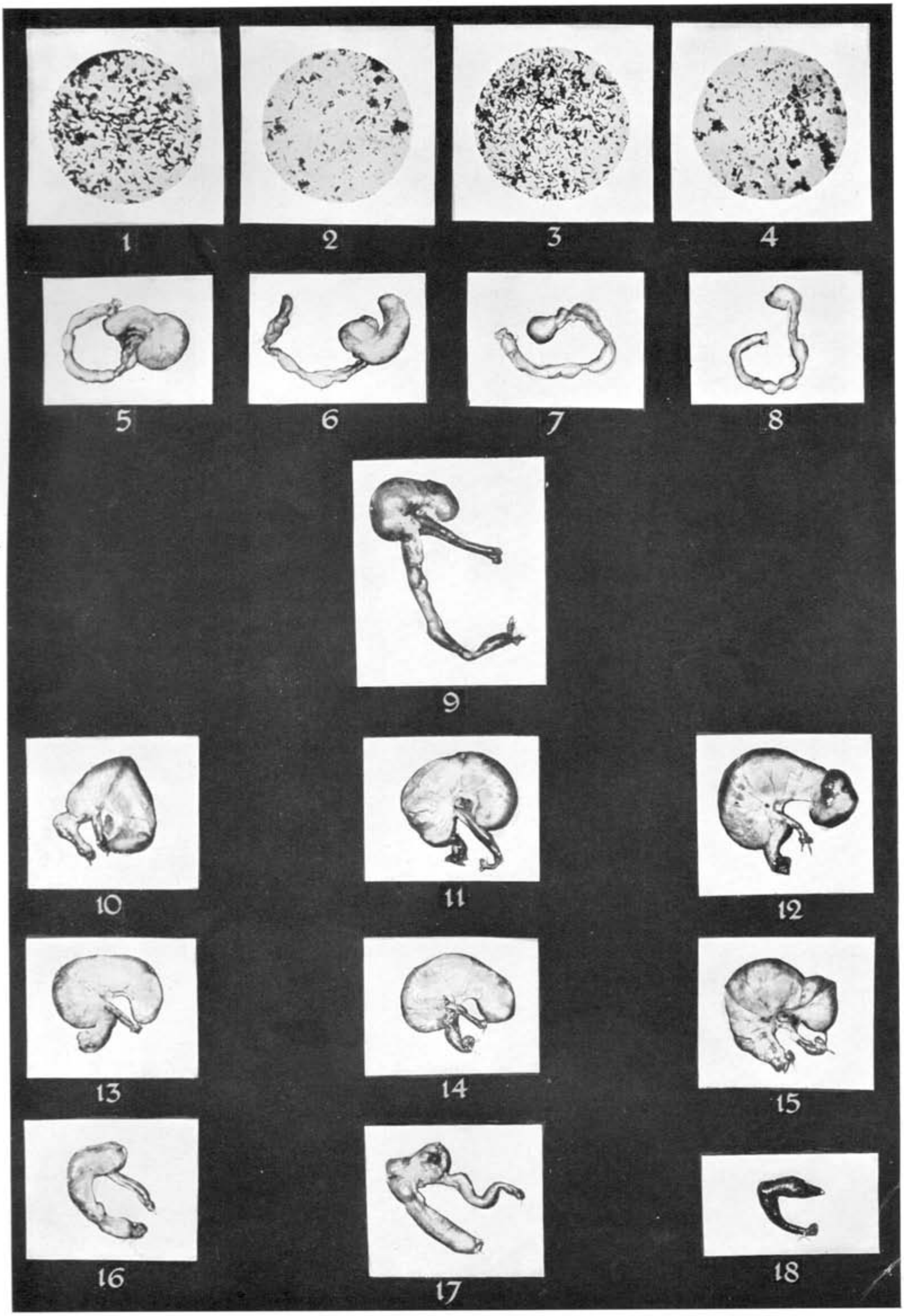
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