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Biotin Transport and Accumulation by Cells of *Lactobacillus plantarum*

I. General Properties 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*. I. General properties of the system. J. Bacteriol. **90**:843-852. 1965.—Resting cells of *Lactobacillus plantarum* were saturated with bound biotin by incubation in phosphate buffer with biotin and glucose for 2 hr. This bound biotin was stable to wide changes in temperature, *p*H, and reaction time. Free biotin could be eluted from the cells by suspending them in cold water or saline. Immersing the cells in boiling water for 30 sec released all free biotin. Recoveries of added biotin exceeded 92%. Free biotin uptake by bound biotin-saturated cells occurred by two mechanisms. One process was independent from temperature (Q_{10}, 1.25), *p*H, cellular metabolism, and inhibition by iodoacetate. The other mechanism was dependent upon temperature (Q_{10}, 2.58; optimum, 37 C), *p*H (optimum, 7.5), and active cellular metabolism, and was inhibited by iodoacetate. Activation energies of 3,700 and 13,800 cal per mole, respectively, were observed for glucose-independent and -dependent free biotin uptake. Both processes exhibited approximately the same degree of inhibition by homobiotin. Higher concentrations of homobiotin were required to inhibit growth than to inhibit free biotin uptake. Intracellular-extracellular ratios as high as 600 were established in the absence of glucose, whereas ratios of nearly 4,000 occurred in the presence of glucose.

Living cells are surrounded by a semipermeable membrane which controls the penetration of molecules to and from the cell interior. On the basis of results obtained from numerous lines of investigation, several mechanisms of transport across cell membranes have been described (Cirillo, 1961; Cohen and Monod, 1957; Heinz and Walsh, 1958; Kepes, 1960).

Knowledge of the conditions affecting the penetration of vitamins into living cells is quite limited. Perhaps the first reports of vitamin penetration into microbial cells were those of Fink and Just (1941*a*, *b*) and Sperber and Renvall (1941), who studied vitamin B_1 accumulation in yeast cells. Active transport of thiamine by cells of *Saccharomyces cerevisiae* (Ziro, 1955), *Staphylococcus aureus* (Citron and Knox, 1954), and *Lactobacillus fermenti* (Neujahr, 1963) has been described. Sanders and Leach (1964) characterized the uptake of lipoic acid by cells of *Streptococcus faecalis*(10 C1) as an energy-requiring, temperature-dependent transport system which was easily saturated with lipoic acid and appeared to exist, at least in part, in the lipoprotein fraction of the cell membrane. The accumulation of bound biotin by cells of *L. plantarum* has been described (Lichstein and Ferguson, 1958; Lichstein and Waller, 1961).

The studies reported here concern the uptake and accumulation of free biotin by bacterial cells. The problem of substrate metabolism during experimental manipulations was overcome by presaturating the cellular binding sites with biotin, thereby allowing direct measurement of accumulated free biotin.

MATERIALS AND METHODS

Organism and growth conditions. L. plantarum strain 17-5, which was employed in all studies, was maintained in stab culture on APT medium (Case Laboratories, Chicago, Ill.) by serial transfer every 2 to 4 weeks, incubated at 30 C overnight, and then refrigerated. Cells for experimental use were grown in the medium of Wright and Skeggs (1944) containing 5×10^{-4} μg of biotin per ml; the medium was modified by the substitution of cysteine for cystine, and the addition of folic acid (0.5 mg per liter) to improve growth. NaCl was omitted because the Difco Vitamin Free Casamoin Acids used as a source of amino acids contains 38% NaCl. Glucose was sterilized separately by auto-

claving and was added aseptically to the sterile medium. Each 100 ml of the experimental medium was inoculated with 0.39 mg (dry weight) of cells grown for 16 to 18 hr at 30 C in 5 ml of the modified Wright-Skeggs medium (except that glucose was autoclaved with the other medium constituents), washed twice with sterile saline, once with sterile distilled water, and suspended in the latter. The culture was then incubated at 30 C for 43 to 46 hr. The bacterial cells were harvested by centrifugation, washed twice (once with 200 ml of saline and once with 200 ml of distilled water), and then suspended in distilled water.

Saturation of biotin binding sites. The washed-cell suspension was incubated with biotin [2×10^{-2} μg/mg (dry weight) of cells] in the presence of 1.0% glucose and 0.1 M phosphate buffer (*p*H 6.8) at 37 C for 2 hr. The bacterial cells were recovered by centrifugation, the packed cell pellet and container were rinsed thoroughly with distilled water, and the cells were washed three times in acidified distilled water (*p*H 4.5 to 5.0, 37 C) to remove accumulated free biotin. These cells were suspended in distilled water to a density of 3 to 11 mg (dry weight) per ml and were used in free biotin uptake experiments.

