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Biotin Transport and Accumulation by Cells of *Lactobacillus plantarum*

II. Kinetics of the System

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ABSTRACT

WALLER, JAMES R. (University of Cincinnati, Cincinnati, Ohio), AND HERMAN C. LICHSTEIN. Biotin transport and accumulation by cells of *Lactobacillus plantarum*. II. Kinetics of the system. J. Bacteriol. **90**:853-856. 1965.—Bound biotin-saturated cells of *Lactobacillus plantarum* accumulated free biotin by a time-dependent process exhibiting substrate saturation phenomena in the presence and absence of glucose. Apparent K_m and V_{max} values determined in the presence and absence of glucose, respectively, from Lineweaver-Burk plots were found to be 31.5 and 7.72 mμM (K_m) and 9.72 and 3.26 μμmoles per mg per min (V_{max}). Free biotin transport per se appeared to be an energy-independent, mediated process, whereas the accumulation of large intracellular vitamin concentrations was energy-dependent. Internal free biotin was quantitatively converted to bound biotin. The rate and extent of bound biotin formation was slower than free biotin uptake, and dependent upon intracellular free biotin levels up to a saturating concentration.

In the preceding paper (Waller and Lichstein, 1965), we described some properties of free biotin uptake by *Lactobacillus plantarum* and concluded that vitamin uptake proceeds in the presence and absence of glucose by two apparently distinct processes. Energy-dependent uptake exhibited characteristics of an enzyme-mediated reaction, and intracellular vitamin levels nearly 4,000-fold greater than those found in the external menstruum were observed.

Since active and passive processes seemed to be involved in free biotin uptake, it was felt that kinetic analysis of the system(s) might yield information concerning the mechanism(s) of transport. The results of such studies suggest that the vitamin is transported across the cell membrane by a carrier mechanism in the presence and absence of cellular metabolism.

MATERIALS AND METHODS

The methods used were those described in the preceding paper (Waller and Lichstein, 1965) except that the effect of time on free biotin accumulation was studied as follows. The cells were separated from the reaction mixture by forcing the suspension through a Swinny hypodermic adapter fitted with a Seitz filter pad or a Millipore HA membrane filter at the desired time intervals. The clear filtrate was collected and analyzed for free biotin. The amount of free biotin accumulated was determined from the difference between the cleared reaction mixtures and the uninoculated controls.

RESULTS

Effect of time on free biotin accumulation. In the presence of glucose, resting cells of *L. plantarum* rapidly accumulated free biotin (Fig. 1). Vitamin uptake was linear during the first 15 min of the reaction and reached completion in 45 min. In the absence of glucose, biotin uptake proceeded for the first 5 min, decreased by 50% in the next 10 min, and finally rose slowly to the 5-min level (13 to 30% of that attained in the presence of glucose). The glucose-independent results resemble the overshoot phenomenon described by Sanders and Leach (1964) for lipoic acid uptake and are consistent with the existence of an exit reaction, activated by a certain level of intracellular free biotin, which establishes an equilibrium with the uptake reaction.

Effect of biotin concentration. In the presence of glucose, the rate of free biotin accumulation increased with increasing external concentration up to about 130×10^{-4} μg of biotin per ml (Fig. 2). Higher vitamin levels did not affect the reaction rate. These data suggest the existence of a substance of limited capacity (carrier, transporter) with which the biotin molecule must enter into a physical combination to transverse the cell membrane. A Lineweaver-Burk plot of the data

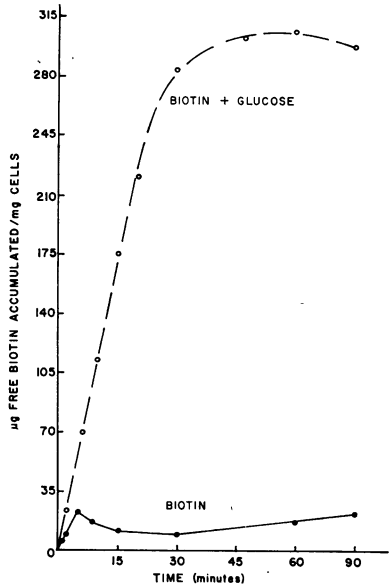

FIG. 1. *Influence of time on free biotin uptake. Temperature, 37 C; pH, 7.5; glucose, 1%; biotin concentration, 200 × 10⁻⁴ μg/ml.*

