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STUDIES ON THE METABOLISM OF d-BIOTIN BY CELLS OF *LACTOBACILLUS ARABINOSUS*

A dissertation submitted to

The Graduate School

of the University of Cincinnati

in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

1964

by

Jerome Birnbaum

B. S. Brooklyn College, 1961

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INTRODUCTION

The development of knowledge concerning the physiology and metabolism of the B vitamins has lagged behind the wealth of information compiled for amino acids and carbohydrates. However, the biosynthesis and metabolism of the B vitamins have become the subject of much investigation in recent years. This is due largely to the advent of newer, more precise techniques for the detection and quantitation of these vitamins.

This laboratory has been engaged actively in such investigations with the B vitamin, biotin. Emphasis has been placed on studies of biotin permeation in *Lactobacillus arabinosus*, and on biotin biosynthesis in *Escherichia coli*. It is the purpose of this thesis to describe in detail experiments on the fate of the d-biotin molecule once it has permeated cells of *L. arabinosus* (17-5). Reported here, is a system having enzymic properties which converts d-biotin to other forms (vitamers) of the vitamin. These vitamers are not utilizable for growth by *L. arabinosus* but can be used by other biotin requiring organisms such as *Saccharomyces cerevisiae* (Fleischmann 139). Several parameters of the d-biotin converting system and its physiological control are described.

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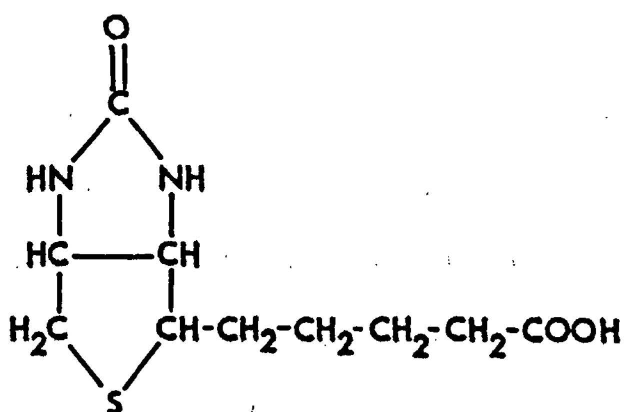
HISTORICAL REVIEW

The stimulating effect of small amounts of extracts of natural materials on the growth of yeast was described first in 1901 by Wildiers (1), who gave the name "bios" to this growth promoting substance. Bios was shown subsequently to be a group of factors, one of which was isolated from dried Chinese chick egg yolk in 1936 by Kogl and Tonis (2), who characterized the compound as the methyl ester, crystallized it, and named it "biotin". du Vigneaud and co-workers isolated biotin from liver (3, 4) and completed the proof of structure. The biotin molecule is indicated by the following formula:



Biotin

The identification of biotin with the curative factor (vitamin H) for egg white injury in animals helped to merge the originally independent studies dealing with a microbiological yeast factor (biotin) and a vitamin-like substance for animals (vitamin H).

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A. Functions of Biotin

It appeared probable to early workers that biotin, by analogy with other members of the B vitamin group, acted in biochemical systems as part of the coenzyme moiety of the functional holoenzyme. The name "biotoprotein" was suggested by some investigators to indicate the enzymatically active complex of biotin and protein (5).

A number of enzymatic reactions have been discovered and analyzed in which biotin appears to participate in a direct or indirect manner. The most conclusive results were obtained by the study of microbiological systems or tissues obtained from biotin deficient animals. Later workers employed also isolated enzyme systems. The evidence so far accumulated indicates that biotin functions in several enzymatic processes in which the common denominator is not easily determined, and which involve several, perhaps independent, metabolic functions.

The first metabolic interrelation noted was between biotin and aspartic acid, and was inferred from the sparing action of the amino acid on the biotin requirement of yeast and a variety of bacteria (6, 7, 8). Studies of the biotin-aspartate interrelationship furnished sufficient proof for the assumption that biotin, or rather a biotin coenzyme is concerned with the condensation of CO_2 and pyruvate to form oxalacetate, or in the decarboxylation of oxalacetate (9, 10). Aspartate could be formed from oxalacetate via transamination.

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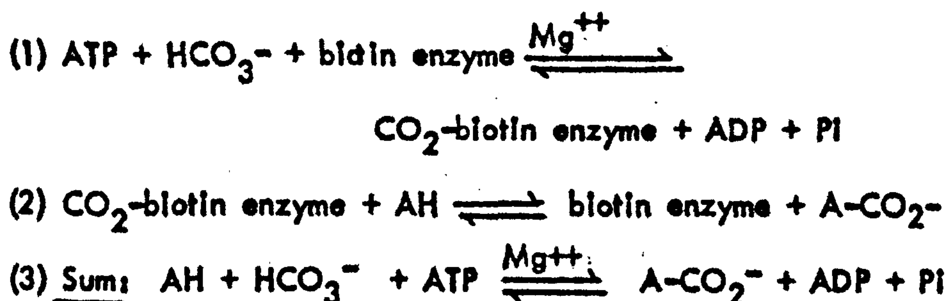
Shive and Rogers (11) have suggested that the decarboxylation of oxalosuccinic acid to alpha-ketoglutaric acid and CO_2 is also a biotin dependent decarboxylation reaction.

More recently, biotin requiring reactions have been divided into two groups (12). The first group including beta-methylcrotonyl (12), the acetyl (13), the propionyl (14), and pyruvate carboxylase (15, 16) reactions, comprises those reactions in which CO_2 is fixed by an acceptor (AH) at the expense of adenosine triphosphate (ATP):

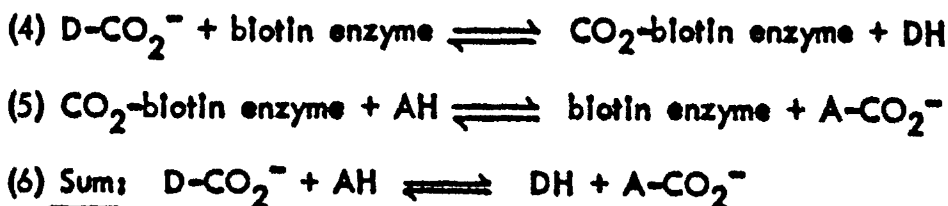


$$(1)\ ATP + HCO_3^- + \text{biotin enzyme} \xrightleftharpoons{Mg^{++}} CO_2\text{-biotin enzyme} + ADP + Pi$$

$$(2)\ CO_2\text{-biotin enzyme} + AH \rightleftharpoons \text{biotin enzyme} + A\text{-}CO_2^-$$

$$(3)\ \underline{\text{Sum:}}\ AH + HCO_3^- + ATP \xrightleftharpoons{Mg^{++}} A\text{-}CO_2^- + ADP + Pi$$

To the second group belong the transcarboxylation reactions. In contrast to the first group of carboxylase reactions, carboxylation in the second group is achieved without expenditure of ATP energy and is brought about by the simultaneous decarboxylation of a second compound which acts as the CO_2 donor ($D\text{-}CO_2^-$). This type of reaction is depicted by the following general equation:



$$(4)\ D\text{-}CO_2^- + \text{biotin enzyme} \rightleftharpoons CO_2\text{-biotin enzyme} + DH$$

$$(5)\ CO_2\text{-biotin enzyme} + AH \rightleftharpoons \text{biotin enzyme} + A\text{-}CO_2^-$$

$$(6)\ \underline{\text{Sum:}}\ D\text{-}CO_2^- + AH \rightleftharpoons DH + A\text{-}CO_2^-$$

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The first reaction of this type was discovered by Swick and Wood (17) in investigations on propionic acid fermentation by *Propionibacterium shermanii*. These investigators found that extracts of this organism catalyze the reversible transcarboxylation between methylmalonyl CoA and pyruvate, giving rise to propionyl CoA and oxalacetate, and that the reaction is inhibited by avidin, a protein from egg white that binds biotin very strongly.

Lynen has suggested (12) that all enzymatic reactions hitherto known, in which biotin has been demonstrated to be the functional group, are carbon dioxide transferring processes. He further suggests that the reversible uptake and transfer of carbon dioxide is the exclusive function of this vitamin in metabolism, and that other effects observed are probably of a secondary and indirect nature.

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B. The Biotin Vitamers

The rapidly developing recognition of the multiplicity of chemical compounds that possessed a specified vitamin activity created a scientific need for a term that would indicate a biological relationship between all such compounds, naturally occurring or synthetic. The term "vitamer" was proposed (18, 19) to refer to anyone of a number or group of chemically related nutrient compounds that act to overcome a given vitamin deficiency in one or another organism, plant or animal. In view of this definition, the term "biotin vitamers" refers to any compound that replaces biotin in microbial and/or animal systems.

1. The structural and functional relationship of biotin to its vitamers.

The relationship of chemical structure of biotin and its vitamers need not be close. As a matter of fact, many vitamers which have not been elucidated structurally, will replace the biotin requirement in several microbial species (19). Recently, Eisenberg (20) demonstrated that an unknown biotin vitamer synthesized by cells of Phycomyces blakesleeanus was utilized in place of biotin by S. cerevisiae. This vitamer, of unknown chemical structure, was characterized using physico-chemical parameters, and found to have the properties of an amino acid. Also, it is avidin uncombinable. These results suggest that a vitamer need not be structurally similar to biotin in order to be biologically active.

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In contrast to the "amino acid" vitamer described by Eisenberg (20), most biotin vitamers are structurally similar to the parent vitamin. Desthiobiotin, for example, differs only in its lack of a sulfur atom. Using growth as a parameter, Burk and Winzler (19) investigated the biotin replacing activity of desthiobiotin. Their findings showed that desthiobiotin may replace biotin in the growth of S. cerevisiae but that it is inactive for lactobacilli. Lilly and Leonian (21) confirmed these findings by showing that desthiobiotin replaced biotin inthe growth of 25 yeasts, but would not replace biotin in cultures of Lactobacillus casei and L. arabinosus. Another vitamer which shares a close strucutral relationship with biotin is oxybiotin. Oxybiotin, also called O-Heterobiotin (22), differs from biotin in that an oxygen atom replaces the sulfur. Krueger and Peterson (23) showed that oxybiotin has the same biological activity as biotin in promoting the growth of Lactobacillus pentosus, strain 124-2.

Biotin vitamers are represented also by a variety of complexes of the biotin molecule. Biocytin, a complex of biotin and the amino acid lysine, can be utilized in place of biotin by L. casei and S. cerevisiae (24). A protein complex containing biotin, was isolated from hog liver and termed "Soluble Bound Biotin" (SBB) by Thoma and Peterson (25). SBB will act as a biotin vitamer in the growth of L. casei, but neither SBB or biocytin will replace biotin in the metabolism of L. arabinosus. These two complexes, when hydrolyzed with strong

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mineral acid, or subjected to enzymic degradation yield a product that will promote the growth of L. arabinosus in a biotin free medium. The degradative enzyme has the properties of a peptidase and has been termed "biotinidase" (25). Through enzymatic hydrolysis of liver protein with crystalline pepsin, Chang and Peterson (26) obtained soluble bound protein compounds. The purest of these polypeptides contained 50 atoms of nitrogen per mole of biotin, and replaced the vitamin in the growth of yeast. Further hydrolysis did not yield any increase in biotin activity, showing that the polypeptide - biotin complex was incorporated directly into the metabolic machinery of the cells.