Uptake of free biotin. Amounts of 1 ml of 1.0 M phosphate buffer of the desired *p*H, together with biotin or biotin plus glucose (1.0%, final concentration) were added to 16-ml cellulose nitrate tubes as required for the particular experiment. The volume of the reaction mixture was adjusted with distilled water so that addition of the acid-washed bacterial cell suspension brought the total volume to 10 ml. After incubation under the desired conditions, the reaction was stopped by immediate centrifugation in a cold centrifuge head (0 to 4 C). The supernatant reaction mixtures were decanted, and the tubes were drained well and wiped to remove as much adhering biotin as possible (less than 0.05 ml of solution remained in each tube). Free biotin was then extracted from the cells.

Extraction of cellular free biotin. Free biotin was extracted from the bacterial cells by adding 1 ml of hot distilled water to each tube and immersing the tubes in boiling water for 5 to 10 min. Then 9 ml of distilled water were added, the contents of each tube were thoroughly mixed, the cells removed by centrifugation, and the supernatant fluids were assayed for free biotin.

Extraction of cellular bound biotin. After free biotin extraction, the boiled cells were washed twice in saline, and the tubes were drained well and wiped to prevent excessive dilution of the acid added later. For the second washing, the cells were transferred to heavy-walled Pyrex centrifuge tubes (18 by 102 mm). The washed cells were suspended in 0.5 ml of 6 N H_2SO_4, and the tubes were capped with rubber and autoclaved for 1 hr at 121 C to release bound biotin. The hydrolysates were treated with 0.5 ml of 6 N NaOH, brought to 10 ml with distilled water, adjusted to *p*H 6.8, and filtered through Whatman no. 2 paper to remove large particulate matter, the presence of which would increase drift in the subsequent assay procedure. Samples were assayed microbiologically for biotin content.

Microbiological assay procedure. The assay procedure was that of Lichstein and Waller (1961), except that sterilization was accomplished by autoclaving for 7 min at 121 C and the tubes were cooled immediately to reduce carmelization. Standard curves were prepared for each assay.

Determination of intracellular free space. The volumes of packed whole cell pellets were determined by centrifuging a dense aqueous bacterial cell suspension for 3 hr in the cold (4 C) at approximately 2,300 $\times$ *g*, by use of Bauer-Schenk cerebrospinal protein sedimentation tubes and a horizontal "swinging-bucket" head. The density of the cell suspension was ascertained through dry-weight determinations. Pellet weights were calculated from this value.

Assuming that the bacterial cell is composed largely of protein and water, and from the knowledge that the specific volume of protein is between 0.70 and 0.75, a reasonable approximation of intracellular free space can be calculated. Thus, in a wet cell pellet occupying a volume of 0.0318 ml and containing 0.01055 g (dry weight) of cells, the volume occupied by solid cellular material would be 0.007385 ml (0.01055 $\times$ 0.70), and the internal free space would be 0.02441 ml (0.0318 $-$ 0.007385). The value per milligram of cells would be 0.00231 ml (0.02441/10.55). Based on the average of several determinations, the intracellular free space per milligram of cells was calculated to be 0.00221 ml for wet cell preparations of *L. plantarum*. These calculations do not include corrections for the extracellular volume of the cell pellet. Such corrections were obtained from a curve relating the amount of biotin adhering to a boiled cell pellet of known weight to the concentration of biotin in the suspending reaction mixtures.

RESULTS

Some properties of free and bound biotin. The extraction of free biotin from various bacterial cells and tissues has been described (Lichstein and Waller, 1961; Thompson, Eakin, and Williams, 1941). Free biotin is defined as that amount of biotin, assayable with *L. plantarum* strain 17-5, which is released from bacterial cells upon short exposure to boiling water. Earlier studies on bound biotin formation (Lichstein and Ferguson, 1958; Lichstein and Waller, 1961) failed to reveal any cellular form of the vitamin other than bound biotin, since the boiling procedure used to stop the binding reaction released all free biotin from the cells. Data presented in Tables 1 and 2 show that free biotin was easily liberated from the bacterial cells. For example, immersing the cells in boiling water for as little as 30 sec released all cellular free biotin, and suspending them in warm

TABLE 1. *Effect of various conditions on release of cellular free biotin**

Conditions of treatment†	Biotin released		Bound biotin released after acid hydrolysis
	During treatment before boiling	By subsequent boiling	
KPO_4, *p*H 6.8, 37 C			
0.02 M	224‡	210	297
0.20 M	156	344	283
NaCl (0.85%)			
4 C	253	268	257
37 C	276	155	248
Water			
4 C	316	235	261
37 C	384	122	268
Boiling			
30 sec	554	4.0	264
2 min	580	4.1	275
5 min	534	2.5	278
60 min	574	0.4	232
121 C	534	4.5	230

* Washed bacterial cells were incubated under conditions described for saturation of biotin binding sites, harvested by centrifugation in the cold as described under uptake of free biotin, and further treated as indicated.

