FACTORS AFFECTING THE ACCUMULATION OF BIOTIN BY
LACTOBACILLUS ARABINOSUS

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The problem of substrate accumulation in microbial cells has been the subject of considerable investigation in recent years. However, comparatively little is known of the conditions influencing the accumulation of vitamins into microbial cells. One might cite as examples the investigations of Oginsky (1952) with vitamin B12, of Lichstein and Ferguson (1958) with biotin, and of Wood and Hitchings (1959) with folic acid.

The present studies are an extension of those of Lichstein and Ferguson (1958) who reported that under suitable conditions cells of Lactobacillus arabinosus accumulated large amounts of biotin.

MATERIALS AND METHODS

Organism and medium. L. arabinosus strain 17-5 was employed in all studies and was maintained by serial transfer at 10- to 14-day intervals on APT Agar (Case Laboratories, Chicago, Illinois) incubated at 30 C. The experimental medium was that designed by Wright and Skeggs (1944) for the microbiological assay of biotin except that cysteine was substituted for cystine and folic acid was included (50 μg/100 ml). The bacterial cells were grown in this medium at 30 C for the desired period of time, harvested by centrifugation, washed three times with saline, and resuspended in distilled water to the desired turbidity as measured in a Klett-Summerson photoelectric colorimeter fitted with a blue filter (400 to 450 mμ).

Procedure. The washed suspension of bacterial cells was added in the desired amounts to 16-ml cellulose nitrate tubes. To these were added 2.5 ml of 0.02 M phosphate buffer together with biotin and other reagents as required for the particular experiment. The total volume was brought to 10 ml with distilled water. After incubation under the desired conditions, the reaction was stopped by immersing the tubes in boiling water for 3 to 5 min. The bacterial cells were recovered by centrifugation, washed three times with saline, and subjected to acid treatment to release bound biotin. To accomplish this, the bacterial cells were resuspended in 0.5 ml of 6 N H2SO4 in pyrex tubes, the tubes capped with rubber and then autoclaved for 1 hr at 121 C. The hydrolyzates were then treated with 0.5 ml of 6 N NaOH, brought to a volume of 15 ml with distilled water, adjusted to pH 6.8, and filtered through Whatman no. 2 paper to remove large particulate matter, the presence of which might produce erratic results in the subsequent assay procedure.

Microbiological assay for biotin. These assays were carried out in pyrex tubes (16 by 150 mm) covered with aluminum caps. To each tube 5 ml of suitable concentrations of biotin or of unknown sample and 5 ml of double-strength Wright-Skeggs medium were added. The NaCl was omitted from the medium to reduce the possibility of inhibition of bacterial growth due to the high salt concentration of the samples employed for assay. The tubes were sterilized by autoclaving at 121 C for 15 min, cooled, and each tube inoculated with 1 drop of a diluted, washed cell suspension of L. arabinosus. Incubation was carried out at 30 C for 40 to 48 hr and growth measured by use of the colorimeter fitted with a red filter (640 to 700 mμ) using uninoculated media as a blank.

RESULTS

Preliminary inspections conducted with both biotin-deficient and biotin-sufficient cells showed that the latter accumulated considerably more biotin than did the deficient cells. Hence, most of the work herein described was performed with
biotin-deficient cells (5 biotin 6.8; grown with 5 X 10^{-2} \mu g biotin/10 ml of media; age of cells 48 hr; incubation time 60 min).

bacterial cells grown sufficient with respect to biotin (5 X 10^{-2} \mu g of biotin/10 ml of medium).

Inasmuch as the earlier investigations with biotin-deficient cells (Lichstein and Ferguson, 1958) had revealed that the uptake of biotin was quite rapid, it was necessary first to evaluate this aspect with the biotin-sufficient cells employed in the present studies. It was found that the reaction proceeded rapidly with approximately 40 per cent of the accumulation occurring in the first 10 min and completion being reached in about 90 min. Thus, for example, in a typical experiment the following values for biotin accumulation per mg of dry cells were obtained: 12 \times 10^{-4} \mu g in 10 min, 22 \times 10^{-4} \mu g at 30 min, and 29 \times 10^{-4} \mu g after 90 min incubation.

