17209. Biotin-Carbohydrate Interrelationships in the Metabolism of *Leuconostoc.**

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(Introduced by Emmett B. Carmichael.)
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During an investigation of the direct utilization of sucrose by *Leuconostoc*[1] it was found that the organisms apparently did not require biotin when the disaccharide was employed, but the presence of the vitamin was essential for growth when the constituent monosaccharides were used in the media. Because a variation in vitamin requirement as a function of carbohydrate source was not found reported in the literature, and because it appeared to offer the possibility of recognition of a new function of biotin, the observation was investigated further.

Experimental. The basal medium employed had the composition given in Table I. With the exception of the carbohydrate component, the basal medium was made up in 3/2 strength, distributed in 2 ml amounts in 10 × 100 mm test tubes closed with cotton plugs, and sterilized 5 minutes at 15 lb. pressure. The carbohydrate component was made up in triple strength, sterilized by Seitz filtration, and added, aseptically, in 1 ml volumes. If other additives were involved, *e.g.*, oleic acid or egg white, these were included in the carbohydrate solution so that the final volume per tube remained constant at 3 ml.

Four sugar sources were employed: Sucrose, glucose, fructose and acid hydrolyzed sucrose. In the case of the first 3 sugars, the final concentration of 5% consisted of 90% of Seitz sterilized sugar and 10% of the same sugar sterilized in neutral phosphate buffer for 15 minutes at 15 lb. pressure. The resulting 0.5% concentration of heat sterilized sugar served as a source of the stimulatory materials reported to be necessary for the growth of many lactic acid organisms.[2] In the remaining instance sucrose was hydrolyzed with 0.1 N HCl under reflux for 80 minutes, neutralized with KOH and passed through a Seitz filter.

TABLE I.
Composition of Basal Medium.

Constituent	Conc. per liter g	Conc. per liter mg
Carbohydrate	50	
Casein hydrolysate	5	
Cysteine		100
Tryptophane		100
Phenylalanine		50
Tyrosine		50
Adenine		10
Xanthine		10
Guanine		10
Thymine		10
Uracil		10
Nicotinic acid		1
Riboflavin		0.5
Thiamin chloride		0.5
Calcium pantothenate		0.5
Pyridoxine		0.4
p-Aminobenzoic acid		0.1
Folic acid		0.01
NaOAc	25	
NH_4Cl	5	
$MgSO_4 \cdot 7H_2O$		100
NaCl		10
$FeSO_4$		10
$MnSO_4$		10
KH_2PO_4		500
K_2HPO_4		500

Three strains of *Leuconostoc* were used in this investigation: *L. mesenteroides* 683, *L. dextranicum elai,* and *L. dextranicum* 8086 (ATCC). Of the various strains available these were selected because of the wide variation in their respective dextran synthesizing capabilities. *L. dextranicum elai* consistently gives yields of dextran approximating complete conversion of the dextrose half of the sucrose molecule into the polysaccharide. The yields routinely obtained with *L. mesenteroides* 683 and *L. dextranicum* 8086 were, respectively, 65% and 33% of that theoretically

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1 Carlson, W. W., and Whiteside-Carlson, V., *Fed. Proc.*, 1949, **8**, 189.

2 Orla Jensen, S., *J. Soc. Chem. Ind.*, 1933, **52**, 374.

possible from complete polymerization of the dextrose component.

The inocula were prepared by incubating the cultures for 24 hours at room temperature in 5 ml of the complete basal medium (Table I) to which biotin was added in the low concentration of 0.01 μg per liter. The bacterial cells were centrifuged from the medium, resuspended in a double volume of sterile saline, recentrifuged and again resuspended in sterile saline to make a 1:12 dilution of the original culture. By means of a sterile syringe (20-gauge needle) one drop of this diluted inoculum was added to each 3 ml tube of medium. In the case of experiments involving *Leuconostoc* the tubes after inoculation were incubated for 72 hours at a constant temperature of 25° C.

As an organism requiring biotin *Lactobacillus arabinosus* 17-5 was also employed in this investigation for the purpose of comparison and assay. The culture was grown in the same basal medium and the inoculum was prepared in the same manner as described for the *Leuconostoc*. Each tube, however, was incubated at 37°C for 72 hours.

The rate of growth of all organisms was measured by titration of each tube of medium with standard alkali, bromthymol blue being used as indicator. The results, corrected for the blank titration of uninoculated controls, are expressed in terms of ml of 0.01 N acid produced per ml of culture.

