ADAPTATION OF THE PROPIONIC-ACID BACTERIA TO VITAMIN B_1 SYNTHESIS INCLUDING A METHOD OF ASSAY

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In a paper dealing with the nutrition of the propionic-acid bacteria Wood, Andersen, and Werkman (1938) report that one species *Propionibacterium pentosaceum* (49W), can be "trained" to grow as well in the absence of vitamin B_1 as in its presence. Their data show that adaptation occurred after continuous serial transfer in an ammonium sulfate medium containing only ether extract of yeast extract as a stimulant; the organism gradually acquired the ability to dispense with vitamin B_1. They did not determine whether the organism, "trained" to dispense with the vitamin, acquires the ability to synthesize it, or whether its metabolism is diverted so that the vitamin is no longer required. The present study was undertaken to determine which of the two possibilities occurs. It has been found that the organism acquires the ability to synthesize vitamin B_1. A bacteriological method of assay of vitamin B_1 employed in the study is also described.

EXPERIMENTAL

1. Method of assay

The present authors (1938) have shown previously that the addition of vitamin B_1 to cells of *P. pentosaceum* which had been grown in the basal medium of Tatum, Wood and Peterson (1936) in the absence of the vitamin, resulted in an almost immediate increase in their anaerobic pyruvate metabolism as judged by the evolution of CO_2. The assay to be described is based on the application of the above observation.

The stock culture of *P. pentosaceum* was grown on a medium consisting of the following: Glucose, 1 per cent; Yeast extract (Bacto), 0.3 per cent; Peptone (Bacto), 0.2 per cent. It was transferred daily, 4 drops of inoculum being transferred to 25 ml. of the above medium. The incubation temperature was 30°C.

The basal medium of Tatum, Wood and Peterson (1936) was employed together with cystine and hydrolyzed casein as follows: Glucose, 1 per cent; Sodium acetate, 0.6 per cent; Ammonium sulfate, 0.3 per cent; Cystine, 0.0050 per cent; Hydrolyzed casein, 0.075 per cent; Ether extract of 3 grams yeast extract per 100 ml., Speakman's salts in half concentration.

Three hundred ml. of the above were placed in 500 ml. flasks and also 25 ml. and 5 ml. portions tubed.

In preparation for a vitamin B_1 assay the actively growing cells of *P. pentosaceum* from 25 ml. of the yeast-extract peptone medium were aseptically recovered by centrifugation, washed twice with two 10 cc. portions of sterile distilled water and resuspended in 5 cc. of sterile water. One drop of this suspension was used to inoculate a 5 ml. portion of the basal medium of Tatum *et al.* (1936). This culture was incubated 24 hours at 30°C. and at the end of that time the centrifuged cells washed with 10 ml. of sterile water, resuspended in 5 ml., and the entire contents used to inoculate a 25 ml. portion of the basal medium. This in turn was incubated for 24 hours at 30°C. and, after washing in 10 ml. of sterile water, was employed as an inoculum for 300 ml. of the basal medium. The contents of the flask were incubated for 72 hours at 30°C., the cells obtained were washed twice in distilled water, and then employed as test organism for a manometric assay of vitamin B_1. The second washing was carried out in a 12 ml. graduated centrifuge cup and the volume of cell paste was recorded after 20 minutes centrifuging at about 1900 r.p.m. Cells of *P. pentosaceum* can be readily recovered by centrifugation.

The cell paste obtained from a single flask of medium was made up to 12 ml. with M/15 phosphate (pH 5.6) and 1 ml. of this suspension was placed in the main vessel of each Warburg cup. Nine milligrams of pyruvic acid as sodium pyruvate were

employed as a substrate and placed in the side arm. Varying amounts of vitamin B_1 contained in a volume of 0.1 ml. of water were added to the cells in the main vessel. After shaking in the water bath at 30°C. for 30 minutes, the contents of the side cup were tipped into the main vessel and readings taken. The atmosphere was nitrogen, from which any oxygen had been removed by passage over hot copper. The total volume contained in the cups was in all cases 2.3 ml. Simple Warburg manometers

TABLE 1

Effect of varying vitamin B_1 concentration on anaerobic pyruvate metabolism of P. pentosaceum

EXPERIMENT NUMBER	YIELD OF CELL PASTE	GAMMAS VITAMIN B_1 ADDED								
		0	0.25	0.125	0.0625	0.0312	0.0156	.0078	.0039	.002
		CO_2 evolved, mm^3								
Part 1										
1	0.50	42	366	338	305	157	113	85	57	56
2	0.50	40	375	333	253	198	111	84	65	53
3	0.48	48	351	316	263	176	123	98	69	59
4	0.48	46	353	296	249	199	102	98	76	63
5	0.49	43	354	314	247	193	122	98	72	62
6	0.50	40	348	317	253	169	147	84	66	56
7	0.45	58	319	287	235	171	107	81	79	64
Part 2										
11	0.54	50	292	254	217	135	105	84	74	66
12	0.50	55	300	265	238	146	114	87	66	66
13	0.51	68	300	278	235	147	114	104	57	51
14	0.52	69	293	273	246	145	110	90	62	62
15	0.49	46	297	269	211	146	104	84	79	65
16	0.50	40	322	290	249	166	112			

were employed and the details of the manometric technique used is described by Dixon (1934).