The specific factors studied first were the influence of temperature and pH and the effect of cell and substrate concentrations. The data presented in figure 1 demonstrate the effect of temperature on the uptake process. A sharp optimum was observed at 37 C with activity falling off rapidly on either side of this point. Virtually no reaction took place at 4 C or at 45 C. The Q_{10} between 30 C and 37 C was approximately 1.8. The nature of the curve and the calculated Q_{10} value are characteristic but not necessarily indicative of enzyme-mediated reactions.

The optimal pH for the accumulation of biotin (figure 2) was approximately 6.8, with activity falling off rapidly on either side of the optimum. Thus, the effect of pH is also consistent with the participation of an enzyme system in the control of biotin uptake by these bacterial cells. These results are similar to those reported for glutamic acid permeability by Gale (1947) in Streptococcus faecalis and by Holden and Holman (1959) employing L. arabinosus. However, pH can affect substrate accumulation by virtue of alterations.

**Figure 1.** Effect of temperature on the uptake of biotin by cells of Lactobacillus arabinosus (pH 6.8; biotin concentration 5 \times 10^{-2} \mu g/ml; cells grown with 5 \times 10^{-2} \mu g biotin/10 ml of media; age of cells 48 hr; incubation time 60 min).

**Figure 2.** Effect of pH on the uptake of biotin by cells of Lactobacillus arabinosus (temperature 37 C; other conditions as in figure 1).

**Figure 3.** Effect of concentration of bacterial cells on rate of biotin uptake (temperature 37 C; other conditions as in figure 1).
in the ionizable groups, thus changing the configuration of the molecules.

The results of rate studies are also in keeping with enzyme-controlled systems. In figure 3, the rate of biotin accumulation is plotted against the concentration of bacterial cells. It is clear that the rate of vitamin uptake was directly proportional to the concentration of cells present, except at high cellular concentrations where the rate of accumulation leveled off. These results are similar to those obtained for the uptake of amino acids (Gale, 1947; Holden and Holman, 1959), sugars (Burger, Hegmova, and Kleinzeiler, 1959), other carbohydrates (Cohen and Monod, 1957), and folic acid (Wood and Hitchings, 1959). A straight line relationship was obtained when the reaction velocity was plotted against the substrate concentration, except that at high extracellular substrate levels the curve fell off abruptly (figure 4). Once again, these results are in keeping with those obtained for glutamic acid (Gale, 1947; Holden and Holman, 1959) and β-d-thiogalactoside (Cohen and Monod, 1957) accumulation, for which enzyme-mediated transport processes have been suggested.

Consideration was given next to the influence of certain physiological factors on the accumulation of biotin by cells of L. arabinosus. Culture age was studied since this is one of the most important factors affecting the activity of microbial cells. These results are summarized in figure 5. It is manifest that with advancing age the bacterial cells exhibited a rapid decrease in the level of endogenous biotin to a rather constant minimum at about 48 hr. The ability of the bacterial cells to accumulate biotin increased rapidly from 16 to 48 hr after which time there was a precipitous decline.

Finally, the influence of the level of biotin in the growth medium and the effect of glucose were investigated. It is apparent (table 1) that increasing levels of biotin in the growth medium up to an extracellular level of $10^{-1}$ μg/10 ml caused an increase in the intracellular concentration of vitamin. Thus, although maximal growth of the organism was supported by a biotin

![Figure 4](http://jb.asm.org/)  
Figure 4. Rate of biotin uptake by cells of *Lactobacillus arabinosus* at various substrate concentrations (temperature 37 C; other conditions as in figure 1).

![Figure 5](http://jb.asm.org/)  
Figure 5. Effect of age of bacterial cells on the level of biotin and the accumulation of biotin (temperature 37 C; other conditions as in figure 1).