† Reaction volume was 1.0 ml and, unless otherwise stated, time was 60 min.

‡ Expressed as micrograms of biotin $\times 10^{-4}$ per 12.6 mg of cells. Total endogenous free biotin, 1.3×10^{-4} μg/12.6 mg of cells; endogenous bound biotin, 23×10^{-4} μg/12.6 mg of cells.

water effected a 76% loss. Also, considerable losses (up to 28%) of the free vitamin occurred during one wash with cold physiological saline (Table 2). Since less than 0.05 ml of liquid remained in each tube, the reaction medium left in the tubes did not contribute significantly to the biotin content of the saline washings. Thus, that amount of vitamin which appeared in the washing medium must have come from within the cells. These results are in contrast to those obtained by Horecker, Thomas, and Monod (1960) and Wood and Hitchings (1959) in their studies of the uptake of galactose and folic acid, respectively. They agree however, with the work of Britten and McClure (1962) and Piez and Eagle (1958), who observed that amino acid pools in *Escherichia coli* and HeLa cells were depleted rapidly by suspending the cells in water or saline, respectively.

L. plantarum strain 17-5 responds only to *d*-biotin, biotin-*d*-sulfoxide, and oxybiotin among the known forms of the vitamin. Of these, oxybiotin does not occur naturally. Since *L. casei* does not respond to biotin-*d*-sulfoxide (Melville, Genghof, and Lee, 1954), identical values when assayed with both organisms would prove the absence of the sulfoxide. Such results were obtained. Thus, it is concluded that the vitamin easily extracted from the bacterial cells was *d*-biotin. Furthermore, since immersing the cells in cold water or saline (Table 1) is an extremely gentle method of extracting cellular constituents, it is reasonable to assume that any biotin which appeared in the external phase had been present internally in the same form.

Since cells of *L. plantarum* accumulate considerable quantities of bound biotin (Lichstein and Waller, 1961), it was necessary to saturate cellular binding sites before quantitative analyses of accumulated free biotin could be conducted. The validity of subsequent free biotin determinations performed with such cells depended upon the stability of bound biotin to the experimental conditions used. Bound biotin was found to be stable to wide variations in *p*H, temperature, and length of reaction period (Table 3). These results, together with those presented in Table 1 (column 4) show that cellular bound biotin did not contribute significantly, if at all, to the free biotin values obtained by using bound biotin-saturated cells.

Quantitative determinations of free biotin could be made only if the vitamin were not being destroyed or converted into some other form (vitamer) by the bacterial cells during the reaction period. Since average biotin recoveries ranged between 92 and 97%, it was assumed that any free biotin which appeared in the cells or disappeared from the reaction menstruum, was an accurate measure of the free biotin uptake system in *L. plantarum* under the conditions employed.

In a control experiment, free biotin, menstruum biotin, and bound biotin determinations were found to vary from average values by 0.72, 1.88, and 2.94%, respectively (Waller, Ph.D. Thesis, University of Minnesota, Minneapolis, 1964), thus demonstrating the accuracy and reliability of the techniques used.

Energy requirements for biotin accumulation. A prime consideration in classifying a permeation system as active or passive depends upon an energy requirement. It is clear that glucose increased cellular free biotin concentrations more than sevenfold over the values attained in its absence (Table 4). Moreover, the intracellular free biotin concentration increased with the reaction time in the presence of glucose, but did not change significantly when it was omitted. A comparison of the zero and 12-min values shows that active metabolism was required for the accumulation of high free biotin levels. More convincing

TABLE 2. *Effect of washing on free biotin content of cells**

Accumulation temp	Accumulation time before centrifugation	Initial biotin concn ($\mu g \times 10^{-4}$/10 ml)	Cellular free biotin ($\mu g \times 10^{-4}$/12.2 mg of cells)	Total μg of biotin $\times 10^{-4}$ recovered from wash saline†	Total μg of biotin $\times 10^{-4}$ recovered from reaction medium
C	*min*				
4–10	0‡	400	150	63	174
		1,000	175	66	718
37	5	400	228	57	56
		1,000	398	164	298

* Experimental conditions as described under uptake of free biotin (*p*H 7.5 and glucose present).
† Cells washed with 10 ml of 0.85% NaCl at 4 C.
‡ For the 0 min determinations, cold cells were added to cold medium and centrifuged immediately in the cold. Time required for separation of cells and reaction medium was approximately 25 min.