Gyorgy and Rose (27) isolated a "saline soluble", high molecular weight, undialyzable, microbiologically active (for yeast) biotin complex from egg yold.

Apparently biotin vitamers display species specificity, in that a given vitamer will successfully replace biotin in one organism but not another. As shown by Burk and his associates (18) species specificity becomes important in the quantitation of biotin in natural materials since microbiological assay is the only available method for the determination of biotin. The ability of a given species, according to Burk et al. (18), to utilize a vitamer in place of the vitamin is a direct expression of the metabolic competence of the species to incorporate the vitamer per se, or to convert the vitamer to the active

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coenzyme form. The organism which cannot utilize the vitamer in place of biotin is either lacking the metabolic machinery to convert the vitamer to an active coenzyme form, or possesses a permeability barrier to the vitamer. It is possible also that the presence of "biotoprotein" (5), which is highly specific for biotin, will not permit the incorporation of slightly dissimilar compounds. One well substantiated possibility is that a vitamer of one species may behave as an "antibiotin" in the second species. Dittmer, Melville and du-Vigneaud (28) showed that desthiobiotin will replace biotin for S. cerevisiae but will inhibit the utilization of biotin by L. casei, thereby acting as an antibiotin. Subsequently, Lilly and Leonian (21) showed that desthiobiotin can be utilized by 25 different strains of yeast but competitively inhibits the utilization of biotin by L. casei.

In a majority of cases it appears that various vitamers are converted into the parent or prototype vitamin. For example, Burk and Winzler (19) uncovered several avidin - uncombinable vitamers in the urine of many mammalian species. These vitamers, miotin, tiotin and a diaminocarboxylic acid derivative of biotin, were shown to be converted during utilization by S. cerevisiae 139 into avidin-combinable vitamers indistinguishable from biotin. Likewise desthiobiotin, an avidin combinable vitamer, has been shown (28, 29) to be converted by this strain of yeast into a vitamer that is presumably

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biotin as measured by microbiological assay. While studying the biological properties of biotin sulfoxides in various microorganisms, Melville, Genghof, and Lee (30) showed that this vitamer was as effective as biotin in promoting the growth of _L. casei_ and _L. arabinosus_ in biotin free media. The use of chromatographic analysis provided evidence that the sulfoxides are converted in part to biotin during their utilization by these microorganisms. However, Krueger and Peterson (23) have shown that oxybiotin is not converted into biotin, but is incorporated into the protein of cells of _L. pentosus_ (124-2), and used as the coenzyme without undergoing further structural modification. Thus, oxybiotin although not a natural product may replace biotin in metabolic reactions.

2. _Metabolism of biotin and its vitamers._

The demonstration of biotin activity for several microorganisms of such analogs of biotin as oxybiotin (22, 31, 32, 33, 34) and desthiobiotin (21, 28, 29, 35, 36, 37) aroused interest in the manner by which these compounds function in cell metabolism. The role of desthiobiotin has been elucidated fairly well. Desthiobiotin was found to be as potent as biotin for a number of microorganisms, mostly yeasts and fungi (21, 28, 29, 35, 36, 37) and is converted into biotin by these organisms. Tatum (36) has shown that desthiobiotin is probably

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an intermediate in the biosynthesis of biotin. This was concluded by studies of a mutant strain of *Penicillium chrysogenum* (62078) which could synthesize desthiobiotin but could not convert it to biotin. The original strain synthesizes biotin and also converts desthiobiotin to biotin. Dittmer and du Vigneaud (38) showed that desthiobiotin acts as an inhibitor of both biotin and oxybiotin (34) for *L. casei*, but has no effect on the growth of *L. arabinosus* and *Rhizobium trifolii* (21) either alone or in the presence of biotin.

The role of oxybiotin in the metabolism of microorganisms was unsettled in the microbiological literature for a few years. Rubin *et al.* (34) reported data obtained by utilizing the differences in activity of oxybiotin and biotin for *S. cerevisiae*, which indicated that oxybiotin (O-Heterobiotin) was converted into biotin or some other compound of comparable activity for *S. cerevisiae* by the organism during growth. Hofmann and coworkers (31, 39) using the method of Rubin *et al.* (34), as well as three differential assay procedures developed by them, seemed to have solved the problem by showing conclusively that yeast assimilates oxybiotin as such, and does not convert it to biotin. Lichstein (40) studied the biotin requirements of several species of propionibacteria and found that oxybiotin may be substituted for biotin in the growth of these organisms. However, the growth curve in media containing oxybiotin in place of biotin exhibited a considerably increased lag period as compared to the culture con-

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taining biotin. It was suggested therefore, that the lag might be a reflection of the time necessary to convert oxybiotin to biotin. In view of these findings it is reasonable to assume that the question of whether oxybiotin is converted to biotin or is used by the growing organism directly may be a matter of species specificity. The assimilation of an analogue not found in nature, and its apparent use metabolically by the cell in place of the natural compound is a unique phenomenon.

Whether or not any part of a given vitamin is destroyed in metabolism has not been investigated extensively. Leonian and Lilly (29) were unable to recover all the added biotin and desthiobiotin from the cell-free medium or cells of several yeasts and molds that were provided a great excess of these compounds. In investigating the metabolism of oxybiotin by yeast, Axelrod *et al* (39) recovered more than 70 per cent of the oxybiotin from the yeast cells. Also, McIlwain and Hughes (41) obtained a recovery of from only 2 to 5 per cent of the supplied pantothenic acid from cultures of hemolytic streptococci that were given an excess of the vitamin.

A more extreme example with a somewhat different aspect is the microbic utilization of vitamins as the sole source of carbon. Metzger (42) reported the decomposition of panththenate, present as the only carbon source, by several *Pseudomonas* species. Likewise, Koser

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and Baird (43) reported the destruction of nicotinic acid, when this was present as the sole source of carbon, by *Pseudomonas fluorescens* and *Serratia marcescens*. Foster (44) described an organism capable of oxidizing riboflavin to lumichrome, and Mirick (45) isolated a soil organism capable of oxidizing p-aminobenzoic acid. Since vitamins have been shown to serve as sole carbon substrates, it was not unreasonable to find that an excess of an "essential" vitamin could also be metabolized. Krueger and Peterson (23) showed that when *L. pentosus* 124-2 was grown in the presence of excess biotin or oxybiotin some of the vitamin was either metabolized or inactivated in such a way that it was not detectable by the procedure used. Assays were run also using *S. cerevisiae* as the test organism and the results were found to be in close agreement with those of *L. pentosus* or *L. arabinosus*. This observation offered evidence that the biotin which could not be recovered was really metabolized and not merely slightly altered, since many biotin analogues are known to have activity for yeast.

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MATERIALS AND METHODS

Organisms and Stock Cultures

L. arabinosus (strain 17-5) was employed as the experimental organism as well as in the microbiological assay for biotin. It was maintained on APT agar (Case) as a stab culture. The medium contains the following ingredients:

	Per cent
Tryptone (Case)	1.25
Yeast extract (Case)	0.75
Sodium citrate	0.5
Dextrose	1.5
Tween 80	0.02
NaCl	0.5
KH_2PO_4	0.5
$MgSO_4$	0.08
$FeSO_4$	0.004
$MnCl_2$	0.014
Na_2CO_3	0.125
Agar (Case)	1.5

S. cerevisiae, Fleischmann strain 139 (Baker's yeast) was employed also in the microbiological assay for biotin. The yeast was

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maintained as a slant culture on ACG agar, which contains the following:

	Per cent
Casitone (Difco)	1.0
Yeast extract (Difco or BBL)	1.0
Dextrose (reagent grade)	0.5
K_2HPO_4	0.5
Agar (Difco)	1.5

The organisms were transferred every 14 to 21 days, incubated at 30 C for 18 to 24 hours and then refrigerated.

Experimental Media

a) Growth medium   The growth of *L. arabinosus* was accomplished in the medium of Wright and Skeggs (46) containing 1×10^0 μg of biotin per 10 ml and modified as follows: cysteine substituted for cystine, and folic acid added to a concentration of 0.5 mg/l to improve growth. Glucose was autoclaved separately to prevent carmalization. The ingredients of the complete modified medium used in these studies were:

Vitamin free casamino acids (Difco)	6 g
Sodium acetate · $3H_2O$	10 g
DL-Tryptophan	200 mg
L-Cysteine	145 mg
K_2HPO_4	0.5 g

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KH_2PO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	200 mg
$MnSO_4 \cdot 4H_2O$	10 mg
$FeSO_4 \cdot 7H_2O$	10 mg
Gaunine HCl	5 mg
Xanthine	5 mg
Uracil	5 mg
Adenine	1.7 mg
Riboflavin	1 mg
Thiamine, HCl	1 mg
Calcium pantothenate	1 mg
Para-amino benzoic acid	0.1 mg
Folic acid	0.5 mg
Nicotinic acid	1 mg
Pyridoxine HCl	2 mg
Glucose	20 g
H_2O	1000 ml

The medium was adjusted to pH 6.8 and autoclaved for 15 minutes at 121 C.

b) L. arabinosus biotin assay medium The medium employed for the microbiological assay of biotin using L. arabinosus was that of Wright and Skeggs, similar to the growth medium, but

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differing in the following manner: no biotin was added, the medium was prepared in double strength concentrations, and glucose was added before sterilization.

c) <u>L. arabinosus inoculum medium</u> For preparation of inoculum cells for growth studies, Wright-Skeggs medium containing 10^0 µg of biotin/10 ml was employed. The inoculum medium for the microbiological assay was modified by the addition of 5×10^{-3} µg in place of 10^0 µg of biotin/10 ml.

d) <u>S. cerevisiae biotin assay medium</u> The medium used in the microbiological assay for biotin by yeast was that of Hertz (47). The medium was devoid of added biotin and was prepared at double strength concentrations. The contents of the concentrated medium are as follows:

Sucrose (reagent grade)	34 g
$(NH_4)_2SO_4$	5 g
KH_2PO_4	3.4 g
DL-aspartate	340 mg
$CaCl_2$	450 mg
$MgSO_4 \cdot 7H_2O$	450 mg
$MnCl_2$	1.8 mg
$FeCl_2$	0.2 mg
H_3BO_3	1.8 mg
$ZnSO_4$	1.8 mg

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$CuSO_4 \cdot 5H_2O$	0.18 mg
KI	0.2 mg
Inositol	9 mg
Calcium pantothenate	0.5 mg
Pyridoxine HCl	1 mg
Thiamine HCl	1 mg
Purified vitamin free casamino acids	7 g
H_2O	1000 ml

Difco vitamin free casamino acids were purified by the following procedure: 14 g of casamino acids were dissolved in 100 ml of water, 2-3 grams of Norite A were added, and the suspension was adjusted to pH 3.5. The solution was shaken for 2 hours on a rotary shaker at room temperature, after which the suspension was filtered through No. 1 Whatman paper. Fifty ml of the filtrate was used in the preparation of one liter of double strength medium.

e) S. cerevisiae inoculum medium The medium used for the preparation of yeast inoculum cells was the same as that used for the stock culture, namely ACG broth.