**TABLE 1**

<table>
<thead>
<tr>
<th>Biotin/10 ml Growth Medium</th>
<th>μg</th>
<th>μg biotin × 10⁴/mg dry cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁻⁴</td>
<td>10⁻³</td>
</tr>
<tr>
<td>Endogenous...</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>+ Biotin...</td>
<td>1.3</td>
<td>12.6</td>
</tr>
<tr>
<td>+ Biotin + glucose...</td>
<td>48.1</td>
<td>52.0</td>
</tr>
</tbody>
</table>

Glucose concentration 1 per cent; temperature 37 C; other conditions as for figure 1.
concentration of 3.5 to 5.0 $\times 10^{-3}$ $\mu$g/10 ml of medium, the bacterial cells accumulated considerably more biotin when cultured in the presence of amounts of vitamin in excess of this quantity. Biotin accumulation by suboptimally grown cells ($10^{-1}$ $\mu$g/10 ml) proceeded quite readily, reaching a level approximating that found in the biotin-sufficient cells. However, the most deficient cells employed ($10^{-4}$ $\mu$g/10 ml) exhibited little ability to accumulate biotin unless glucose was present, in which case the final intracellular concentration of biotin reached far exceeded the level attained by the optimally grown cells. An explanation for this high level is not yet available, but several factors might be considered. For example, the deficient cells might possess larger numbers of sites capable of binding biotin. Also possible is a difference in the levels of enzymes concerned with the breakdown of bound biotin (Toma and Peterson, 1954) or the degradation of free biotin (Baxter and Quastel, 1953). The marked stimulatory effect of glucose on the uptake process by biotin deficient cells suggests that energy is required for this process. The absence of a glucose effect in the biotin sufficient bacterial cells may reflect adequate endogenous sources of energy. Although not given in the table, it is pertinent to note that glucose stimulation of biotin accumulation decreased with increasing age of the cells. Maximal stimulation was recorded with 24-hr cells whereas cells which were 48 hr or older exhibited essentially no stimulation by the presence of this carbohydrate. This may also be related to differences in endogenous stores of energy.

**DISCUSSION**

The results herein presented provide the basis for a clearer understanding of the factors involved and the mechanism of biotin accumulation by cells of *L. arabinosus*. Data obtained from studies of the uptake process itself suggest that the accumulation is dependent on enzyme mediated reactions. Moreover, the process is stimulated under certain conditions by an exogenous source of energy, and in a previous paper (Lichtenstein and Ferguson, 1958) it was shown that the stimulatory effect of glucose was inhibited by iodoacetate. Such results suggest the participation of an active process. However, the assignment of true active transport to an accumulation process requires the establishment of a concentration gradient, as well as proof that the intracellular substrate exists in the same form as that offered extracellularly. Such proof is obviously difficult to obtain in the present case since biotin was converted to and measured in a bound form.

It appears reasonable to conclude that an active process is involved in biotin accumulation by cells of *L. arabinosus*. The site of the enzymatic participation is the unresolved question. It may indeed be in the permeability process itself, or it may be concerned with the binding of the vitamin to active sites within the bacterial cell. Work is in progress in an attempt to resolve this question.

**SUMMARY**

The results of studies concerned with conditions affecting the uptake of biotin by cells of *Lactobacillus arabinosus* are presented. Rate studies, including the effects of time, substrate and cell concentration yielded results consistent with those characteristic of enzyme mediated reactions. Sharp temperature and pH optima were exhibited at 37 C and pH 6.8, respectively.

Glucose stimulation of biotin accumulation appeared to depend both on the degree of vitamin deficiency and the age of the bacterial cells. The endogenous level of intracellular bound biotin increased as the concentration of biotin in the growth medium was increased. Moreover, the endogenous concentration of bound biotin decreased with increasing age of the bacterial cells.

Unexpected were the very high levels of biotin observed in initially biotin-deficient cells after incubation with the vitamin under certain conditions. Possible explanations for this finding are offered.

**REFERENCES**