TABLE 3. *Stability of bound biotin*[a]

*p*H[b]	Bound biotin[c]	Temp[d]	Bound biotin[c]	Time[e]	Bound biotin[c]
		C		*min*	
Control	100.5	Control	76.6	Control	79.0
2.3	97.5	10	75.1	0	78.5
5.6	100.5	20	74.1	5	82.5
7.4	100.5	37	76.6	30	82.0
10.6	100	45	74.1	90	81.0
		62	70.1		

[a] Stability was determined by comparing bound biotin content of cells before (control) and after each free biotin uptake experiment.
[b] Saturation time, 120 min; temperature, 37 C; reaction time, 30 min.
[c] Micrograms of biotin $\times 10^{-4}$ per milligram of cells.
[d] Saturation time, 120 min; *p*H, 6.8; reaction time, 15 min.
[e] Saturation time, 90 min; *p*H, 7.5; temperature, 37 C.

evidence was the demonstration that iodoacetate inhibited glucose-stimulated free biotin accumulation (Table 5). The inhibitor did not completely eliminate vitamin uptake, and little or no effect was noted in the absence of glucose, suggesting the presence of endogenous energy reserves or an energy-independent uptake system.

Effect of temperature. The effect of temperature on free biotin accumulation depended upon the presence or absence of glucose (Fig. 1). In the absence of carbohydrate, some free vitamin entered the cells by a temperature-insensitive, apparently energy-independent mechanism exhibiting a Q_{10} of 1.25 between 20 and 30 C. In contrast, glucose-stimulated vitamin uptake proceeded by a temperature-sensitive, energy-dependent mechanism which exhibited an optimum at 37 C and a Q_{10} of 2.58 between 20 and 30 C. It is significant that even at 0 C, in the presence or absence of glucose, a certain level of intracellular free biotin was attained.

The activation energies of the free biotin accumulating process were determined from an Arrhenius plot of the temperature data (Fig. 2). The activation energy of the temperature-insensitive, glucose-independent reaction was calculated to be about 3,700 cal per mole of free biotin accumulated, whereas the value for the temperature-sensitive, glucose-stimulated process was approximately 13,800 cal per mole. This 10,100-cal difference represents the energy required for activation of some mechanism responsible for accumulation of the large quantities of intracellular free biotin depicted in Fig. 1 and 2 and in Table 4.

These results agree favorably with those reported by others. For example, Gale (1953) showed that lysine accumulation by certain gram-positive cocci was temperature- and energy-independent (Q_{10}, 1.4), whereas glutamic acid uptake was completely inactive at 3 C even in the presence of glucose, and exhibited Q_{10} values of 1.9 to 2.9 with increasing temperatures. Folic acid accumulation by certain lactic acid bacteria (Wood and Hitchings, 1959) is completely dependent upon an energy source, whereas sugar transport in yeasts proceeds by an energy-independent mechanism exhibiting characteristics of

TABLE 4. *Effect of glucose on free biotin accumulation**

Time	Amt ($\mu g \times 10^{-4}$) of biotin/mg of cells	
	Biotin	Biotin + glucose
min		
0†	43‡	37
12	32	230
24	44	338

* Temperature, 37 C; *p*H, 7.5; biotin concentration, 1,107 $\times 10^{-4}$ μg/ml.

† Cells and menstruum at 0 to 4 C immediately centrifuged in the cold (0 to 2 C).

‡ Pre-experimental free biotin value, 2×10^{-4} μg of biotin per mg of cells.

TABLE 5. *Effect of iodoacetate on free biotin accumulation**

Iodoacetate concn	Amt ($\mu g \times 10^{-4}$ of biotin)/mg of cells (dry wt)	
	Biotin	Biotin + glucose
M		
0	34	86
1×10^{-3}	39	26
5×10^{-3}	34	23

* Temperature, 37 C; *p*H, 6.8; biotin concentration, 200 $\times 10^{-4}$ μg/ml; time, 30 min. Endogenous = 0.1 $\times 10^{-4}$ μg/mg of cells.

carrier-mediated transport (Cirillo, 1961). Britten and McClure (1962) showed that amino acid pool formation in *E. coli* occurs very slowly, if at all, at 0 C or in the absence of glucose. However, an exchange of intracellular pool constituents with the extracellular phase did occur at 0 C, although at a considerably reduced rate. In general, diffusion type reactions exhibit Q_{10} values of 1.4 or lower, and Q_{10} values approaching 2.0 or higher are usually considered indicative of enzyme-mediated reactions.

Free biotin accumulation in *L. plantarum* therefore resembles amino acid pool formation in *E. coli* in that both systems exhibit a low level of transport that is temperature-independent and proceeds in the absence of an exogenous energy supply, whereas high intracellular levels are established only as a result of active metabolism.