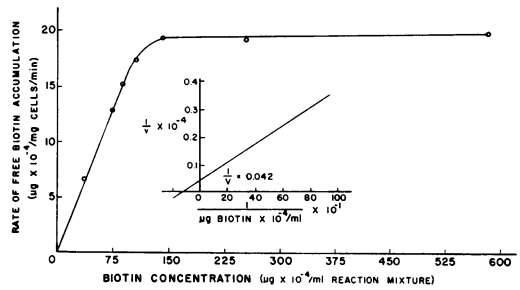

FIG. 2. *Effect of biotin concentration on free biotin uptake in the presence of glucose. Experimental conditions as for Fig. 1, except that cells were separated by centrifugation in the cold.*

(insert, Fig. 2) produced a straight line from which apparent K_m [77 × 10^{-4} μg of biotin per ml (31.5 mμM)] and V_{max} [23.8 × 10^{-4} μg (9.72 μμmoles) of biotin per mg of cells per min] values were calculated.

In the absence of glucose, free biotin uptake exhibited many properties characteristic of diffusion, except that it was inhibited by homobiotin to the same degree as was glucose-stimulated accumulation (Waller and Lichstein, 1965). This suggested that here too a component of finite capacity was involved. If biotin enters the bacterial cells by diffusion and subsequently is adsorbed to protoplasmic constituents, the rate of uptake should be proportional to the extracellular vitamin concentration. Demonstration that the rate of glucose-independent vitamin uptake was readily saturated (Fig. 3) provided further evidence that this reaction also was a mediated process and not diffusion followed by adsorption. Apparent K_m and V_{max} values obtained from Lineweaver-Burk plots of the data (insert, Fig. 3) were, respectively, 18.8 × 10^{-4} μg of biotin per ml (7.72 mμM) and 8 × 10^{-4} μg (3.26 μμmoles) of biotin per mg of cells per min.

Conversion of intracellular free biotin into bound biotin. The effects of time, glucose, and biotin concentration on intracellular free biotin and bound biotin formation are presented in Fig. 4. By comparing the levels of free and bound biotin in the presence and absence of glucose when external biotin was 40 × 10^{-4} μg/ml, it can be seen that internal free biotin was quantitatively converted to bound biotin. Glucose increased the rate of bound biotin formation (see 5- and 10-min values for free and bound biotin with and without glucose) at the lower vitamin concentration, but did not affect the maximal level attained, indicating that extracellular biotin was limiting. When the external vitamin was increased to 100 × 10^{-4} μg/ml, bound biotin formation was affected only slightly in the absence of glucose whereas both the rate and extent of free biotin uptake increased markedly (see 5- and 10-min values). Since between 10 and 60 min the decrease in intracellular free biotin did not equal the increase in bound biotin, it appears that external biotin replaced that internal free biotin which was converted to bound biotin. Glucose increased the rates of free biotin uptake and bound biotin formation (compare 5-min free and bound biotin values in the presence and absence of glucose) and the maximal level of bound biotin attained (compare 60-min bound biotin values). Again, a quantitative relationship exists between the decrease in internal free biotin and the increase in

