

CXXIX. SYNTHESIS OF VITAMIN B₁ BY INTESTINAL BACTERIA OF THE RAT

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MANY types of micro-organisms have been tested by various workers for their ability to synthesize "vitamin B" (= complex). In such studies the organisms were grown in various nutrient media and the "vitamin B" was detected by its growth-promoting action or by curative tests on experimental animals such as the rat, the mouse or the pigeon. Thus "vitamin B" has been reported to have been produced by Pfeiffer's bacillus [Damon, 1923], *Mycobacterium smegmatis*, *Mycobact. phlei* and *Mycobact. moelleri* [Damon, 1924], *Monila candida* [Hoet *et al.* 1924], *B. vulgatus* [Scheunert & Schieblich, 1923], various types of *Torula*, *Oospora*, *Actinomyces* and *Eubacteriales* [Sunderlin & Werkman, 1928], *B. ellenbachiensis* and *B. prodigiosum* [Schieblich, 1931], *B. lactis aerogenes* and *Vibrio alcaligenes* [Schieblich, 1933]. On the other hand, the following micro-organisms (among others) have been found to produce no vitamin: *Lactobacillus bulgaricus* and *Amylomucor* B [Wollman, 1921; Wollman & Vagliano, 1922], *Bact. lactisacidi*, *B. mycoides*, *B. subtilis* and *Micrococcus agilis* [Slanetz, 1923], *Bact. coli* [Cooper, 1914], the tubercle bacillus [Cunningham, 1924].

Considering the above results together, it seems reasonable to believe that different types of bacteria may vary in their ability to synthesize "vitamin B". This view receives direct support from the experiments of Schieblich [1931], who showed that there was a well marked difference in the extent of the synthesis of "vitamin B" by various bacteria. Thus, when grown in a suitable culture medium, *B. vulgatus* was found to produce large amounts of the vitamin B complex, *B. mycoides* smaller amounts and *B. mesentericus* very small quantities only. These conclusions were based on growth tests on rats.

An examination of the literature also shows that studies of the synthesis of "vitamin B" by a specific organism have produced somewhat contradictory results. To take one example, *Bact. coli* was reported to be unable to synthesize "vitamin B", by Cooper [1914], Damon [1921], Eijkman *et al.* [1922], and Weill *et al.* [1922], while positive results were claimed by Kuroya & Hosoya [1923] and by Sunderlin & Werkman [1928]. Of the various causes which may account for this lack of agreement the following are perhaps the most important: (1) the use of different methods for detecting the presence of the vitamin, (2) the administration of insufficient amounts of micro-organisms for the feeding test and (3) the use of different culture media by the various workers.

Most of the work mentioned above dealt probably with the bacterial synthesis of the "vitamin B" complex as a whole rather than with its separate components. More recently, however, the synthesis of vitamin B₁ by *B. vulgatus* has been reported by Guha [1932]. Various other investigators have presented evidence which suggested that vitamin B₁ is synthesized in the intestinal tract of the rat [Roscoe, 1931; Guerrant *et al.* 1935; Whipple & Church, 1935; Leong, 1937].

The work described in this paper was designed: (1) to study the extent of the synthesis of vitamin B₁ by the intestinal bacteria of the rat when grown in broth medium and (2) to determine whether the synthesized vitamin is secreted by the organisms into the surrounding culture medium or is retained within the bacteria. Employing the bradycardia method for the assay of vitamin B₁ [Drury & Harris, 1930; Birch & Harris, 1934; Leong & Harris, 1937], data concerning the rate and the extent of this bacterial synthesis in terms of the International Unit have been obtained.