Effect of pH. The data in Fig. 3 portray the effects of *p*H on free biotin accumulation. The gross difference between the curves again suggests the existence of two distinct mechanisms for free biotin accumulation by cells of *L. plantarum*. Glucose-independent accumulation was affected only slightly, especially between *p*H 4.0 and 9.0. A similar effect on vitamin B_{12} uptake by cells of *L. leichmanii* was noted by Davis and Chow (1952). Such results are typical of mechanisms involving diffusion, which generally exhibit regular, proportional changes or little or no effect with respect to *p*H variations.

The results obtained in the presence of glucose revealed that biotin accumulation was *p*H-sensitive and had an optimal range between *p*H 5.6 and 8.1 with definite peaks at *p*H 5.6 and 7.4. The existence of marked *p*H sensitivity and a limited optimal range is in agreement with results on the uptake of folic acid (Wood and Hitchings, 1959), thiamine (Ziro, 1955) and amino acids (Britten and McClure, 1962; Holden and Holman, 1959), and is generally considered indicative of reactions involving enzyme participation.

Intracellular free biotin concentrations. An important criterion for establishing the "active" nature of a transport process is the presence of large concentration differences (gradients) between the internal and external cellular environments, with a movement of molecules into the region of high concentration. Such an uphill movement necessarily involves the selective retention of molecules on one side of a semipermeable barrier. Since osmotic work is involved in concentrative processes, the participation of active cellular metabolism is required to estab-

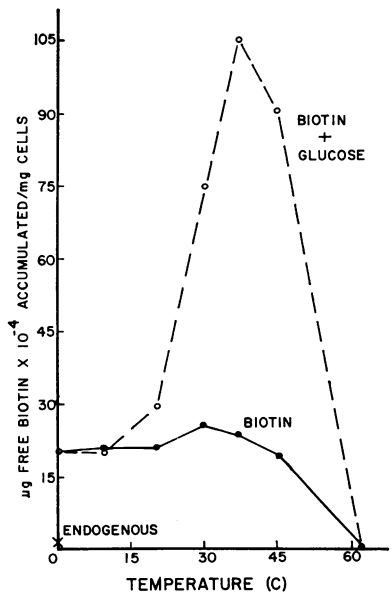

FIG. 1. *Effect of temperature on free biotin accumulation. Conditions: pH, 7.5; time, 15 min; biotin concentration, 200 $\times 10^{-4}$ μg/ml. Endogenous = free biotin present in cells prior to mixing with reaction menstruum.*

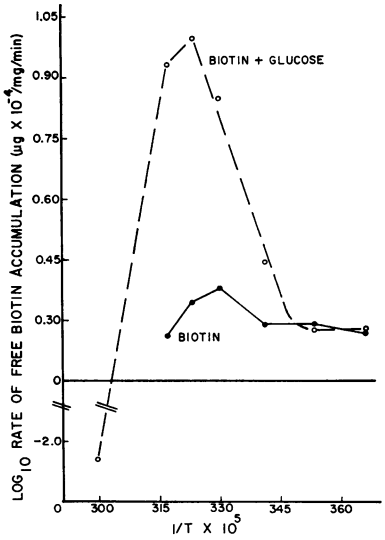

FIG. 2. *Arrhenius plot of temperature data.*

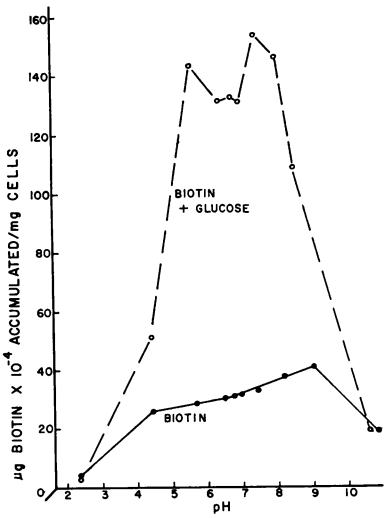

FIG. 3. *Effect of pH on free biotin accumulation. Temperature, 37 C; time, 30 min; biotin concentration, 200 × 10⁻⁴ µg/ml.*

lish and maintain intracellular concentrations in excess of the external environment.