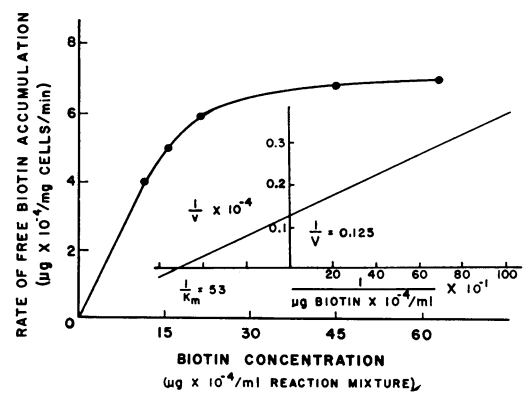

FIG. 3. *Effect of biotin concentration on free biotin uptake in the absence of glucose. Conditions as for Fig. 2.*

bound biotin at the lower vitamin level. Since the rate of free biotin uptake exceeded the rate of bound biotin formation in both the presence and absence of glucose, it can be concluded that free biotin uptake was not rate-limiting for bound biotin formation at sufficiently high external biotin concentrations. It is obvious, however, that bound biotin formation was dependent upon the level of intracellular free biotin, and that extracellular vitamin was transferred into the bacterial cells and then converted, quantitatively, into bound biotin by an apparently enzyme-mediated reaction dependent upon cellular metabolism (Lichstein and Waller, 1961). Kosow and Lean (1962) showed that biotin is covalently bound to protein by an adenosine triphosphate-dependent enzyme system to form propionyl holocarboxylase. Since biotin has been shown to be the prosthetic group in several CO_2-transferring reactions (*see* Ochoa and Kaziro, 1961) and since Kosow and Lane (1961) suggested that numerous biotin-containing apocarboxylases other than propionyl carboxylase probably were formed in their reaction mixtures, it may be that the bound biotin formation observed during these and previous studies (Lichstein and Ferguson, 1958; Lichstein and Waller, 1961) reflects an enzymatic production of biotin-containing enzymes.

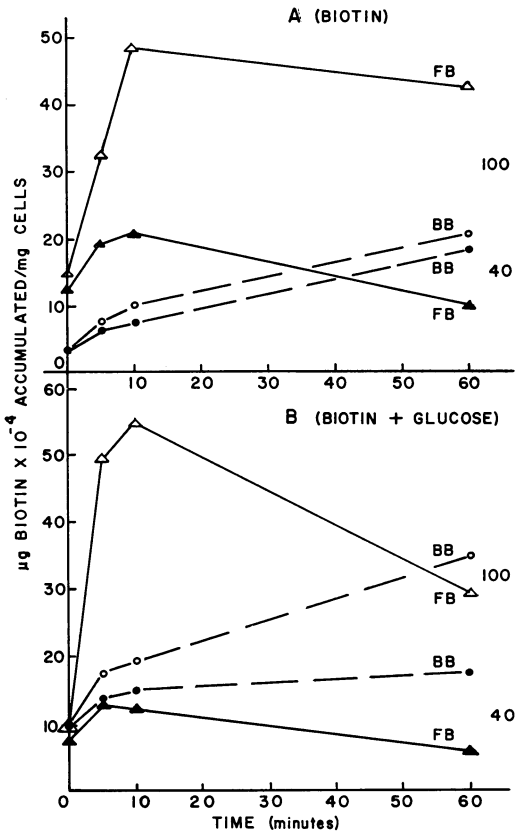

FIG. 4. *Conversion of intracellular free biotin into bound biotin in the presence and absence of glucose. Washed bacterial cells from a 45-hr culture were incubated with 40 × 10^{-4} or 100 × 10^{-4} μg of biotin per ml with and without glucose (1%) for 0, 5, 10, and 60 min in 0.1 M phosphate buffer (pH 6.8) at 37 C. The reaction was stopped by cooling the tubes for 30 sec to 1 min at −10 C, followed by immediate centrifugation in the cold. Symbols: △ = free biotin (FB; initial external concentration, 100 × 10^{-4} μg/ml); ▲ = free biotin (initial external concentration, 40 × 10^{-4} μg/ml); ○ = bound biotin (BB) (initial external concentration, 100 × 10^{-4} μg/ml); ● = bound biotin (initial external concentration, 40 × 10^{-4} μg/ml). Figure 4A = no glucose; Fig. 4B = plus glucose.*

TABLE 1. *Biotin uptake at limiting concentrations with and without glucose**

Initial external concn (μg × 10^{-4}/ml)	External concn after 15 min†	
	Biotin	Biotin + glucose
11.8	1.4	1.1
15.4	2.1	1.5
21.5	3.2	2.0

* Temperature, 37 C; *p*H, 7.5.