Dry Weight Determinations

Free intracellular and bound biotin are expressed on the basis of mg dry weight of cells. A simple and rapid procedure was used for the estimation of dry weight of cells per unit volume. The determination

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was accomplished in the following manner: L. arabinosus was grown in a large volume of Wright-Skeggs medium until a maximum cell crop was attained. The cells were separated from the menstruum, washed twice in H_2O, and resuspended in a given volume of distilled water. The resulting suspension was diluted to several cell densities. Turbidity measurements were made of each dilution with a Klett-Summerson Photoelectric Colorimeter set at 420 mμ, and using distilled water as a blank. Aliquots of each dilution were taken for dry weight determinations after drying at 80-90 C for 24 hours. The results are expressed in two ways: 1) -by the weight of the dry cells in mg per Klett unit and; 2) -graphically, by plotting the turbidity in Klett units against the mg of dry cells per ml of suspension. Once this was accomplished, a dry weight determination could be made by reading the turbidity of a sample at 420 mμ and either multiplying by the number of mg of dry cells per Klett unit, or interpolating from the dry weight curve (Figure 1).

Growth Studies with L. arabinosus

Growth studies were performed with this organism so that correlations could be made between the physiological character of the cells and their biotin content.

To several Erlenmeyer flasks (125 ml volume) were added 50 ml of modified Wright-Skeggs medium containing 10^0 μg of

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Figure 1. Standard curve relating turbidity to dry weight of cells of *Lactobacillus arabinosus*.

(Conditions as given under materials and methods.)

Turbidity = Klett units at 420 mμ

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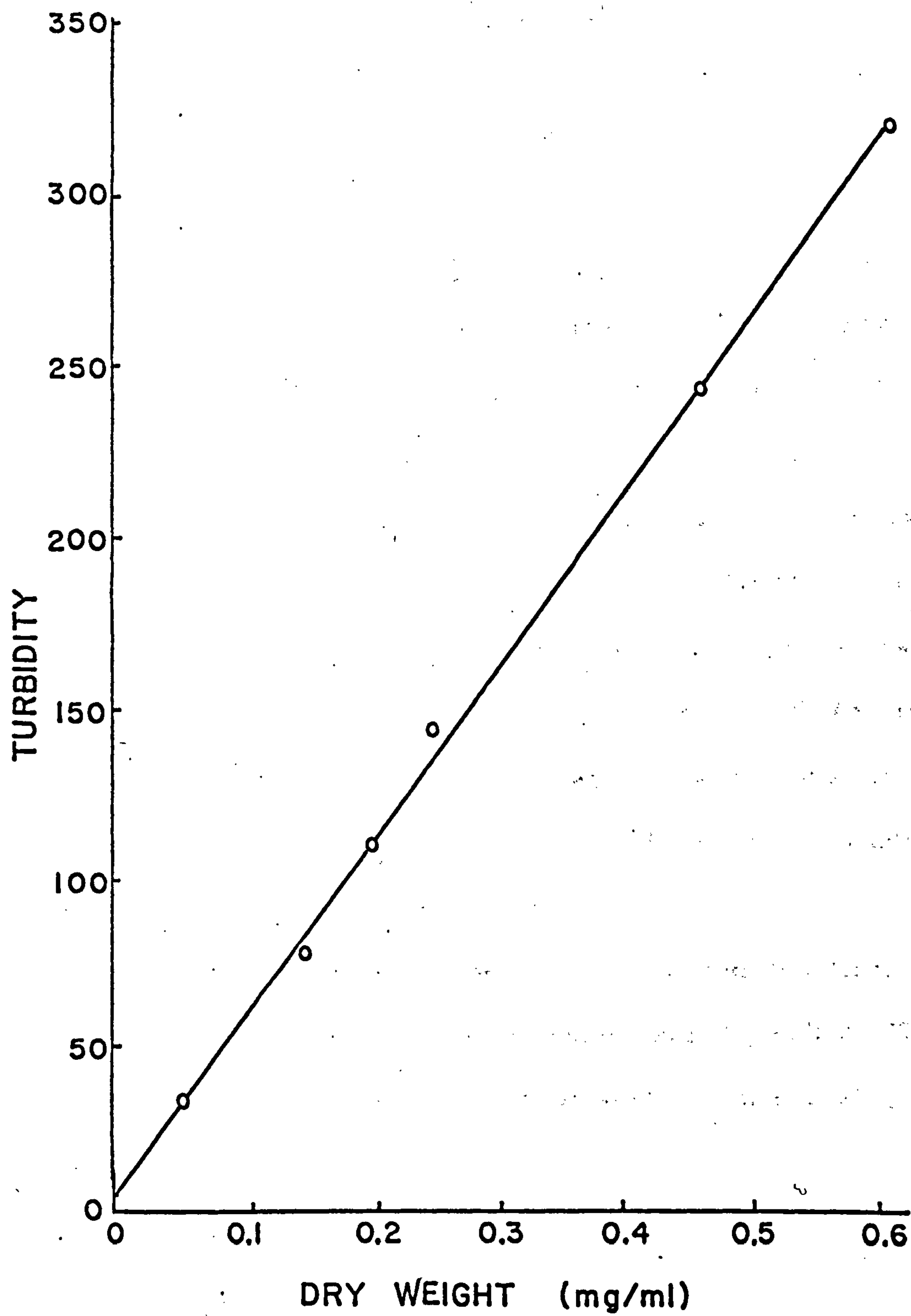

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biotin/10 ml, and inoculated with *L. arabinosus* equivalent to 0.2 mg dry cells. The flasks were incubated at 30 C for various lengths of time under stationary conditions. The amount of growth was evaluated as follows:

a) Turbidity measurement Ten ml of culture suspension were transferred to a Klett tube and read directly in a Klett-Summerson photoelectric colorimeter at 660 mμ (red filter) using an uninoculated tube of medium as the blank.

b) Dry weight determination Ten ml of culture suspension were transferred to a 16 x 150 mm screw cap tube (Pyrex) and quickly frozen at approximately -20 C until ready for use. After cold storage the tubes were thawed and centrifuged to separate the cells from the growth menstruum. The cells were washed twice with distilled water and suspended in 10 ml of water. Aliquots were dried at 80 C overnight and weighed on an analytical balance.

c) Viable count One ml aliquots of suitable dilutions were added separately to sterile Petri dishes containing melted APT agar. After mixing and hardening, these plates were inoculated at 30 C for 48 hours and the colonies counted.

Preparation of Experimental Cells

Each 100 ml of Wright-Skeggs growth medium was inoculated with 0.4 mg dry weight of cells and incubated for 24 hours at 30 C.

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After incubation the cells were removed from the growth menstruum by centrifugation, washed twice with cold saline, and resuspended in the desired reaction mixture at a cell density of 1 mg/ml.

The contents of the reaction mixture varied somewhat with each experiment, but in general the suspension consisted of single strength Wright-Skeggs medium, 0.1 M phosphate buffer, and 200 units of biotin (a unit is defined as 1 x 10^{-4} μg of biotin per unit weight or volume). The reaction mixtures were incubated at varying temperatures and for different periods of time depending upon the experimental protocol.

Preparation of Samples for Biotin Analysis

After the desired incubation period, the reaction vessels were centrifuged at 2 C (3090 x g) for 10 minutes. The supernatant was recovered and frozen for "menstruum biotin" analysis at a later time. The centrifuge tube was rinsed quickly to remove any menstruum biotin remaining on the walls, and the pellet resuspended in a known volume of water. Aliquots were taken for dry weight determinations and the remainder of the suspension was placed in a boiling water bath for 5 to 10 minutes. After boiling, the suspension was centrifuged in the cold (2 C) for 10 minutes, and the supernatant frozen and saved for "free biotin" analysis. The pellet was washed twice with distilled water, suspended in 6N H_2SO_4, and autoclaved for 60 minutes at 121 C. After autoclaving, an equal volume of 6N NaOH was added to the

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acid suspension and adjusted to a known volume with water. The neutralized suspension was then filtered through Whatman #1 paper to remove any cell debris that might alter the results of the biotin assays. The final filtrate represented the "bound biotin" fraction.

All fractions to be assayed for biotin with L. arabinosus were adjusted to pH 6.8, while samples assayed with S. cerevisiae were adjusted to pH 4.0.

Microbiological Assay Procedures

a) Biotin assay with L. arabinosus The assay vessels were 16 x 150 mm Pyrex tubes covered with aluminum caps. Five ml or less of suitable concentrations of biotin (ranging from 1×10^{-4} µg to 5×10^{-3} µg per sample) or unknown sample, were added to the tubes, the total volume being adjusted to 5 ml with distilled water. To this were added 5 ml of double strength Wright-Skeggs medium. The tubes were sterilized by autoclaving at 121 C for 7 minutes, cooled, and each inoculated with 1 drop of a suspension of L. arabinosus prepared as follows: cells were grown for 18-24 hours in 5 ml of inoculum broth at 30 C, washed twice in sterile water, resuspended in 5 ml of the same menstruum, and approximately 0.1 ml of this suspension diluted with 15 ml of water. After inoculation the contents were mixed by shaking and incubated at 30 C for 45 to 48 hours. Growth was measured turbidimetrically in a Klett-Summerson photoelectric colorimeter at 660 mµ (red filter) using uninoculated medium as a blank. Each tube

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was shaken vigorously prior to reading in order to suspend the bacterial cells.

b) Biotin assay with *S. cerevisiae* The assay vessels were 18 x 150 mm Pyrex tubes covered with aluminum caps. Three ml or less of suitable concentrations of biotin (ranging from 4×10^{-5} µg to 1×10^{-3} µg per sample), or unknown were added to the tubes, the total volume being adjusted to 3 ml with distilled water. The tubes were sterilized by autoclaving at 121 C for 7 minutes, cooled, and to each tube were added 3 ml of double strength Hertz medium. The medium was inoculated previously with 0.5 ml of a suspension of *S. cerevisiae* (turbidity at 420 mµ equal to 40 Klett units) per 100 ml of double strength medium. The control tube received 3 ml of uninoculated medium. The contents were mixed by shaking and incubated for 21 to 24 hours at 30 C. Growth was measured turbidimetrically at 420 mµ using the uninoculated medium as a blank. Each tube was shaken vigorously before reading to suspend the yeast cells.