Free biotin accumulation by the bacterial cells resulted in large intracellular concentration gradients in the presence or absence of glucose (Table 6, columns 5 and 6). In the absence of carbohydrate, the internal free biotin concentration remained relatively constant. However, in the presence of glucose the intracellular vitamin concentration rose markedly with both increasing time and external biotin concentration, reaching a maximal value of 153,000 × 10^{-4} µg of biotin per ml of intracellular free space (IFS). This value was nearly sixfold higher than the maximal level attained without glucose (25,800 × 10^{-4} µg/ml of IFS). Since, at the outset of the experiment, the maximal external biotin concentration was 1,107 × 10^{-4} µg/ml and the minimal intracellular level was 1,000 × 10^{-4} µg/ml, vitamin accumulation at all extracellular levels proceeded against definite concentration gradients. When glucose was present in the reaction mixture, the intracellular free biotin level rapidly increased until the external vitamin concentration was exceeded by nearly 4,000-fold (column 8). Three points are especially pertinent: (i) vitamin accumulation took place against definite concentration gradients, (ii) intracellular concentrations many times greater than the extracellular concentrations were established, and (iii) a source of energy was required for maximal free biotin accumulation. Thus, the data presented in Table 6 show that the glucose-stimulated free biotin-accumulating system satisfies three of the most basic requirements for definition as an active transport process, and strongly indicate that in the presence of glucose cells of *L. plantarum* accumulate free biotin by a mechanism involving active transport.

Vitamin accumulation in the absence of glucose proceeded against an apparent concentration gradient (Table 6, column 5) resulting in positive intracellular-extracellular (I–E) ratios. The mere presence of a positive I–E ratio does not necessarily indicate the involvement of active transport. Such gradients could be established under the influence of a Donnan type equilibrium. However, in such a case, the final equilibrium ratios should, if anything, increase with increasing external concentrations (*see* Gortner, Gortner, and Gortner, 1950), and the ratios established under the influence of simple diffusion should remain constant. The data in column 7 show that the distribution ratios decreased with increasing external concentrations, under conditions both conducive (37 C) and inhibitory (0 C) to active cellular metabolism. Thus, it does not appear that free biotin accumulation in the absence of glucose occurs by means of active transport or simple diffusion, nor does it appear that a Donnan type equilibrium was responsible for the observed intracellular free biotin levels.

Effect of homobiotin on the accumulation of free biotin. Most transport systems have been shown to possess a high degree of substrate specificity. The demonstration that a structurally related compound will interfere with a transfer reaction is considered indicative of combination with some specific site, probably at the cell membrane, since

TABLE 6. *Intracellular-extracellular (I-E) distribution of free biotin*[a]

Reaction time	Extracellular concn ($\mu g \times 10^{-4}$ ml)			Intracellular concn ($\mu g \times 10^{-4}$/ml of IFS[b])		Distribution ratio (I-E)	
	Initial	At time indicated		Biotin	Biotin + glucose	Biotin	Biotin + glucose
		Biotin	Biotin + glucose				
min							
0[c]	36	34	35	18,500[d]	18,500	544	530
	1,107	1,110	1,052	19,500	16,700	17.5	15.8
12	36	36	13	13,100	43,900	364	3,310
	1,107	1,133	990	14,500	104,000	12.8	105
24	36	34	12	20,400	47,000	600	3,920
	1,107	1,152	1,008	25,800	153,000	21.6	152

[a] Temperature, 37 C; *p*H, 7.5.
[b] Intracellular free space. See Materials and Methods.
[c] Cells and menstruum at 0 to 4 C immediately centrifuged in the cold (0 to 2 C).
[d] Initial intracellular free biotin concentration was approximately $1,000 \times 10^{-4}$ μg/ml of IFS.

TABLE 7. *Effect of homobiotin on free biotin accumulation**

Conditions	Homobiotin-biotin ratio					
	0	100	1,000	10,000	25,000	100,000
0 C						
Biotin	4.3†	3.6 (16)‡		1.2 (72)	0.7 (84)	0.5 (88)
Biotin + glucose	5.5	8.9	2.6 (53)	1.7 (69)		0.7 (88)
37 C						
Biotin	5.2	4.7 (10)	2.4 (54)	1.7 (67)	1.4 (73)	
Biotin + glucose	19.0	21.3	8.8 (54)	2.4 (87)	1.7 (91)	0 (100)

* Temperature, 37 C; *p*H, 7.5; time, 15 min; biotin concentration 50×10^{-4} μg/ml. We are indebted to Hofmann-La Roche for a gift of homobiotin.
† Micrograms of biotin $\times 10^{-4}$/mg of cells.
‡ Numbers in parentheses indicate per cent inhibition when compared with control without homobiotin.

transport by diffusion should be independent of the presence of any other related or unrelated compound as long as cellular permeability is not affected. The results obtained from studies of free biotin accumulation with the use of homobiotin (side chain possesses five CH_2 groups instead of four as in the biotin molecule) are summarized in Table 7. It can be seen that free biotin accumulation was inhibited progressively with increasing homobiotin concentrations. At a homobiotin–biotin (HB–B) ratio of 1,000:1, vitamin uptake was inhibited by 54% in the presence or absence of glucose; a ratio of 25,000:1 produced nearly complete inhibition of vitamin accumulation. Varying the reaction temperature from 0 to 37 C did not influence the inhibition patterns significantly. Thus, this homologue effectively inhibited free biotin uptake, suggesting that free biotin transport into cells of *L. plantarum*, in the presence and absence of glucose, occurs by means of a specific transport system(s). The results also show that the biotin transport system(s) has a much lower affinity for homobiotin than for the vitamin molecule itself.