1. RATE AND EXTENT OF SYNTHESIS OF VITAMIN B₁ BY INTESTINAL BACTERIA

Material and method. As culture medium ordinary bullock's heart extract broth (pH 7.2) was used. We found that this broth medium contained traces of vitamin B₁, the exact amount of which was determined after adsorption on acid clay in accordance with the technique previously described by Harris & Leong [1936]. This factor did not vitiate our results, as we carried out quantitative estimations of vitamin B₁ in the medium both before and after inoculation. However, a separate experiment was undertaken in which a specially prepared vitamin-B₁-free broth was used for growing the bacteria, with the object of confirming the results of our main experiment. It was thought advisable to employ ordinary broth in the main experiment in preference to a specially devitaminized broth, as the former would perhaps procure a more natural growth of the bacteria.

The organisms were obtained from the pooled caecal contents of three adult piebald rats. The animals weighed about 200 g. (about 6 months old) and were reared on the laboratory stock diet, consisting mainly of brown bread, meat, cabbage and milk.

Bacteriological examination of caecal contents. Microscopic and cultural examination of the caecal contents used for inoculation showed the presence of the following organisms: (1) enterococci, about 5%; (2) lactobacilli, about 1%; (3) various clostridia which presumably did not grow to any great extent in our aerobic mixed cultures; (4) organisms of the *Bact. coli* group, which constituted the rest.

Preliminary experiments. These need not be detailed here and were undertaken to find the most convenient quantities of culture medium to provide an amount of vitamin B₁ suitable for assay by the bradycardia method. It was found that 150 ml. of broth constituted a suitable amount. Our specimens were prepared and analysed in triplicate in order to obtain more reliable results.

Main experiment

Three rats were killed by coal gas, their caecal contents were mixed together and made into a thin suspension in sterile saline. After this suspension had been left to stand for $\frac{1}{2}$ hr. to allow coarse particles to settle, 21 sterilized flasks of 250 ml. capacity (each containing 150 ml. broth) were each inoculated with 1 ml. of the supernatant fluid and incubated at 37°. The cultures were allowed to grow for $\frac{1}{2}$, 1, 2, 3, 4, 6 or 9 days, and after each period 3 flasks were removed and the bacterial mass and supernatant liquid in each specimen were assayed separately for vitamin B₁. The cultures were centrifuged at 4000 r.p.m. for 1 hr. and the deposited bacteria were dried *in vacuo* at room temperature over P₂O₅. The dried deposits were then weighed and assayed. The supernatant liquids were passed through a Seitz filter and the pH was determined colorimetrically.

The vitamin B₁ content of each specimen of supernatant liquid was determined by the bradycardia method after adsorption of the vitamin on acid clay. The results of this experiment are given in Table I and depicted in Fig. 1.

Table I. *Synthesis of vitamin B₁ by intestinal bacteria grown in 150 ml. broth medium*

Days of incubation		Cure of bradycardia, days (individual tests)	Vitamin B ₁ content, i.u. (average of tests)	Total vitamin B ₁ content in culture and supernatant liquid, i.u.
	Sterile broth (pH 7.2)	3, 3, 3, 5	1.6	—
½	Culture	2, 3, 4	1.3	2.6
	Supernatant liquid (pH 7.3)	1, 3, 4.5	1.3	
1	Culture	3, 4, 5.5	1.9	3.7
	Supernatant liquid (pH 7.5)	3, 4, 4.5	1.8	
2	Culture	3, 4, 4.5	1.7	2.5
	Supernatant liquid (pH 8.1)	1, 2, 2.5	0.8	
3	Culture	2, 3, 3	1.1	2.4
	Supernatant liquid (pH 8.4)	3, 3, *	1.3	
4	Culture	1, 1, 2	0.5	1.8
	Supernatant liquid (pH 8.4)	2, 2, 5	1.3	
6	Culture	0.5, 1, 1.5	0.4	1.2
	Supernatant liquid (pH 8.6)	1, 3, *	0.8	
9	Culture	0.5, 0.5	0.2	0.6
	Supernatant liquid (pH 8.6)	1, 1	0.4	

* Test rat died.