Standard curves were prepared for each assay and plotted on semi-log graph paper. Figures 2 and 3 represent typical growth responses to graded concentrations of biotin by *L. arabinosus* and *S. cerevisiae* respectively. A linear response for the *L. arabinosus* assay was obtained in the range of 5×10^{-4} to 3.5×10^{-3} µg of biotin per sample. For the *S. cerevisiae* assay the linear range was between 8×10^{-5} and

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Figure 2. Response of Lactobacillus arabinosus to graded amounts of biotin.

No additions = turbidity of 15.

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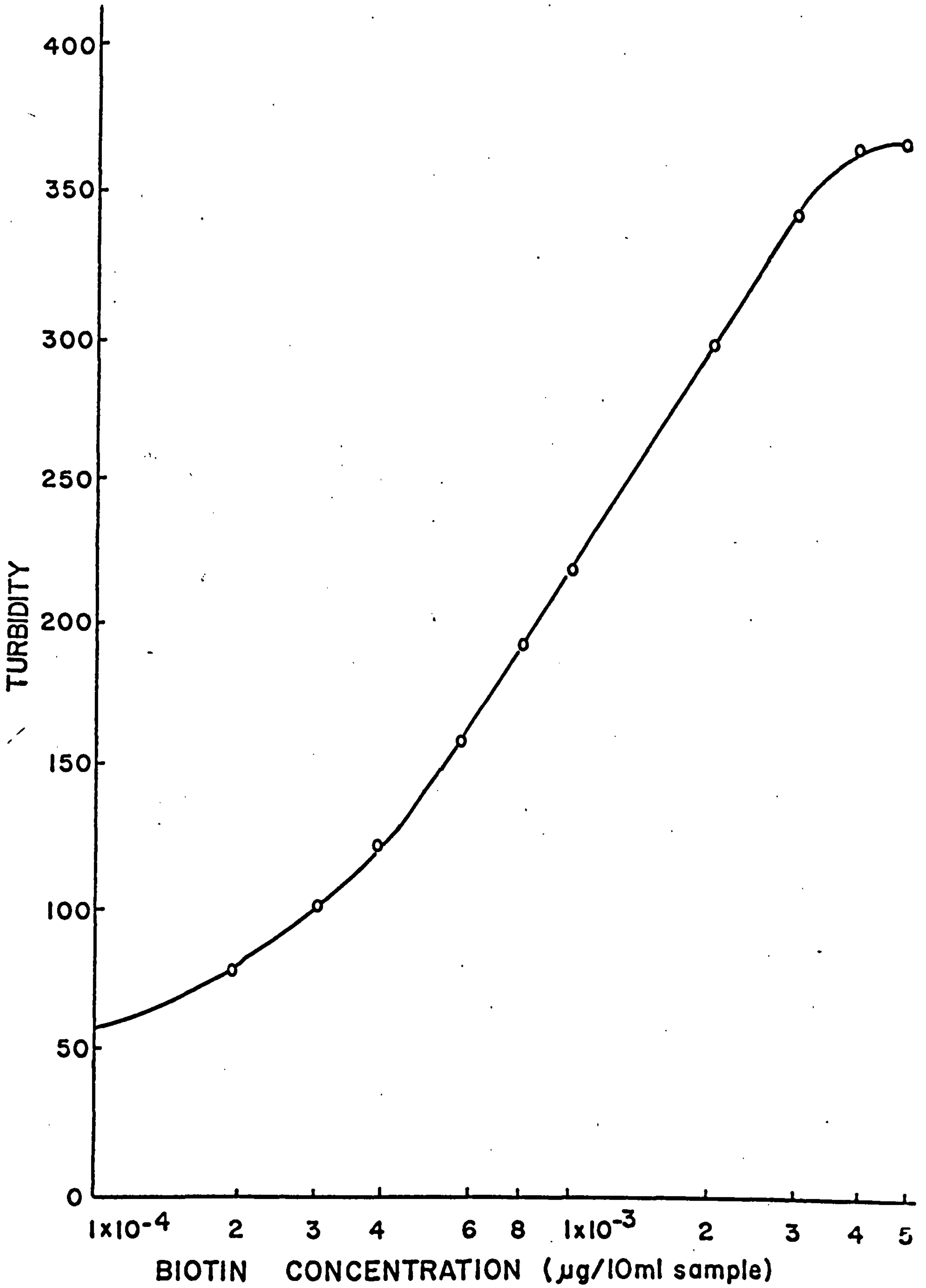

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Figure 3. Response of *Saccharomyces cerevisiae* to graded amounts of biotin.

No additions = turbidity of 25.

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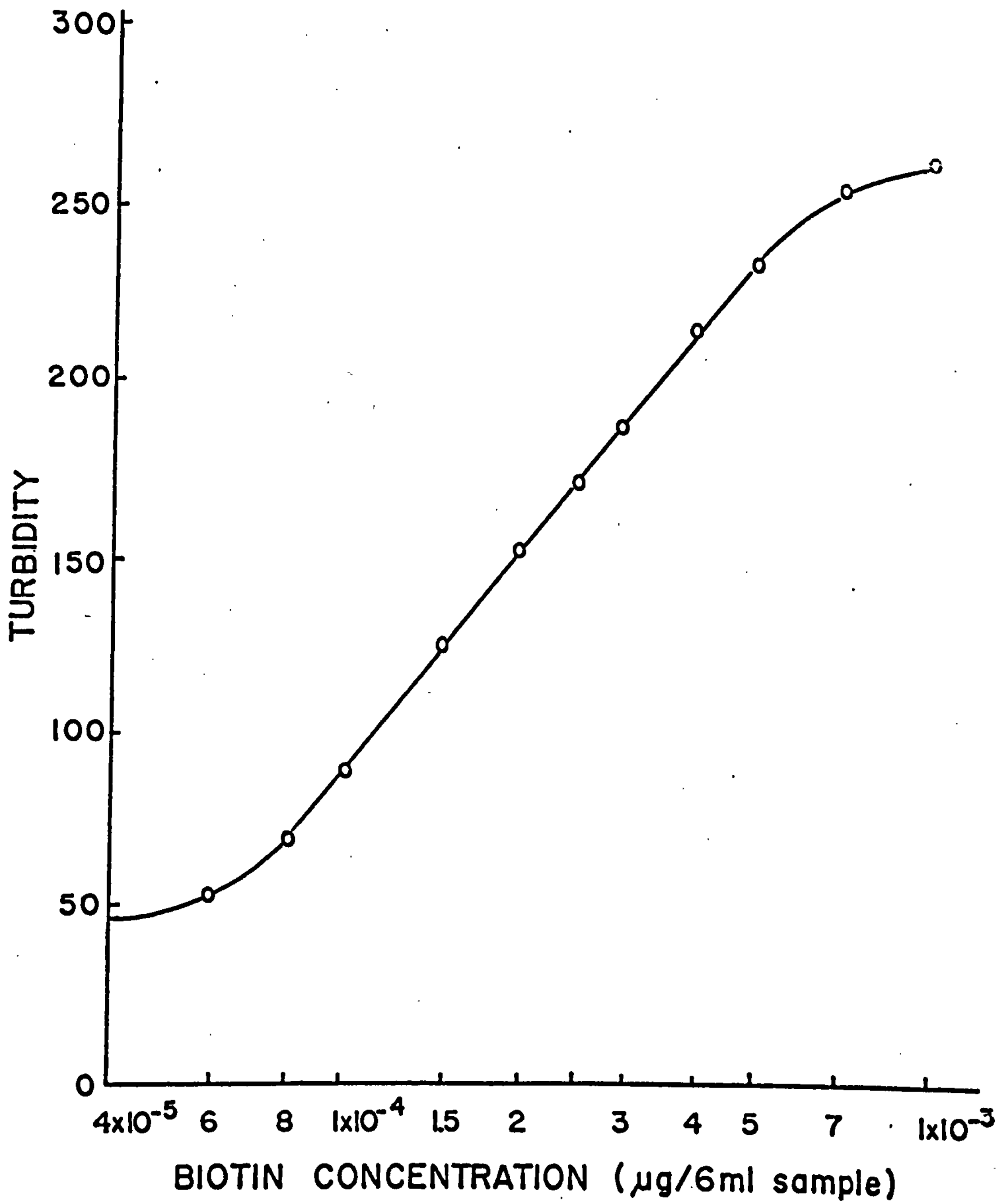

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5×10^{-4} µg of biotin per sample. The biotin concentrations of unknown samples were obtained by interpolation from the standard curves.

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RESULTS

A. Growth Studies

1. Growth curves. Bacterial growth was followed by means of turbidimetric measurements, dry weight determinations and viability counts (figures 4, 5 and 6). The primary purpose of this study was to ascertain the time of initiation and approximate duration of the logarithmic, stationary, and decline phases of the growth cycle for this organism. This would permit also correlations of the effect of culture age and culture conditions upon the free intracellular and bound biotin content of cells of *L. arabinosus.* Inasmuch as large inocula were used (0.4 mg dry cells/100 ml) in order to simulate the experimental conditions, no evaluation of the lag phase was possible.

In general, there was excellent agreement among the methods of evaluation in regard to the time of commencement, duration, and termination of each growth phase. The logarithmic phase of growth levelled at 15 to 16 hours, and the "true" stationary phase appeared to begin at about 20 hours. The period between 16 and 20 hours will be designated as the late log or early stationary phase for convenience. The conditions at this period become of prime importance in later experiments.

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Figure 4. Growth curve of *Lactobacillus arabinosus.*

Growth determined turbidimetrically.

Temperature of incubation 30 C;

turbidity = Klett units at 660 mμ.