Results and Discussion. The presence of biotin in sucrose has been reported by various investigators.[3,4] To verify the observation that *Leuconostoc* require biotin for growth in glucose and fructose media, but not in a sucrose medium, a sample of the disaccharide was hydrolyzed with acid to invert sugar. Kreuger and Peterson[5] have shown that autoclaving for 2 hours at 15 lbs pressure with 2 N acid did not destroy biotin added to media. It therefore seemed safe to assume that the conditions employed here, 80 minutes refluxing at atmospheric pressure with 0.1 N acid, would not destroy any biotin present in the sucrose samples.

The ability of the 3 strains of *Leuconostoc* to grow in media containing sucrose, or the same sucrose hydrolyzed by acid, was determined both in the presence and absence of added egg white. The results given in Table II show that whereas satisfactory growth occurred both in the sucrose and hydrolyzed sucrose media, added egg white was strongly inhibitory in the medium containing invert sugar. In contrast, in the sucrose medium, egg white inhibited growth only partially and then to a degree approximately inversely proportional to the yield of dextran. *L. dextranicum* 8086 with a dextran yield of 33%, was markedly inhibited by egg white, whereas *L. dextranicum* elai, which forms a theoretical yield of the polysaccharide, was insensitive to the biotin-binding action of egg white. *L. mesenteroides* 683 occupied a middle position both which respect to dextran yield and inhibition by egg white. Thus, to the degree that a strain of *Leuconostoc* utilizes sucrose via the mechanism resulting in dextran synthesis, it appears to be free of a requirement for biotin.

The ability of the *Leuconostoc* strains to grow in glucose or fructose media in the presence or absence of egg white also was determined. The low level of growth observed in the basal media without added biotin was suppressed by egg white in the cases of *L. dextranicum* 8086 and *L. mesenteroides* 683. Growth of *L. dextranicum* elai was increased by the added egg white. Shorb[6] has reported the presence of small amounts of vitamin B_{12} and the T. J. factor in egg white. In separate experiments, *L. dextranicum* elai was found to be stimulated slightly by vitamin B_{12}† and to respond strongly to tomato serum as a source of the T. J. factor. The observed stimulation by egg white thus appears to be due to its T. J. factor content.

Reversal of egg white inhibition by added biotin also was demonstrated. In order to

[3] Jackson, W. R., and Macek, T. J., *Ind. Eng. Chem.*, 1944, **36**, 261.

[4] Hall, H. H., Paine, H. S., and Fabian, F. W., *Food Research*, 1947, **12**, 99.

[5] Kreuger, K. K., and Peterson, W. H., *J. Bact.*, 1948, **55**, 693.

[6] Shorb, M. S., *Science*, 1948, **107**, 397.

† We are indebted to Merck and Company, Rahway, N. J., for a sample of crystalline vitamin B_{12}.

TABLE II.
Effect of Egg White, Biotin and Oleic Acid on the Growth of *Leuconostoc* in Various Carbohydrate Media.

Additions to basal medium	Test organism	ml 0.01 N NaOH per ml medium			
		Sucrose	Hydrolyzed sucrose	Glucose	Fructose
None	*L. dextranicum* 8086	12.3	9.7	1.1	1.5
	L. mesenteroides 683	9.7	11.6	1.5	2.0
	L. dextranicum elai	7.2	5.8	0.7	0.3
Egg white 0.2 ml per tube	*L. dextranicum* 8086	2.4	0.7	0.1	0.1
	L. mesenteroides 683	5.7	0.4	0.6	0.4
	L. dextranicum elai	7.1	2.5	1.9	2.6
Egg white 0.2 ml per tube Biotin 0.01 μg per ml	*L. dextranicum* 8086	—	12.0	11.2	8.5
	L. mesenteroides 683	—	7.2	7.7	13.4
	L. dextranicum elai	—	7.3	3.5	7.8
Oleic acid 5 μg per ml	*L. dextranicum* 8086	11.6	8.4	4.0	2.5
	L. mesenteroides 683	11.9	11.3	4.7	6.2
	L. dextranicum elai	0.2	0.9	0.1	0.9
Egg white 0.2 ml per tube Oleic acid 5 μg per ml	*L. dextranicum* 8086	8.6	0.2	0.1	0.1
	L. mesenteroides 683	7.7	0.3	0.1	0.2
	L. dextranicum elai	1.9	2.0	0.2	0.9

eliminate variables such as activity of the inoculum, the data for Table II were all collected at one time. Tubes corresponding to the addition of both egg white and biotin to the sucrose medium were not included in this experiment. From results of other experiments, however, it can be stated that added biotin reverses such variable inhibition of growth in sucrose media as occurs with the various strains of *Leuconostoc*. Slight stimulation over the growth observed in the basal medium without added biotin was customarily found, traceable to the presence of stimulatory factors in egg white.