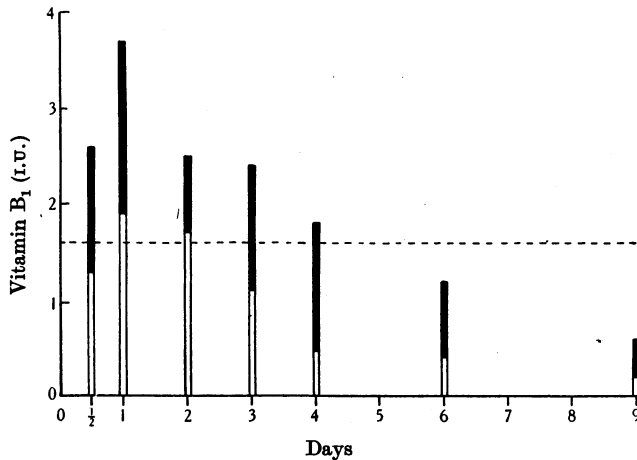


Fig. 1. Synthesis of vitamin B₁ in broth medium. ■ Amount of vitamin B₁ in supernatant liquid. □ Amount of vitamin B₁ in dried bacterial growth. ---- Amount of vitamin B₁ in original sterile medium.

Rate of synthesis of vitamin B₁. It will be seen from Fig. 1 that there has been an appreciable synthesis of vitamin B₁ in the ½, 1, 2 and 3-day cultures, as the combined vitamin B₁ content in each bacterial growth and its supernatant fluid exceeded that originally present in the sterile medium. The combined vitamin content of the 4-day culture and its supernatant liquid was not appreciably higher than that present in an equivalent amount of the sterile medium.

These results demonstrate that the quantity of the vitamin found in the different cultures varied with the age of the culture. Under the conditions of this experiment, the greatest synthesis of the vitamin occurred during the first 3 days of incubation. When this period was exceeded the amount of vitamin B₁ in the bacterial growth was found to be much smaller. Inspection of Fig. 1 will show that there had been a distinct diminution in vitamin B₁ content after the 4th day of incubation.

Amount of vitamin B₁ in bacteria. The dry weight and the vitamin B₁ content of each bacterial mass are shown in Table II. The weights of the bacterial growths after varying periods of incubation were found to be fairly constant,

Table II. *Vitamin B₁ content of cultures of intestinal bacteria*

Age of culture days	Dry wt. of culture g.	Cure of bradycardia, days (av. of 3 tests)	Vitamin B ₁ in dried bacteria I.U./g.
$\frac{1}{2}$	0.12	3.0	11
1	0.12	4.2	16
2	0.13	3.8	13
3	0.13	2.7*	9
4	0.13	1.3	4
6	0.12	1.0*	3
9	0.11	<0.5*	<2

* Average of 2 tests.

i.e. about 0.13 g. It will be noted that in the 1-day culture, the dried bacteria contained about 16 I.U. of vitamin B₁ per g. while the vitamin content of the cultures was found to decrease when grown for longer periods. Thus the 9-day culture contained less than 2 I.U. per g.

It is realized that the cultures examined in these studies consisted of a mixture of many types of micro-organisms. It is of interest to compare the vitamin B₁ potency of such intestinal bacteria (the richest sample containing about 16 I.U. per g.) with that of yeast, which is perhaps the richest known natural source of this vitamin. The highest values to be found in the literature for yeast are 15 I.U. per g. (quoted from Cowgill, 1934, and based on the relationship that 20 "milligram-equivalents" equal 1 I.U.) and 23 I.U. per g. [Baker & Wright, 1935].

Destruction of vitamin B₁ during incubation. It will be seen from Fig. 1 that the vitamin B₁ content of the bacterial growth remained at a high level for the first 3 days of incubation, and then decreased rapidly until less than 0.2 I.U. was found in the 9-day growth. There was also a distinct decrease in the amount of vitamin B₁ in the supernatant liquids when the time of incubation was longer than 6 days. These observations suggest that a destruction of the vitamin had occurred when the micro-organisms were grown for periods longer than 3 days. A destruction of the vitamin during the first 3 days might also have occurred, although it was not possible to demonstrate this point. The following factors may have accelerated the destruction of the vitamin: (1) the high temperature of incubation and (2) the increasing alkalinity of the culture media as a result of bacterial metabolism. In Table I are given the figures showing the gradual rise in pH values of the supernatant liquids as the period of incubation was prolonged.