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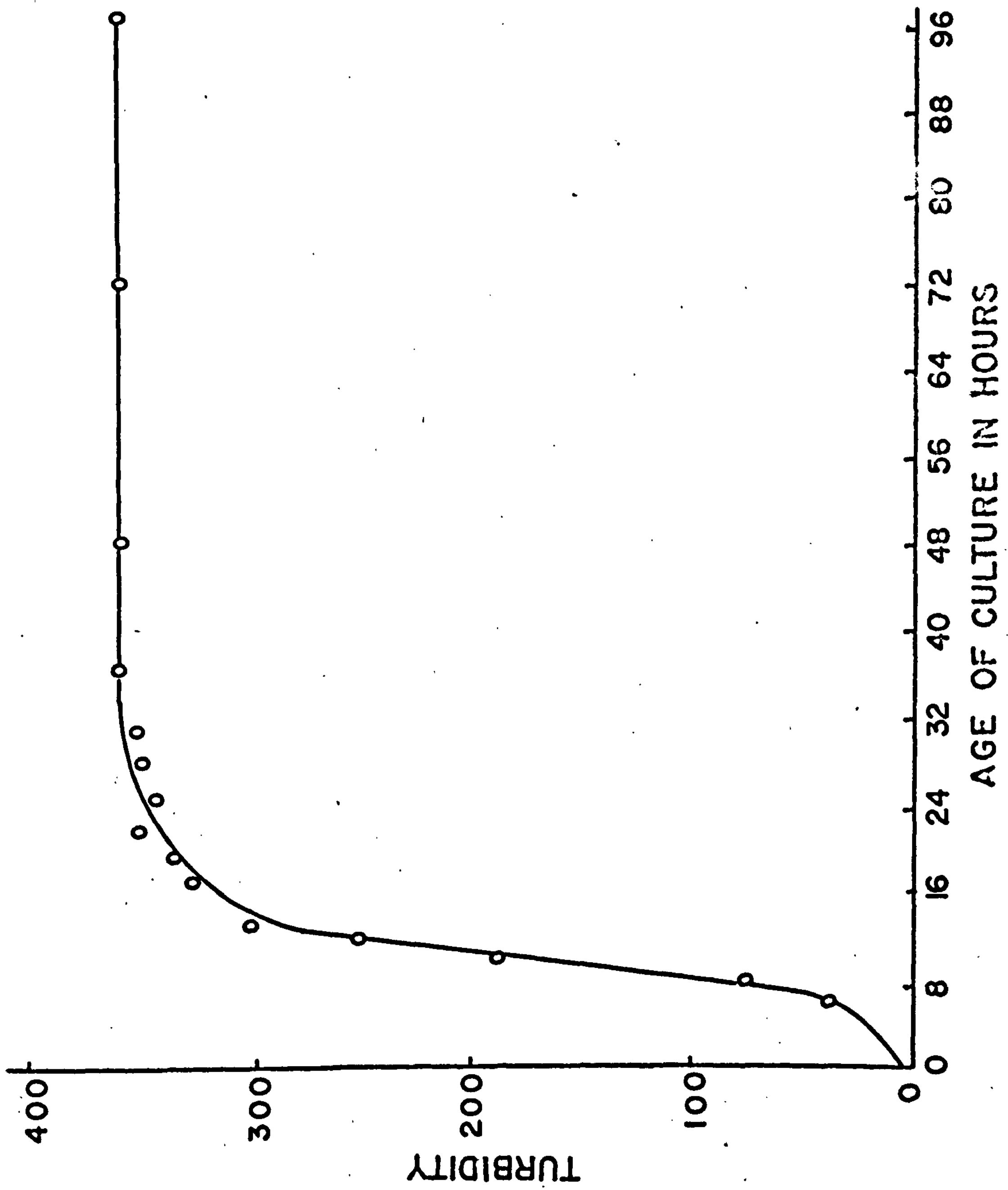

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Figure 5. Growth curve of *Lactobacillus arabinosus.*

Growth determined by increase in dry weight.

Temperature of incubation, 30 C.

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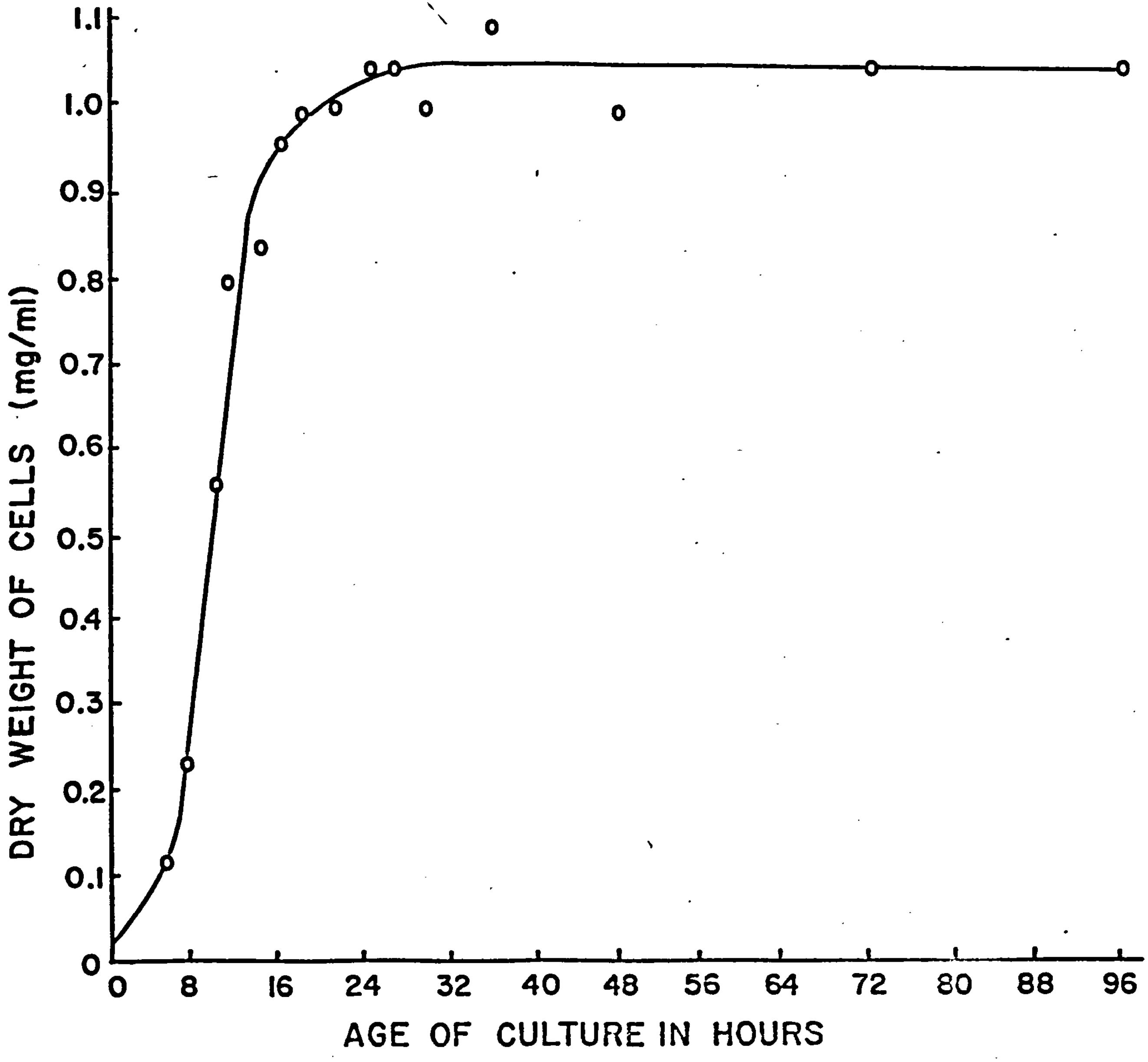

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Figure 6. Growth curve of *Lactobacillus arabinosus.*

Growth determined by viable cell count.

Temperature of incubation, 30 C.

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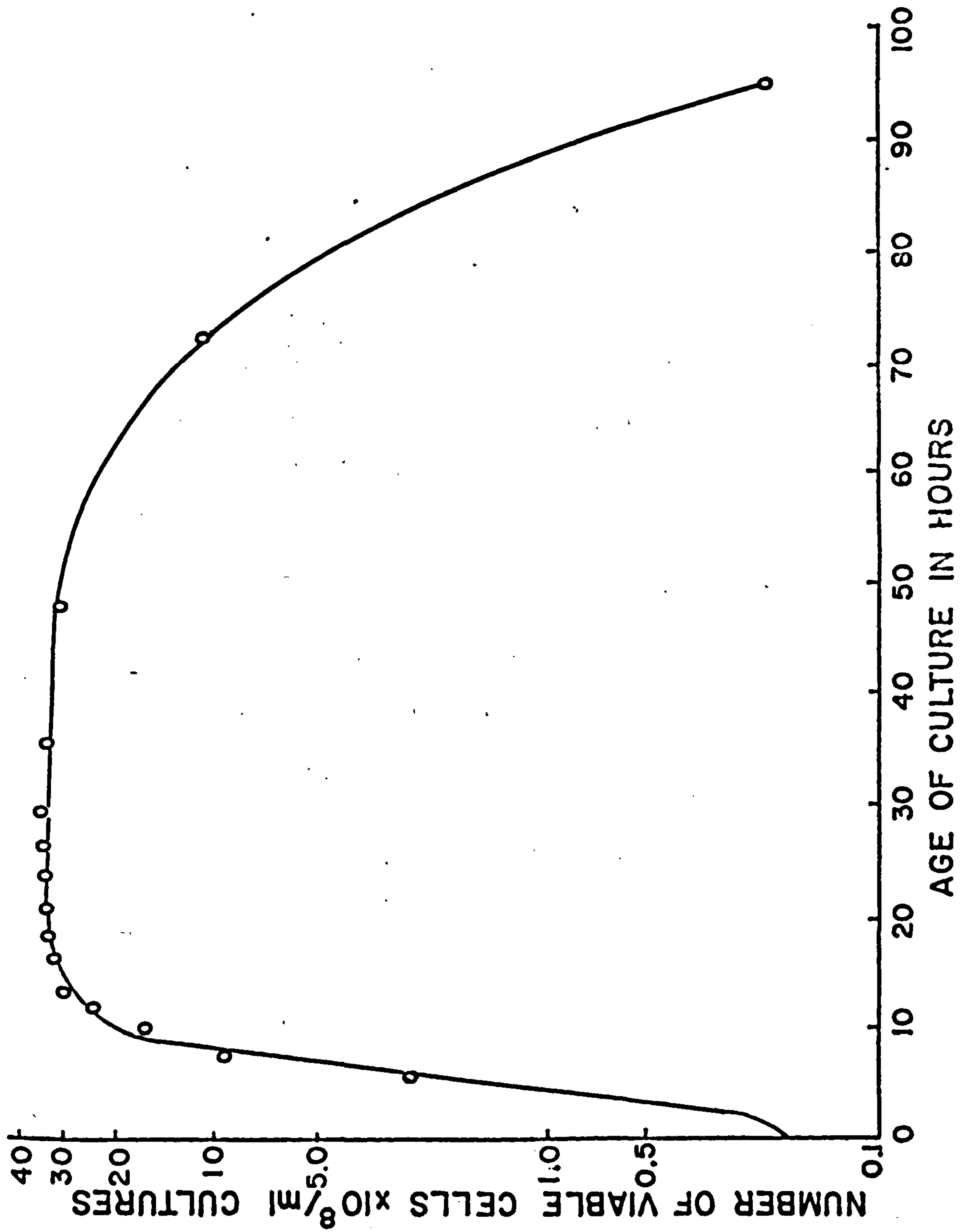

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The stationary phase of growth which appeared at about 20 hours continued until approximately 48 hours of incubation. From 48 to 72 hours the cells appeared to die slowly, with a steady rate of decline becoming evident after 72 hours. At 96 hours the number of viable cells remaining was the same as the number initially inoculated into the growth medium (figure 6). Though viable cell count began to fall sharply after 48 hours, the turbidity and dry weight measurements (figures 4 and 5 respectively) showed no decrease, thus indicating that cell death occurred without cell lysis.