Results of several runs at various times are shown in table 1. The cells employed in part 1 of the table were grown in a medium containing ether extract of yeast prepared separately from that used in part 2. The results shown were obtained at the conclusion of a $2\frac{1}{2}$ hour run and are corrected for endogenous CO_2 production.

The results show that the response of cells grown in the manner described to the addition of varying quantities of synthetic vitamin B_1[1] is sufficiently consistent to warrant its use in the assay of the vitamin. A fairly accurate approximation of the vitamin content of a material may be obtained if extracts are tested in several dilutions with their vitamin content within the range of the test described.

The manometric determination described is affected neither by the presence of the pyrimidine[2,3] or thiazole[3,4] fraction of vitamin B_1 nor by nicotinic amide in the amounts tested (up to 10 gammas).

As two advantages of this method over animal assays may be mentioned, the short period actually involved in the assay (about 4 hours including $2\frac{1}{2}$ for the manometric run) and the small amount of material required for a single assay, 0.025 to 0.05 grams of dried bacterial cells being sufficient for an assay.

Other methods employing microorganisms which have been described in the literature are the growth tests of West and Wilson (1938) who used *Staphylococcus aureus* and of Schopfer and Jung (1936) who used the mold, *Phycomyces blakesleeanus*; a fermentation test with yeast as the test organism has been described by Schultz, Atkin and Frey (1937).

2. Adaptation of P. pentosaceum to synthesis of vitamin B_1

Cultures of *P. pentosaceum* which had been carried on the basal medium, described earlier, for (1) only a few transfers and (2) until they had been "trained" were compared as to their vitamin B_1 content and their activity on pyruvate. It has already been shown (Silverman and Werkman, 1938) that the vitamin is essential in the anaerobic pyruvate metabolism of this group of organisms.

A transfer of the organism was made from the yeast extract-peptone medium after washing twice, to the basal medium of

[1] Kindly supplied by the Winthrop Chemical Company.

[2] Pyrimidine fraction = 2 methyl 5 bromoethyl 6 aminopyrimidine hydrobromide.

[3] Thiazole fraction = 4 methyl 5 β hydroxyethylthiazole.

[4] Kindly supplied by Merck and Company.

Tatum, *et al.* (1936). It was carried through eight transfers, using only a single drop as inoculum, into 5 ml. portions of medium; in the ninth transfer the cell contents of the 5 ml. portion were used to inoculate 25 ml. of the basal medium; and in the tenth transfer, the entire cell contents of the 25 ml. portion were used to inoculate 300 ml. of medium. The cells were not washed during the transfers on the basal medium.

In a manner similar to the above, a culture taken from a yeast-extract peptone medium was carried through three transfers on the basal medium—from 5 ml. to 25 ml. to 300 ml. Here, and in the above, transfers were made at 24-hour intervals and the 300 ml. portions were incubated for 72 hours. Hereafter, the cells carried ten transfers on the basal medium lacking vitamin B_1 will be referred to as "trained", those carried only three transfers, as "untrained." It was found that cells of *P. pentosaceum* after passing through three transfers show a marked depletion of vitamin B_1; however, by the tenth transfer the ability to synthesize the vitamin is definitely manifested.

In comparing the yield of cell material from 300 ml. of medium we find that we obtain twice the volume of the "trained" cells in comparison to the "untrained." Wood, Andersen and Werkman (1938) have already indicated the increase in acid production from glucose as the organism becomes adapted.

Both the "trained" and "untrained" cells were assayed for vitamin B_1 content. Extracts from cells which had been dried in a vacuum desiccator at room temperature for 24 hours were prepared by suspending 0.1 gram of dried cells in 5 ml. of water, the suspension kept in a boiling water bath for 5 minutes, and then clarified by centrifuging. The supernatant fluid was then assayed for vitamin B_1. By taking the average value found for several dilutions of both the "trained" and "untrained" cells we find the "trained" cells have a vitamin B_1 content of 6.25 gammas per gram of dry cells and the "untrained" cells contain but 0.40 gamma per gram of dry cells. The extraction of the vitamin with water was, of course, incomplete; but aqueous extraction should remove comparative amounts of the vitamin and so justify the comparisons made.