† Equilibrium was established under these conditions.

DISCUSSION

It is becoming increasingly evident that most if not all molecules are transported into the bacterial cell by means of some finite component(s), presumably located in the cell membrane, with which a physical combination must be established during the transfer process. Intracellular accumulation against a concentration gradient requires cellular energy except under certain conditions, e.g., during "counterflow" (Cirillo, 1962). However, the actual transport of a molecule across the cell membrane is apparently energy-independent (*see* Koch, 1964), and, indeed, sugar transport into yeast cells has been shown to be an energy-independent, mediated transport (Cirillo, 1961). The kinetic data given in the present paper, together with the previous demonstration that transport occurs in the absence of glucose and at 4 C (Waller and Lichstein, 1965), show that biotin transport, per se, is an energy-independent, mediated process. It appears that the accumulation of high intracellular levels of free biotin is the energy-dependent step in the vitamin uptake system, since glucose-stimulated uptake was inhibited by 10^{-3} M iodoacetate and did not occur in the cold (Waller and Lichstein, 1965).

Helmreich and Kipnis (1962) suggested that the link between cellular metabolism and amino

acid transport into lymph node cells involves the transition of the carrier from a state of high affinity for the penetrant on the outer surface of the cell to one of low affinity on the inner surface. If this were so, an activated carrier would actually exhibit lower affinity for the penetrant and could release it within the cell at an elevated level (thus eliminating the need for a separate carrier-penetrant complex splitting enzyme) thereby establishing an intracellular concentration exceeding that present in the external environment.

The effect of homobiotin on biotin uptake (Waller and Lichstein, 1965) suggests that both glucose-dependent and -independent processes are mediated by the same carrier system and that the apparent affinity of the carrier for the vitamin during biotin-carrier complex formation is similar or identical under both conditions. Assuming then that a single carrier system is involved and that K_m refers to dissociation of the carrier-biotin complex, the apparent K_m values obtained for free biotin uptake in the presence and absence of glucose (31.5 and 7.7 mμM, respectively) are compatible with a decrease in the apparent affinity ($1/K_m$) of the carrier for the vitamin molecule upon activation. If, in the presence of the sugar, the carrier's affinity at the external surface of the membrane were much lower (higher K_m) than in its absence, less vitamin should have been removed from the menstruum. On the other hand, a carrier with a higher affinity should remove considerably more biotin. Instead, removal in the presence and absence of glucose at equilibrium was nearly identical (Table 1), indicating apparently similar affinities for the biotin molecule under both conditions. The data also suggest that the transition from high to low affinity in the presence of glucose occurs after formation of the carrier-biotin complex and prior to or at the time of release of free biotin into the internal environment. Although the kinetic data presented are compatible with the proposed model for vitamin transport, they do not exclude other possible models.

Although there are many similarities between the free biotin transport system in *L. plantarum* and galactoside transport in *Escherichia coli*, certain notable dissimilarities do exist, especially with respect to exit mechanisms. Osborn, McLellan, and Horecker (1961) and Koch (1964) suggested that free (inactive) transporter substance (carrier) could combine with intracellular galactosides and thereby form the first step in exit. It was assumed that under accumulating conditions the transporter is present entirely in an activated form and unable to combine with internal substrate (Kepes, 1960); therefore, internal galactosides could be retained during washing procedures. In contrast, considerable quantities of internal free biotin were released rapidly when the cells were washed with saline or suspended in phosphate buffer (Waller and Lichstein, 1965). Preliminary studies on biotin exit (Waller, Ph.D. Thesis, University of Minnesota, Minneapolis, 1964) suggest that biotin release proceeds by a process more complex than diffusion. The mechanism of free biotin exit from cells of *L. plantarum* is currently under investigation.

ACKNOWLEDGMENTS

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