2. Menstruum and cellular biotin levels during growth.

Preliminary experiments were designed to follow the course of biotin accumulation in growing cells of *L. arabinosus* as assayed with both *L. arabinosus* and *S. cerevisiae*. Typical results obtained for free intracellular, bound, and menstruum biotin at several periods of growth are presented in table I. When *L. arabinosus* was employed as the assay organism maximum values for free and bound biotin were obtained at 24 hours of growth. From 24 to 96 hours a gradual decrease, totalling 33 per cent, was obtained for the free biotin fraction. Bound biotin, when assayed with *L. arabinosus*, exhibited a sharp drop (28 per cent) between 24 and 48 hours, and remained close to the 48 hour level up to 96 hours of growth. Menstruum biotin which accounts for the major portion of the biotin

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TABLE I

Menstruum and Cellular Biotin During Growth of Lactobacillus arabinosus

Hours of Growth	24		48		72		96	
Assay Organism	L.A.[1]	S.C.[2]	L.A.	S.C.	L.A.	S.C.	L.A.	S.C.
Free Biotin ($x 10^{-4} \mu g/mg$)	12.7	14.3	10.8	15.6	9.6	12.5	8.5	10.2
Bound Biotin ($x 10^{-4} \mu g/mg$)	25.5	25.5	18.3	25.5	17.5	25.7	17.0	22.3
Menstruum Biotin ($x 10^{-4} \mu g/ml$)	760	925	770	925	765	920	755	930
Per Cent Recovery	79.7	96.4	81.2	96.9	79.5	96.2	78.3	96.5

[1] L.A.= Values obtained employing L. arabinosus as the assay organism.

[2] S.C.= Values obtained employing S. cerevisiae as the assay organism.

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presented initially to the growing organisms, remained level throughout growth. The recovery of biotin with the L. arabinosus assay method ranged from 78 to 81 per cent of that provided initially to the organism.

Striking differences were obtained when S. cerevisiae was employed as the assay organism. Values for free biotin ranged from 50 to 28 per cent higher at 48 and 96 hours respectively than those obtained with the lactobacillus assay method. In contrast to the lactobacillus assay, the yeast assay did not exhibit a sharp decrease in bound biotin from 24 to 48 hours, but remained level throughout 96 hours of growth. Values for menstruum biotin were 155 to 175 units/ml higher with S. cerevisiae than with L. arabinosus. The difference in menstruum biotin between the two assays was seen at all four growth periods. The total recovery of biotin with the yeast assay was 96 to 97 per cent primarily because of higher values obtained for menstruum biotin.

Since only d-biotin was present initially in the cultures, the assay values with both organisms should not differ beyond experimental error, unless some of the d-biotin was converted to another form of the vitamin (vitamers) during growth. If such vitamers were biologically less active or totally inactive to L. arabinosus but still utilizable by S. cerevisiae, differences would appear in the assay values. This

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hypothesis can be used to explain the data obtained (table I). Thus a conversion of biotin to biotin vitamers inactive for L. arabinosus appears to occur before 24 hours of growth. The vitamers accumulate in the growth menstruum resulting in the difference between the two assays (155 to 170 units/ml). The vitamers do not appear intracellularly at 24 hours as evidenced by the close similarity of free and bound biotin values with both assays. However, the vitamer does appear within the cells at 48 hours, as shown by the significantly higher values for free and bound biotin when assayed with S. cerevisiae.

Of interest are the relatively low values obtained for free intracellular biotin in proliferating cells, as compared to the high levels of biotin accumulated in resting cells of L. arabinosus as reported by Lichstein and Waller (43). These results are explained in part by findings in later experiments.

It seemed desirable to ascertain more precisely the time at which conversion of d-biotin to vitamers occurred. The course of free, bound, and menstruum biotin was followed beginning at 12 hours of growth, which represents the middle of the logarithmic phase. No differences were detected between the two assays for free or bound biotin for any period between 12 and 23 hours, indicating the presence of only d-biotin in these fractions. However, marked differences were noted in regard to menstruum biotin (figure 7). As assayed with S. cerevisiae the level remained constant at about 930 units/ml from

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Figure 7. Menstruum biotin in cultures of Lactobacillus arabinosus.

(Wright-Skeggs medium; initial pH 6.8; incubation temperature 30 C; initial biotin concentration 1,000 units/ml)

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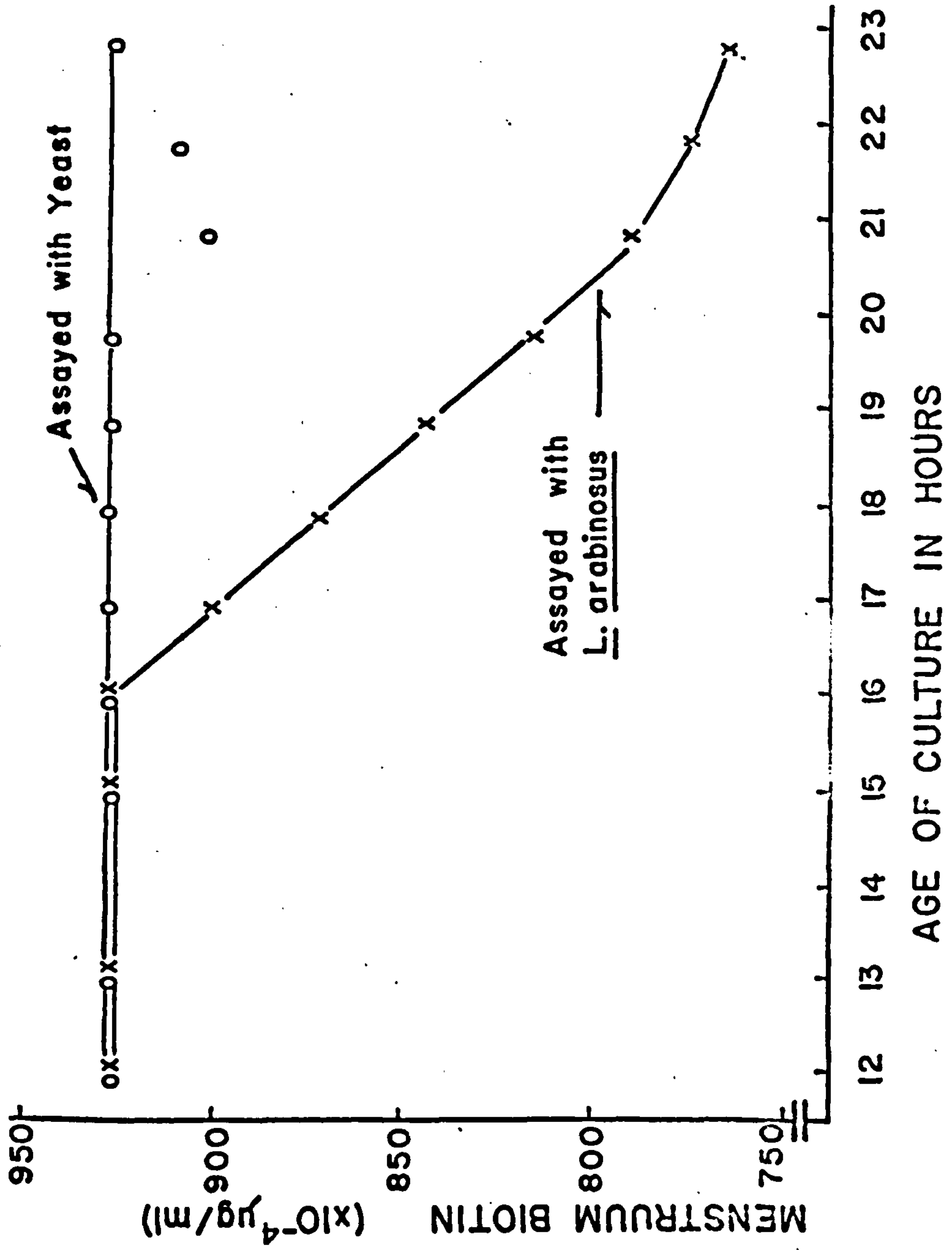

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12 to 23 hours. As measured with L. arabinosus, menstruum biotin remained at the same level until 16 hours of growth at which time it began to drop markedly. It appears therefore that the conversion of d-biotin begins at about 16 hours and is most active up to 20 hours, i.e., the late log and early stationary phases. The significance of this will become evident when the physiological control of this system is discussed. It is probable on the basis of the data given in table I that the conversion process reaches conclusion by approximately 24 hours. Conversion of biotin appears to reach an equilibrium in favor of d-biotin, i.e. 80 - 85 per cent d-biotin and about 15 per cent biotin vitamers.

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B. Studies on the Converting Activity of Non-proliferating Cells of *L. arabinosus*

The experimental evidence described demonstrates that *L. arabinosus* will convert d-biotin to one or more biotin vitamers which cannot be utilized for growth by this organism. The conversion appears in the late log and early stationary phases, and reaches an equilibrium at about 23 to 24 hours. If the converting system was enzymatic in nature, it should exhibit typical characteristics of enzyme systems, *i.e.*, temperature and pH optima, requirement for energy, etc. It was therefore necessary to prepare a reaction mixture in which the experimental cells could be suspended, and in which the parameters of the converting system could be studied.

First attempts to formulate a reaction mixture employed 0.1 M acetate buffer at pH 4.7. This pH was chosen because it approximated the hydrogen ion concentration of the growth medium at the time of conversion. Added also was 1 per cent glucose on the assumption that an energy source was necessary for maximum biotin converting activity. The substrate was d-biotin (200 units/ml). Twenty four hour cells were added at a density of 1 mg dry weight/ml, and incubated at 30 C for 6 hours. Aliquots were taken for analysis of menstruum and free intracellular biotin with both *L. arabinosus* and *S. cerevisiae*. Any difference between the two assays would represent an index of converting activity. Bound biotin was not included for

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assay because 24 hour cells grown in 10^0 µg biotin/10 ml have their biotin binding sites completely saturated with d-biotin (Table I).

The results with this reaction mixture are graphically represented in figure 8. No significant differences were seen between the two assays for menstruum biotin over the 6 hour incubation period. Small, consistently higher values for free biotin were obtained with the S. cerevisiae assay compared to the L. arabinosus assay. This was due probably to carry over of biotin vitamers from the growth cultures and does not represent biotin converting activity. Thus, this reaction menstruum did not satisfy the requirements for biotin conversion. Plate counts over the 6 hour incubation period showed very little cell death, so that acetate appears not to be toxic to the cells.

Subsequent experiments employed the same reaction mixture with the addition of 0.05M K_2HPO_4. Phosphate was included in an attempt to satisfy the requirements of the biotin transport system, since Lichstein and Waller (48) had reported excellent biotin accumulation in cells of L. arabinosus when phosphate buffers were employed. Figure 9 illustrates the results obtained using the acetate buffer reaction mixture modified by added phosphate. At the end of one hour of incubation a sharp decrease in menstruum biotin was evident with both assays. Correspondingly a marked increase in free intracellular biotin, equaling the decrease in menstruum biotin can be seen. Free biotin decreased slowly over the next 5 hours of incubation with a corresponding steady

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Figure 8. Menstruum and free intracellular biotin in cells of Lactobacillus arabinosus when incubated with biotin and glucose in 0.1 M acetate buffer.

