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Effect of Size of Inoculum on the Apparent Vitamin Requirements of Lactic Acid Bacteria.*

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The vitamin requirements of 23 lactic acid bacteria, as reported recently by Shankman, Camien, Block, Merrifield and Dunn,[1] were, in many cases, contrary to those previously reported. For example, biotin, nicotinic acid and pantothenic acid, which have previously been found essential for all lactic acid bacteria tested[2,3,4] were reported as unessential for growth of several organisms, including *Lactobacillus arabinosus* and *Lactobacillus casei.* These findings were especially unexpected inasmuch as the latter two organisms have been widely used for determination of each of these vitamins. These authors attributed their results to use of an exceptionally complete basal medium, which permitted synthesis (though limited, in some cases) of vitamins required for growth in less complete media.

An investigation to determine the cause for these variable findings was undertaken. In their experiments, Shankman, *et al.* used an exceptionally heavy inoculum, which had been grown in a medium rich in vitamins. It was considered possible, therefore, that their results were complicated in some cases by carry-over of sufficient vitamin with the inoculum to permit growth.

Experimental. A series of tests was made in a medium[5] similar to those used for vitamin assay, which contained hydrolyzed casein as the nitrogen source. The inoculum was grown in 10 ml of the complete medium. After 18 hours, cells were centrifuged, resuspended in 30 ml of 0.9% sodium chloride solution, and 1 ml of this suspension was used to inoculate 10 ml of the basal medium which lacked the vitamin under investigation. This inoculum is similar to that used by Shankman *et al.*[1] Additional series were inoculated with 1 ml of 1:7, 1:70, or 1:700 dilutions of this inoculum. These 4 inocula are referred to as inoculum A, B, C and D, respectively. Turbidity was measured at one or 2 day intervals over a period of 7 days. Typical data are given in Table I. In the complete medium, supplemented with all essential vitamins, heavy growth was obtained with all inocula. In the nicotinic acid and pantothenic acid-deficient media, inoculum A permitted heavy growth; inoculum B much lighter growth, and inocula C and D essentially no growth. These data indicated that growth in vitamin-deficient media was due to introduction with the heavy, washed inoculum of sufficient quantities of vitamin to permit heavy growth. An alternative explanation would be that only a few cells capable of vitamin-synthesis were present among the population of the inoculum culture, and that with heavy inocula, but not with light inocula, enough of these were transferred to the deficient medium to initiate growth.

It is possible to decide between these two explanations by subculture. If the cells which grow in the deficient medium synthesize their own vitamins, they should continue to grow in the deficient medium on subculture. If on the other hand, growth in the deficient medium results from carry-over of vitamins with

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1 Shankman, S., Camien, M. N., Block, H., Merrifield, R. B., and Dunn, M. S., *J. Biol. Chem.*, 1947, **168**, 23.

2 Snell, E. E., *J. Bact.*, 1945, **50**, 373.

3 Peterson, W. H., and Peterson, M. S., *Bact. Rev.*, 1945, **9**, 49.

4 Knight, B. C. J. G., in Harris, R. S., and Thimann, K. V., *Vitamins and Hormones*, New York, 1945, **3**, 106.

5 Rabinowitz, J. C., Mondy, N. I., and Snell, E. E., *J. Biol. Chem.*, 1948, **175**, 147.

TABLE I.
Effect of Size of Inoculum on Growth of *Lactobacillus arabinosus* in Vitamin-Deficient Media.

	% of incident light transmitted*							
	Inoculum A		Inoculum B (1:7 dilution of A)		Inoculum C (1:70 dilution of A)		Inoculum D (1:700 dilution of A)	
Medium	24 hr	168 hr	24 hr	168 hr	24 hr	168 hr	24 hr	168 hr
Nicotinic acid deficient	53	38	84	83	92	90	93	90
Pantothenic acid deficient	53	38	88	82	93	91	95	95
Complete	20	17	23	18	32	20	72	17

* Distilled water = 100.

TABLE II.
Growth in Subcultures from Vitamin-Deficient Media.