2. RETENTION OF THE SYNTHESIZED VITAMIN B₁ IN THE BACTERIAL CELLS

An interesting question is whether the synthesized vitamin can diffuse out of the bacterial cells into the culture medium. In a study of the formation of vitamin B₁ by *B. vulgatus*, Guha [1932] showed that the synthesized vitamin was not secreted into the surrounding medium (as judged by curative tests on pigeons). The following experiment was carried out in an attempt to obtain information on this point with regard to intestinal bacteria.

Medium. Ordinary broth was brought to pH 10 by the addition of NaOH and autoclaved for 4 hr. under a pressure of 1½ atmospheres. The broth was then readjusted to pH 7.2 by the addition of conc. HCl. It was hoped to destroy all the vitamin B₁ in the broth by this treatment. The results of several separate determinations showed that this autoclaved broth contained no appreciable amount of vitamin B₁ (less than 0.4 I.U. was found in 500 ml.).

Method. Six 150 ml. samples of this medium were then inoculated with a suspension (in normal saline) of the caecal contents from a normal piebald rat. These were incubated aerobically at 37° and examined for vitamin B₁ after intervals of 1 and 3 days. Triplicate samples of the 1-day culture were taken and the bacterial growth was separated from the supernatant liquid by centrifuging followed by filtration through a Seitz filter as before. Each culture was assayed separately. In testing the supernatant liquids the filtrates from the 3 specimens were combined, made up to the original volume (i.e. 450 ml.) with water, and adsorbed on acid clay. A similar procedure was adopted for the 3-day cultures.

Results. The results are shown in Table III. It was found that during the period of incubation (i.e. 1-3 days) no detectable amounts of vitamin B₁ had appeared in the supernatant liquid. At the same time, however, a synthesis of

Table III. *Synthesis of vitamin B₁ by intestinal bacteria grown in vitamin-B₁-free broth*

Period of incubation days		Cure of bradycardia, days (individual tests)	Vitamin B ₁ content I.U.
	Sterile broth 500 ml. (vitamin B ₁ -free)	<1, <1, <1	<0.4
1	Culture*	1, 1.8, 3.5	0.8
	Supernatant liquid, 450 ml.	<1	<0.4
3	Culture*	4, 4, 6	2.2
	Supernatant liquid, 450 ml.	<1	<0.4

* Culture from 150 ml. medium.

the vitamin had occurred, for the separated bacterial growths were found to contain demonstrable amounts (0.8 I.U. in the 1-day culture and 2.2 I.U. in the 3-day culture). These observations suggest that there was no diffusion of vitamin B₁ from the bacterial cells into the surrounding medium although the intestinal bacteria of the rat could synthesize vitamin B₁ in broth which was practically free from it.

The question may be raised as to why the rat develops B₁ avitaminosis when the vitamin can be synthesized by its intestinal flora. Our experiments indicate that no diffusion of the vitamin out of the bacterial cells takes place, so that no absorption would be expected from the caecum of the rat. When the bacterial growth was fed to the animal it was then able to digest and absorb the vitamin.

SUMMARY

Quantitative studies of the synthesis of vitamin B₁ (in terms of the International Standard) by intestinal bacteria grown in ordinary broth medium have been made.

Under the conditions described, the greatest amount of vitamin B₁ was formed in the 1-day culture. The richest culture was found to contain about 16 I.U. per g. A destruction of the vitamin was observed when the microorganisms were grown for periods longer than 3 days.

No diffusion of synthesized vitamin B₁ from the bacterial cells into the broth medium could be detected.

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