Oleic acid is regarded as a probable product of biotin activity and is stated to be capable of substituting for the vitamin in the case of certain lactic acid organisms.[7,8] The effect of oleic acid, at a level of 5 μg per ml, was determined for the various types of carbohydrate media given in Table II. *L. dextranicum* elai showed a marked sensitivity to oleic acid, growth being almost completely inhibited in all the media employed. With the other two strains of *Leuconostoc*, oleic acid produced little effect in sucrose or hydrolyzed sucrose media. Growth in glucose and fructose was stimulated, demonstrating an ability of the substance to partially replace the biotin requirement of the organisms. Reversal of egg white inhibition by added oleic acid occurred only with *L. mesenteroides* 683 and *L. dextranicum* 8086 in sucrose medium. In the case of *L. dextranicum* elai, the toxicity of oleic acid was reversed to a small extent by added egg white. Failure of oleic acid to reverse egg white inhibition in media containing invert sugar, glucose, or fructose, as compared with the result obtained in the sucrose medium, suggests that utilization of the intact disaccharide follows a different metabolic pathway.

The results given in Table II are taken to indicate that sucrose does contain traces of biotin, these small amounts of the vitamin being important to growth of the organisms in the invert sugar medium, and also in the sucrose medium to the extent that utilization of the disaccharide does not follow the path-

[7] Williams, V. R., and Fieger, E. A., *J. Biol. Chem.*, 1946, **166**, 335.

[8] Boquist, H. P., and Snell, E. E., *J. Biol. Chem.*, 1948, **173**, 435.

TABLE III.
Assay of Biotin Level of Various Carbohydrate Media with *Lactobacillus arabinosus.*

Additions to basal medium	ml 0.01 N NaOH per ml medium			
	Sucrose	Hydrolyzed sucrose	Glucose	Fructose
None	1.4	4.3	1.7	0.7
Biotin 0.00005 μg per ml	4.6	8.8	4.0	5.2
Biotin 0.0005 μg per ml	19.0	23.3	23.3	16.6
Egg white 0.2 ml per tube	0.0	0.2	0.2	0.1
Egg white 0.2 ml per tube Biotin 0.01 μg per ml	23.3	25.2	26.7	20.1

way leading to polysaccharide synthesis.

An attempt was made to estimate the amount of the vitamin in the various carbohydrate media by means of a standard biotin assay organism, *Lactobacillus arabinosus* 17-5. The results, briefly summarized in Table III, show that only a low level of growth was obtained with any of the carbohydrate media. Egg white suppressed this low level of growth, complete reversal of the inhibition being observed with added vitamin. Added to the basal media at a level of 0.00005 μg per ml, biotin caused a definite stimulation of growth, a nearly maximal effect being obtained at a level of 0.0005 μg per ml. These results indicate that *Leuconostoc* respond to very low levels of biotin.

To test the possibility that *Leuconostoc* synthesize biotin, the cultures were grown in the sucrose and invert sugar basal media for 72 hours. The cultures were then sterilized for 15 minutes at 15 lb pressure and aliquots of the resulting solutions added to tubes of the glucose basal medium, after which they were inoculated with *L. arabinosus*. Representative titration figures, as obtained with the material from the growth of *L. mesenteroides* 683 in sucrose and invert sugar media were, respectively, 1.5 ml and 5.2 ml. Thompson[9] has reported that organisms that synthesize biotin release the vitamin into the medium. Since essentially no stimulation of the growth of *L. arabinosus* by *L. mesenteroides* culture solutions was observed, it appears unlikely that *Leuconostoc* synthesize biotin. It is therefore concluded that the observed difference in the biotin requirement of *Leuconostoc* in a sucrose medium, as compared with the requirement in media containing the constituent monosaccharides, is real. Information concerning the metabolic pathway in invert sugar, glucose, or fructose media, as compared with the pathway in a sucrose medium which leads to polysaccharide synthesis, thus may suggest a function for biotin in addition to those presently known.[10]

Summary. With 3 strains of *Leuconostoc* it was found that biotin was required for growth in media containing invert sugar, glucose, or fructose. In a sucrose medium the organisms were free of a biotin requirement to the degree that the disaccharide was utilized via the mechanism resulting in dextran synthesis.

[9] Thompson, R. C., Univ. of Texas Publ. 4237, 1942, p. 87.

[10] Couch, J. R., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., *Arch. Biochem.*, 1949, **21**, 77.

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