	% of incident light transmitted*							
	L. arabinosus				*L. casei*			
	Original		Subculture I		Original		Subculture I	
Medium	24 hr	168 hr	24 hr	168 hr	24 hr	168 hr	24 hr	168 hr
Nicotinic acid deficient	53	40	90	87	53	40	91	91
Complete	18	16	16	14	32	21	44	25
Pantothenic acid deficient	46	46	90	87	57	51	92	92
Complete	19	20	19	19	32	21	30	23
Biotin deficient	36	35	91	91	56	40	91	92
Complete	19	20	18	18	32	21	39	22

* Uninoculated medium = 100.

the inoculum, this should not be sufficient to permit growth in the deficient medium on repeated subculture. In testing this procedure, the inoculum medium, the size of the inoculum, and the basal media used were those described by Shankman, *et al.*[1] Ten ml volumes were used and turbidities were read at 24, 48, 72 and 168 hours. In confirmation of the results of Shankman and coworkers,[1] heavy growth of both organisms occurred in media free of pantothenic acid, nicotinic acid, or biotin (Table II). When heavy growth was obtained in these media, 1.0 ml was transferred to 10 ml of similarly deficient media, and to the same medium with all vitamins added. In the subcultures, no growth occurred without the vitamins, although good growth occurred when these were present (Table II). These results demonstrate that these organisms do not synthesize biotin, pantothenic acid or nicotinic acid in amounts sufficient to permit significant growth under these conditions. They do not, of course, deny the possibility that cultures of these organisms might be obtained by adaptation or selection which would grow in the absence of these vitamins. It is not necessary, however, to postulate this occurrence to explain growth in vitamin-free media when heavy inocula, grown in vitamin-rich media, are employed.

Discussion. When grown in the presence of an excess of certain vitamins, several microorganisms are known to accumulate amounts in the cell far higher than those present when cells are grown with only sufficient of the vitamin to permit maximum growth. *Lactobacillus pentosus,* for example, accumulates up to 15 times as much biotin in its cells when grown with excess biotin as it does when grown with minimal amounts of biotin.[6] Since the latter quanti-

[6] Krueger, K. K., and Peterson, W. H., *J. Bact.*, 1948, **55**, 693.

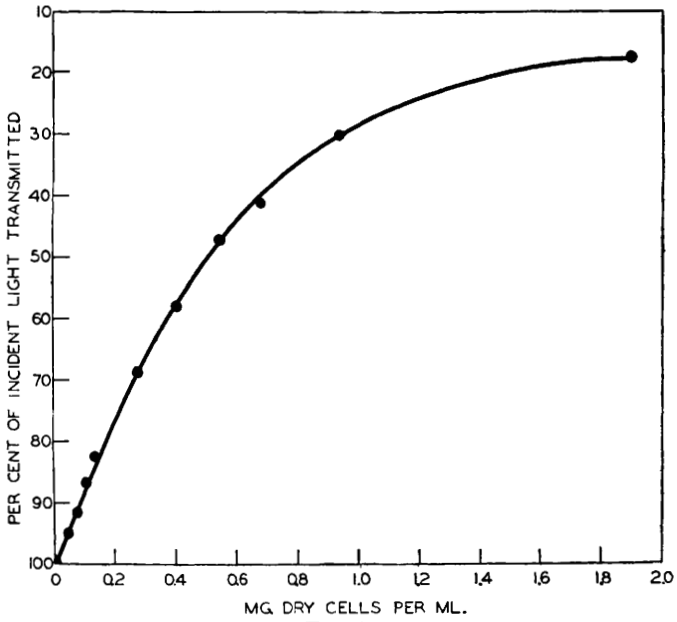

FIG. 1.

The relationship between culture turbidity and weight of cells for *Lactobacillus arabinosus*.

ties are sufficient to permit heavy growth, it would be expected that biotin-requiring cells containing a 15-fold excess over their minimum requirements of biotin would be able to increase in mass as much as 15 times when inoculated into a biotin-free medium before growth would cease for lack of biotin. If the inoculum is sufficiently heavy, increases of this magnitude will appear as heavy growth; with lighter inocula, a similar increase would not suffice to bring the number of cells into the visible range. These relationships are clearly apparent from Fig. 1, which relates light transmission to weight of cells of *Lactobacillus arabinosus* under our conditions. Inoculum A in 10 ml of medium gives a reading of 90, corresponding to .08 mg of dry cells per ml which lend a barely visible turbidity to the medium. A 15-fold increase in mass, which can occur without added biotin, would give 1.2 mg of cells per ml, which appears heavily turbid (galvanometer reading 24). A 70-fold dilution of this inoculum might also increase 15 times in mass, but would still give less than 0.02 mg of cells per ml, an amount too small to be detected visually.

These results emphasize the extent to which experimental results with certain microbiological methods may differ depending upon the size and history of the inoculum. Lack of appreciation of these simple relationships has been the source of very considerable difficulty in applying microbiological methods for vitamin assay.

Summary. Discrepancies in the findings of various investigators relative to the requirement of certain lactic acid bacteria for pantothenic acid, nicotinic acid, and biotin have been resolved. Recognition of the essential nature of these and other vitamins may be prevented by use of heavy inocula, from cultures grown in vitamin-rich media. This appears due to the fact that some organisms store certain vitamins in excess of their requirements, and thus are able to grow to a limited extent when subsequently transferred to media free of the essential vitamin or growth factor.