It was observed further that the HB–B ratio which caused 54% inhibition of vitamin uptake had no effect on the growth of the organism (Table 8), and that considerably higher HB–B ratios (5,000:1 to 10,000:1) were required to cause the same degree of growth inhibition. These results suggest that the rate-limiting reaction for bacterial growth may well be the incorporation of the vitamin into cellular enzymes rather than penetration into the cell. The lower HB–B ratios required for inhibition of biotin uptake, as opposed to those required to similarly affect

TABLE 8. *Effect of homobiotin on growth of Lactobacillus plantarum**

Homobiotin-biotin ratio	Biotin activity ($\mu g \times 10^{-4}$)/tube	Per cent inhibition	Biotin activity ($\mu g \times 10^{-4}$)/tube	Per cent inhibition
0	5.05		10.7	
1,000	5.05	0	10.7	0
5,000	4.1	18.4	6.0	47
10,000	1.95	61.4	2.4	83
20,000	0.15	97.3	0.1	99
50,000	0	100	0	100

* Cells were grown as for a microbiological assay (see Materials and Methods). Various amounts of homobiotin were added to two known levels of biotin (5.05 and 10.7 $\times 10^{-4}$ μg per tube).

growth, also suggest that the intracellular site of biotin action is even more specific for the biotin molecule than the transport system or, perhaps, that only small quantities of biotin are required for cellular functions and that such functions are affected only when the intracellular level of biotin has been reduced sufficiently due to inhibition of the transport system.

DISCUSSION

An overwhelming majority of studies on "free" accumulated substrates have failed to detect any difference between the internal and external phases, indicating that the molecules do indeed exist internally in an unchanged form. However, it was suggested by Britten and McClure (1962) that in *E. coli* the "free" amino acid pool does not contain truly free amino acids, but rather that the molecules are bound in a highly labile manner by the protoplasmic constituents in which they are bathed. These labile bonds supposedly can be broken merely by changing the degree of cytoplasmic hydration. If accumulated substances indeed are bound, they should be osmotically inactive. Sistrom (1958) showed that thiogalactosides accumulated by osmotically sensitive spheres (protoplastlike forms) of *E. coli* were present within these bodies in an osmotically active form. Cirillo (1962) and Wachsman and Storch (1960) showed, respectively, that protoplasts of *S. cerevisiae* and *Bacillus megaterium* accumulated osmotically active sorbose (Cirillo, 1962), glucose, and a variety of organic acids (Wachsman and Storch, 1960), as evidenced by swelling and lysis. Cohen and Monod (1957) calculated that stoichiometric binding of the galactosides accumulated by *E. coli* would require the entire protein content of the bacterial cells for this function alone. They concluded that the sugars must exist free in the intracellular fluids. A similar conclusion was reached by Christensen (1960) for amino acid accumulation in animal cells. In general, then, the available evidence supports the view that many substances are accumulated within living cells in a free form identical to the extracellular species from which they came.

The microbiological assay for biotin with the use of *L. plantarum* is a specific and sensitive test for structural integrity. This organism responds to only three known forms of the vitamin. Of these, *d*-biotin alone was shown to be present in "free" biotin-containing bacterial extracts (Waller, Ph.D. Thesis, University of Minnesota, 1964), and essentially all the *d*-biotin added to the reaction mixture was detectable as *d*-biotin. Furthermore, since "free" biotin could be removed from the cells by extremely mild treatments (Table 1), it is probable that this easily extractable cellular biotin was, in fact, free *d*-biotin.

A comparison of the effects of *p*H on free biotin accumulation with the acidimetric titration curve for *d*-biotin constructed by Melville (1944) shows that *p*H 7.4 (optimum for free biotin uptake) coincides with the equivalence point of the titration curve, indicating that at this *p*H the biotin molecule is entirely in a neutral form. This correlation is consistent with the results of Overgaard-Hansen and Lassen (1959), Christensen and Jones (1961), and Harvey and Collins (1962), who showed, respectively, that only the neutral forms of uric acid, hypoxanthine, and citric acid participated in mediated transport into human erythrocytes and cells of *Streptococcus diacetilactis* and *Leuconostoc citrovorum*. The reduction in free biotin accumulation observed between *p*H 6.0 and 7.0 may represent some effect on the accumulating mechanism, perhaps on the energy-yielding system or on a mechanism for transferring the biotin molecules across the cell membrane, or on the cell membrane itself. Since *p*H changes did not markedly affect glucose-independent vitamin accumulation in the *p*H range from 5.6 to 7.4 (Fig. 3), it can be speculated that the results noted in the presence of the sugar indeed were related to some aspect of the accumulation mechanism.