Since such a large difference in vitamin B_1 content of the

"trained" and "untrained" cells exists we should expect a corresponding difference in the pyruvate metabolism of the cells, inasmuch as the vitamin is essential in biological pyruvate breakdown. This assumption is borne out in figure 1. The "untrained" cells show almost no activity on sodium pyruvate; the "trained" cells show almost a maximum anaerobic pyruvate

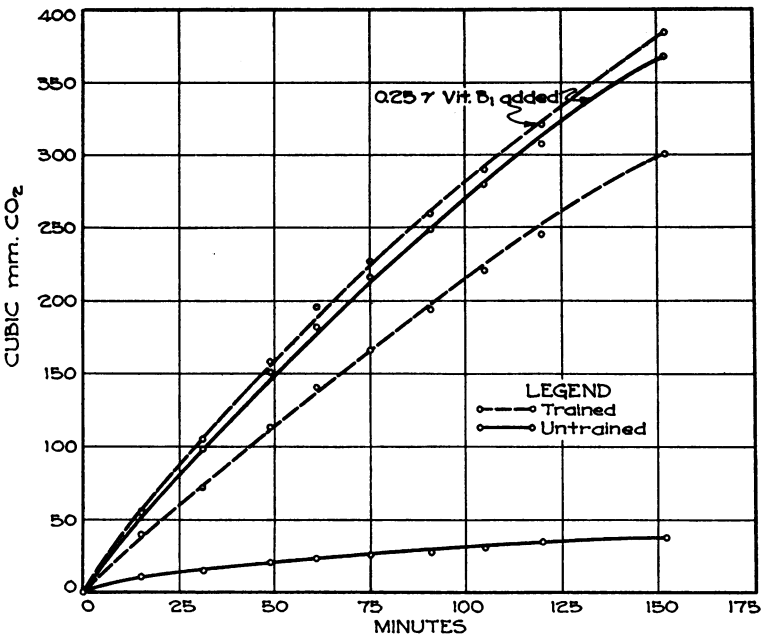

FIG. 1. COMPARISON OF THE ANAEROBIC PYRUVATE METABOLISM OF "TRAINED" AND "UNTRAINED" CELLS OF P. PENTOSACEUM

Cells, 1 ml. of 1:20 suspension (by vol.) in M/15 phosphate, pH 5.6. Substrate, 9 mgm. pyruvic acid. Atmosphere, nitrogen. Temperature, 30°C. Total volume, 2.3 ml. Vitamin B_1 added directly to cell suspension.

metabolism judging from the slight increase on addition of 0.25 gamma vitamin B_1. The addition of 0.25 gamma of the vitamin to the "untrained" cells increases their metabolic rate to substantially that of the "trained" cell suspension containing added vitamin B_1; indicating that at least so far as the anaerobic pyruvate metabolism is concerned, the "trained" cells differ from the "untrained" only by having a higher vitamin B_1 content.

Table 2 recapitulates the differences found in a single series of experiments in which the "trained" and "untrained" cells were compared.

The finding that *P. pentosaceum* can be trained to synthesize vitamin B_1 is somewhat similar to the report of West and Wilson (1938a) that *Rhizobium trifolii* in a purified basal medium can also synthesize the vitamin in quantities to permit normal growth; however, this apparently is not a case of adaptation; for to initiate growth, a trace of the vitamin is required, after which the cells bring about its synthesis.

The mechanism of adaptation may be that of mutation and subsequent selection, or the development of the vitamin B_1

TABLE 2

Comparative yields of cells, their vitamin B_1 content and anaerobic pyruvate metabolism

	YIELD OF WET CELLS	VITAMIN B_1 CONTENT PER GRAM DRY CELLS	ANAEROBIC PYRUVATE METABOLISM
	ml.	*gamma*	*mm³ CO_2/hr.*
"Trained" cells	1.15	6.25	120.3
"Untrained" cells	0.50	0.40	15.2
Ratio $\frac{\text{"trained"}}{\text{"untrained"}}$	2.3	15.6	7.9

synthesizing enzyme system within the original cell, or perhaps, the utilization of a latent mechanism which was always present in the cell, but employed only under stress, i.e., in the continued absence of vitamin B_1. Which mechanism it is, however, cannot be stated at the present time.

SUMMARY

1. A technique for the assay of vitamin B_1 is described which is based on the increased anaerobic pyruvate metabolism of vitamin B_1-deficient cells of *Propionibacterium pentosaceum* occurring on the addition of the vitamin.

2. *Propionibacterium pentosaceum* if transferred continuously on a basal medium free of vitamin B_1 can be "trained" to synthesize this vitamin to satisfy its growth requirements.

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