(Cell density 1 mg/ml; incubation temperature 30 C; age of cells, 24 hrs; biotin concentration about 200 x 10^{-4} μg/ml; 1 per cent glucose; 0.1 M acetate buffer at pH 4.7.)

Menstruum biotin X———X

Free biotin 0 - - - - - - 0

Assayed with L. arabinosus = LACTO

Assayed with S. cerevisiae = YEAST

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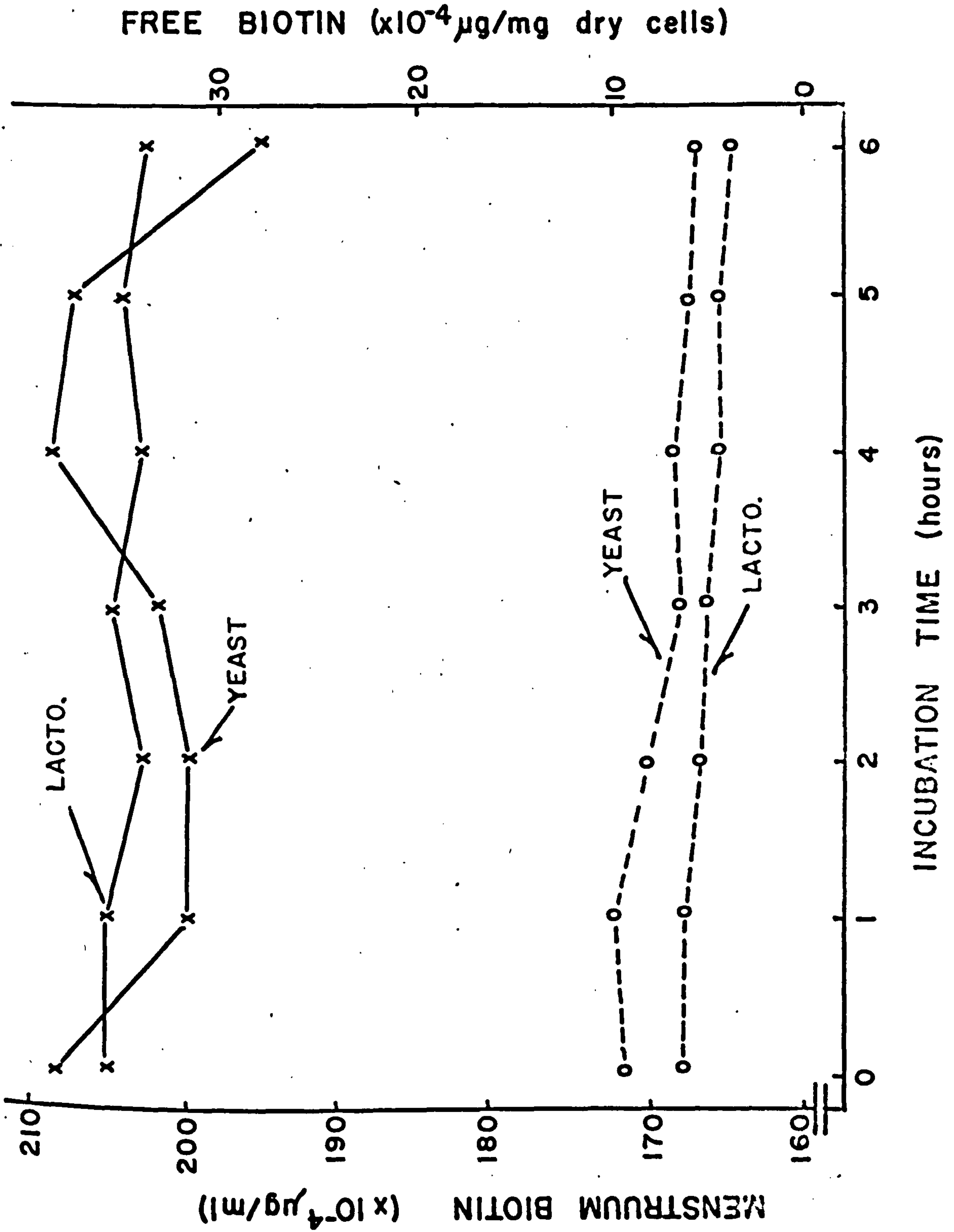

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Figure 9. Menstruum and free intracellular biotin in cells of Lactobacillus arabinosus when incubated with biotin, glucose, and phosphate in 0.1 M acetate buffer.

(Biotin concentration about 200 x 10^{-4} µg/ml; 1 per cent glucose; 0.05 M K_2HPO_4; 0.1 M acetate buffer at pH 4.7.)

Menstruum biotin X ——— X

Free biotin 0 - - - - - 0

Assayed with L. arabinosus = LACTO

Assayed with S. cerevisiae = YEAST

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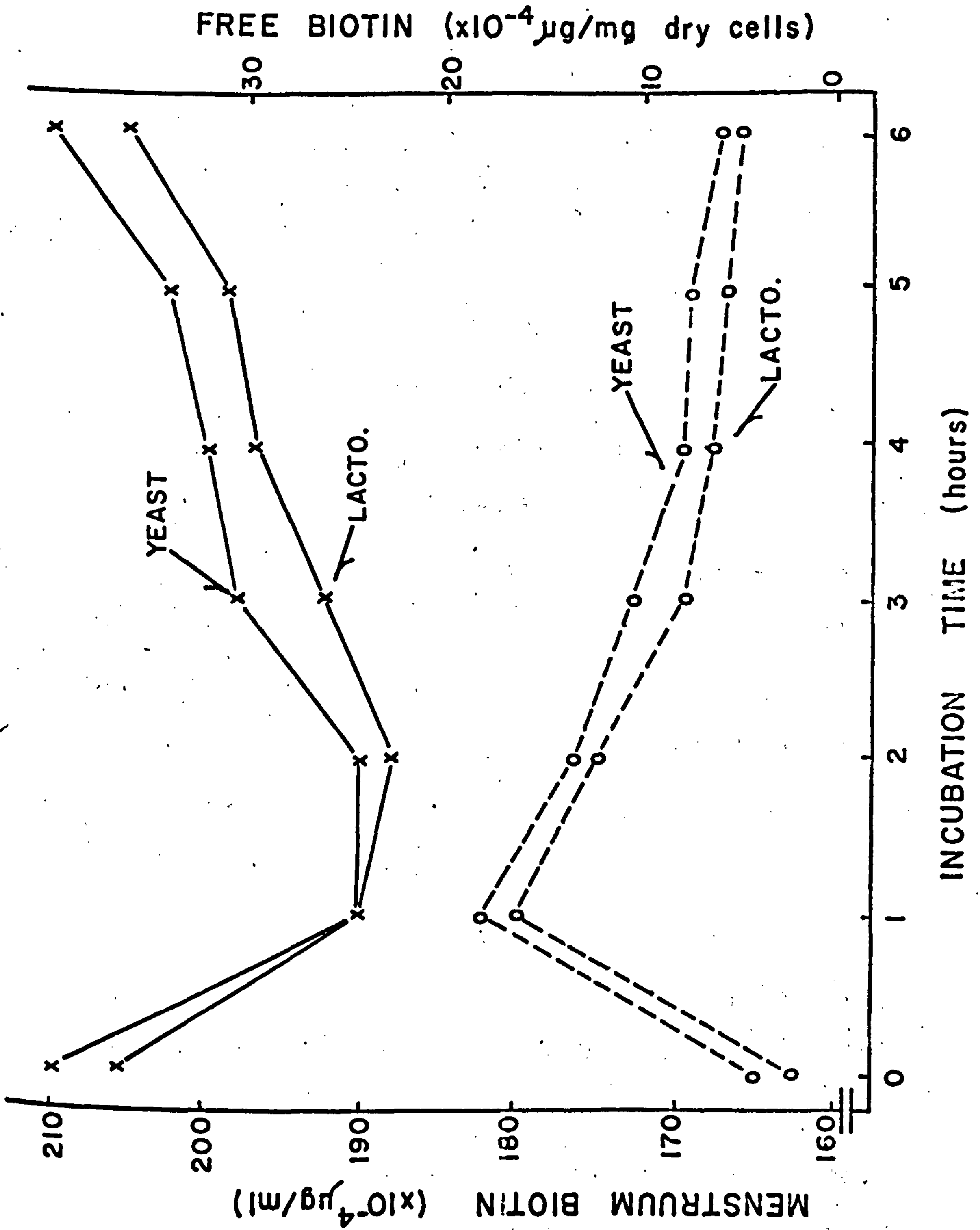

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increase in menstruum biotin until 6 hours at which time the values returned to their initial levels. Throughout the incubation period no significant differences were obtained between the two assays for either free or menstruum biotin. Thus, the addition of small amounts of phosphate aided the permeation of biotin, but did not stimulate conversion.

Further experiments employed 0.1 M phosphate buffer at pH 6.8 in place of the acetate buffer. The results are illustrated in figure 10. Phosphate buffer provided excellent conditions for biotin permeation, as can be seen by the 30 unit decrease in menstruum biotin and the corresponding increase in free biotin. The intracellular free biotin remained at a maximum for one to two hours and then began to pass out of the cells resulting in an increase in menstruum biotin. Throughout the 6 hours of incubation no significant differences were detected using both assays. It seems clear from these experiments that the presence of additional phosphate did not improve the conversion process at either pH 4.7 or 6.8.

In summary of the situation to this point it is manifest that the biotin converting system was demonstrable only in cells actively growing in Wright-Skeggs medium. To study this system more precisely the same inoculum of 24 hour cells was introduced into modified Wright-Skeggs medium. The major changes were reduction of the glucose concentration from 2 per cent to 1 per cent and the introduction of

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Figure 10. Menstruum and free intracellular biotin in cells of *Lactobacillus arabinosus* when incubated with biotin and glucose in 0.1 M phosphate buffer.

(Biotin concentration about 200 x 10^{-4} µg/ml; 1 per cent glucose; 0.1 M phosphate buffer at pH 6.8.)