In general, most substrate accumulations exhibit some degree of dependence upon metabolic energy. Some systems seem to be completely dependent upon an energy source (Gale, 1953; Sanders and Leach, 1964; Wood and Hitchings, 1959; Ziro, 1955), whereas others appear to be stimulated by exogenous energy (Britten and McClure, 1962; Harvey and Collins, 1962; Wood and Hitchings, 1959). In contrast, Burger, Hegmova, and Kleinzeller (1959) and Cirillo (1961) showed that sugar transport in yeast cells is not de-

pendent upon metabolic energy. Also, Gale (1953) and Jacquez (1962) showed, respectively, that the uptake of lysine by certain gram-positive bacteria and of uracil by animal cells was unaffected by the presence of an energy source or metabolic inhibitors.

Free biotin accumulation by cells of *L. plantarum* appeared to exhibit characteristics of both energy-dependent and -independent systems, since a small but significant amount of free biotin was accumulated in the absence of an exogenous energy source, whereas glucose metabolism caused a sevenfold increase in the intracellular free biotin levels attained within the bacterial cells (Table 4). These findings are similar to those of Wood and Hitchings (1959) for the uptake of folic acid and citrovorum factor by *L. casei*. Even though the magnitude of change in their experiments was not so great as that reported here, the existence of an uptake system in *L. casei* exhibiting both independence from and dependence upon metabolic activity was apparent.

The existence of large concentrations of internal substrates has been observed often (Britten and McClure, 1962; Cohen and Monod, 1957; Holden and Holman, 1959; Horecker et al., 1960; Piez and Eagle, 1958; Sanders and Leach, 1964). For example, Horecker et al. (1960) showed that the amount of galactose accumulated by cells of *E. coli* could approach 15% of the cellular dry weight, resulting in intracellular concentrations more than 10,000 times greater than the external concentrations. Britten and McClure (1962), studying amino acid accumulation and pool formation in *E. coli*, observed valine pools exceeding the external concentration by a factor of 18,000. Holden and Holman (1959) reported I–E ratios of 390 for glutamic acid accumulation in *L. plantarum*. Also, a number of reports indicate that some cells do not establish intracellular gradients (I–E) exceeding one. Most notable in this respect are the results of Cirillo (1961) and Burger et al. (1959), who studied sugar transport in yeast cells and concluded that nonactive, carrier transport systems were involved in the transport of monosaccharides into yeast cells. Systems involving the participation of both active and diffusion components have been reported (for example, Heinz and Walsh, 1958; Helmreich and Kipnis, 1962).

It appears that free biotin accumulation by cells of *L. plantarum* proceeded by two distinct mechanisms. One course of uptake exhibited many characteristics of an enzyme-mediated reaction (temperature and *p*H dependence, sensitivity to metabolic inhibitors, energy dependence) and caused the intracellular accumulation of free biotin to levels nearly 4,000-fold greater than the external concentrations. In contrast, the second mechanism possessed many characteristics suggestive of diffusion (independence from temperature, *p*H, and metabolic inhibitors, and an energy requirement). If the energy-independent vitamin transfer proceeded by means of diffusion, definite intracellular gradients should not have been observed, and homobiotin should have had little or no effect on the process. However, positive I–E values were established in the absence of glucose, and vitamin uptake in the presence and absence of glucose was inhibited to the same degree by the homologue. The simplest explanation for the positive I–E value in the absence of glucose would be a highly labile adsorption to protoplasmic constituents. However, in this case a great difference in the effect of homobiotin on uptake in the presence and absence of glucose would be expected, since the intracellular HB–B ratio per binding site would be much greater in the absence of the sugar than in its presence. Thus, the data seem to suggest the existence of some step, common to both systems, requiring the combination of biotin with a component of finite capacity which would mediate the transfer of biotin molecules from a region of lower concentration to one of higher concentration, even in the absence of an energy source. The mechanism by which positive intracellular concentrations are established in the absence of glucose is not known, but a change in the affinity of an assumed carrier during transversal of the osmotic barrier, as suggested by Patlak (1957), could offer an explanation for the observed results.

Additional evidence for carrier-mediated transport of free biotin into cells of *L. plantarum* is presented in the following paper.

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