Menstruum biotin	X ———— X
Free biotin	0 - - - - - 0
Assayed with *L. arabinosus* =	LACTO
Assayed with *S. cerevisiae* =	YEAST

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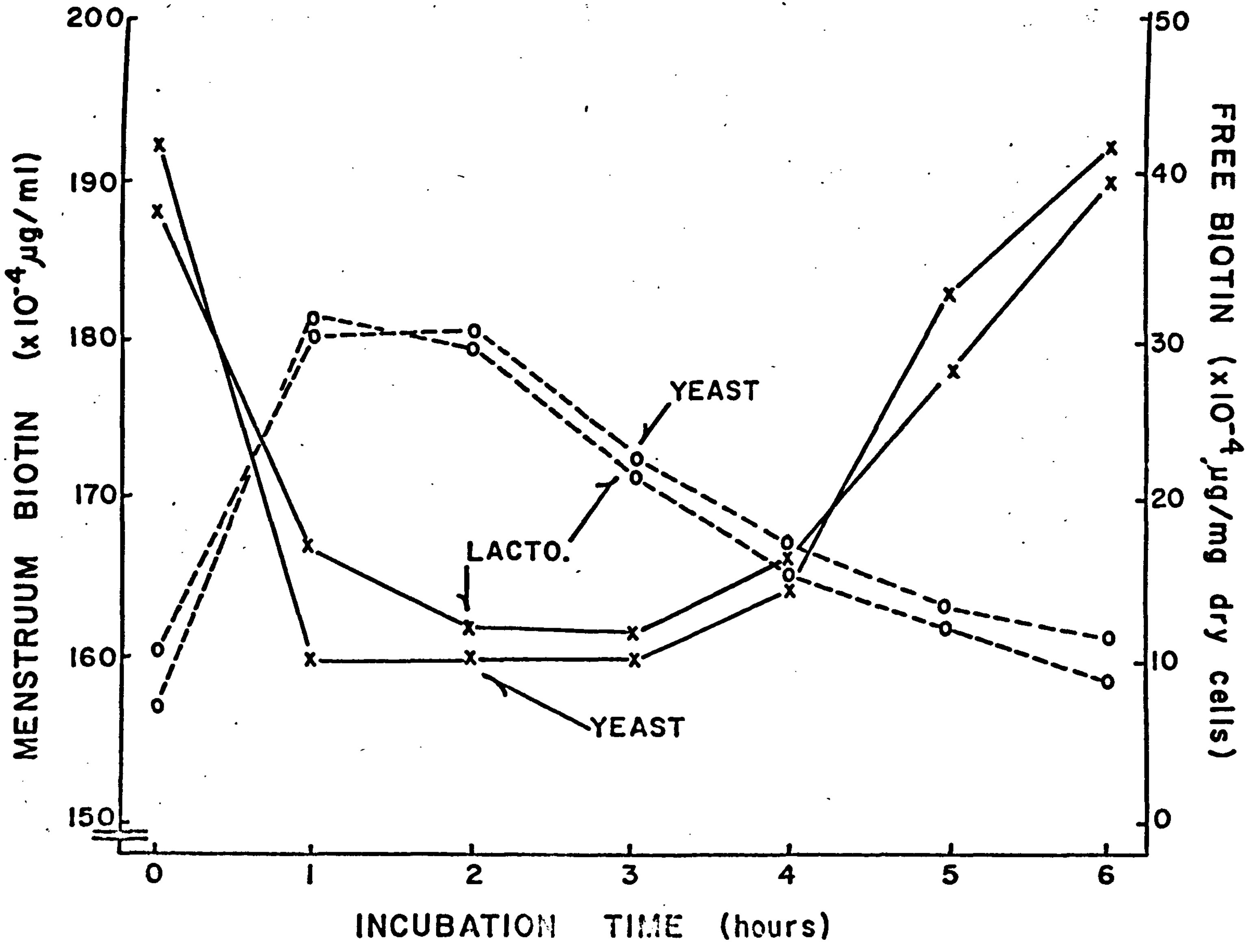

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additional phosphate. These modifications were made in order to prevent large changes in the pH during the experiments. It is seen (Figure 11) that biotin accumulation by L. arabinosus under these conditions was good, but reached a maximum at 3 to 4 hours as compared to one hour when the cells were incubated in phosphate buffer and glucose alone. In this respect Wright-Skeggs medium appears to hinder biotin accumulation to some degree, and may be an explanation for the finding that free biotin levels are low in growing cells. At the end of 4 hours the free biotin level began to fall markedly and the menstruum biotin began to increase when measured with the yeast assay. The L. arabinosus value for menstruum biotin at the end of 6 hours of incubation was about 45 units below that obtained with the S. cerevisiae assay. These results suggest that Wright-Skeggs medium supplies the essential factors required for biotin conversion.

When Wright-Skeggs medium was used to demonstrate conversion during a 6 hour incubation period the cells were not in a resting state since measurements made during this incubation period exhibited significant increases in turbidity. In a typical experiment for example, a cell suspension of 325 Klett units (660 mμ) increased to 365 units at the end of 6 hours. However, plate counts over the six hour incubation period showed no increase in the number of viable cells (38.2×10^8 viable cells/ml initially, and 37.0×10^8

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Figure 11. Menstruum and free intracellular biotin in cells of Lactobacillus arabinosus when incubated in modified Wright-Skeggs medium.*

*(Biotin concentration about 200 x 10^{-4} µg/ml; 1 per cent glucose; 0.1 M phosphate buffer pH 6.8 in addition to that amount commonly present.)

Menstruum biotin X ———— X

Free biotin 0 - - - - - 0

Assayed with L. arabinosus = LACTO

Assayed with S. cerevisiae = YEAST

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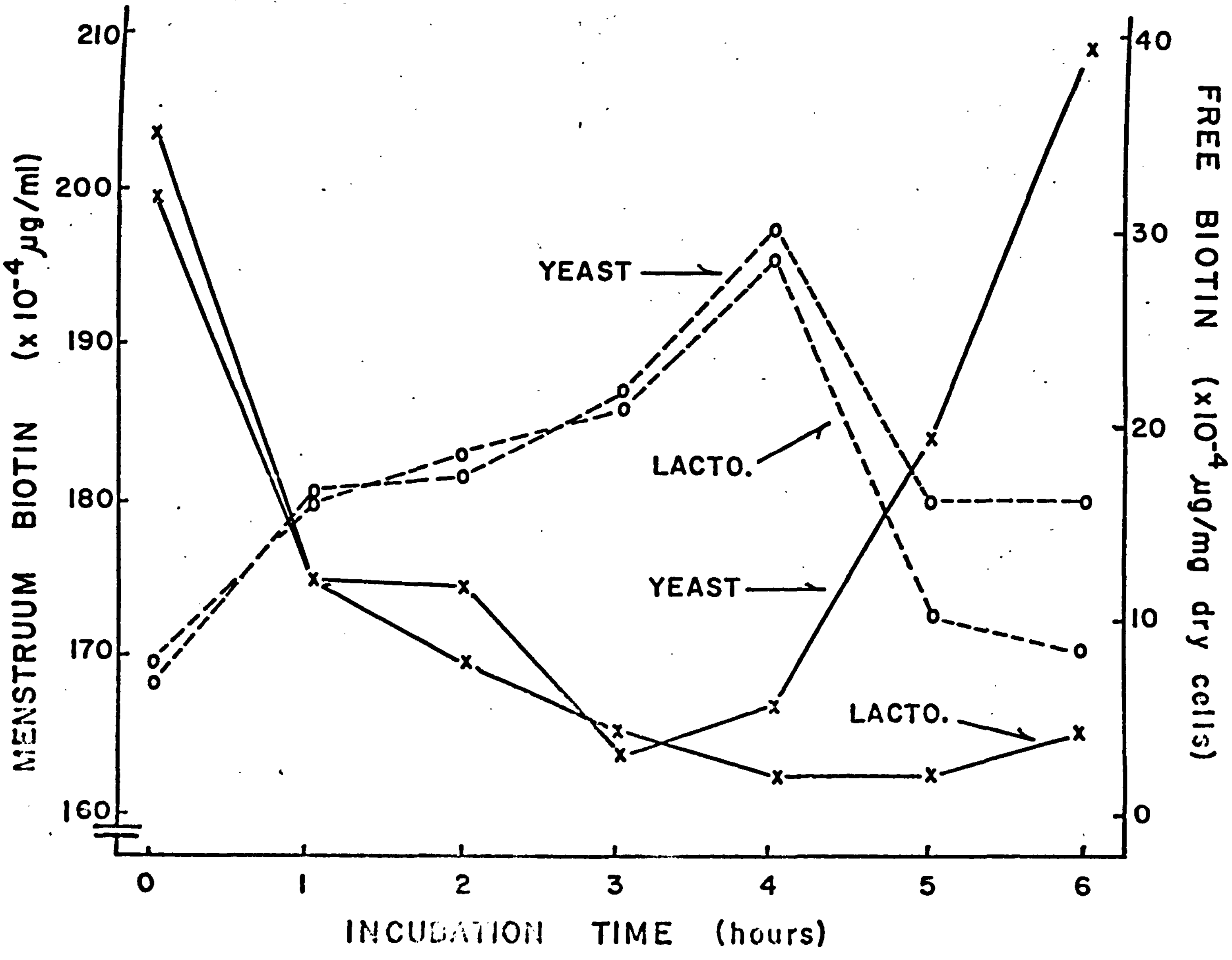

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viable cells/ml at the end of 6 hours). Thus, the cells suspened in this medium are referred to as "non-proliferating" rather than resting, since some small amount of growth is occurring without noticeable reproduction.

The biotin converting activity may be measured by subtracting the *L. arabinosus* assay value for menstruum biotin at any time T from the *S. cerevisiae* assay value, and dividing by the number of mg of dry cells. This relationship may be expressed with the following simple equation:

UNITS OF D-BIOTIN CONVERTED = Ym - Lm/mg

where Ym equals the yeast assay value for menstruum biotin in units/ml, and Lm equals the *L. arabinosus* assay value in units/ml.

Though a small difference in free biotin was observed between the two assays at the end of the incubation period, it can be disregarded in view of the much larger differences between the assays for menstruum biotin (figure 11).

Conversion appeared to commence only after free intracellular biotin was at a maximum. This point became of great importance in the design of future experiments.

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C. Physiological Factors Influencing Biotin Conversion in Cells of L. arabinosus

It is well established that the physiological conditions of living cells as well as their environment play a vital role in the expression of their genetic potential. Some of the physiological factors affecting the conversion of biotin have already been described i.e., free intracellular biotin must be at a maximum, and a factor in Wright-Skeggs medium must be supplied before conversion commences. The present section deals specifically with the effects of temperature, glucose, and pH on the converting system.

1. Effect of temperature.

Enzymes characteristically exhibit a temperature optimum for activity which may in some cases be quite sharp and in other instances quite broad. Biotin conversion if enzymatic in nature, should likewise reveal a marked effect of temperature variation.

Twenty four hour old cultures were frozen at -45 C to stop all metabolic activity, and then thawed at 0 C. After thawing, the cells were separated from the growth menstruum by centrifugation, placed in the modified Wright-Skeggs medium, and aliquots incubated at temperatures ranging from 0 to 60 C. Menstruum biotin analyses performed with both organisms produced the results illustrated in figure 12. The units of d-biotin converted per mg dry cells (Ym - Lm/mg) are plotted against

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Figure 12. The effect of temperature upon the biotin converting activity in cells of *Lactobacillus arabinosus*.

(Modified Wright-Skeggs medium)

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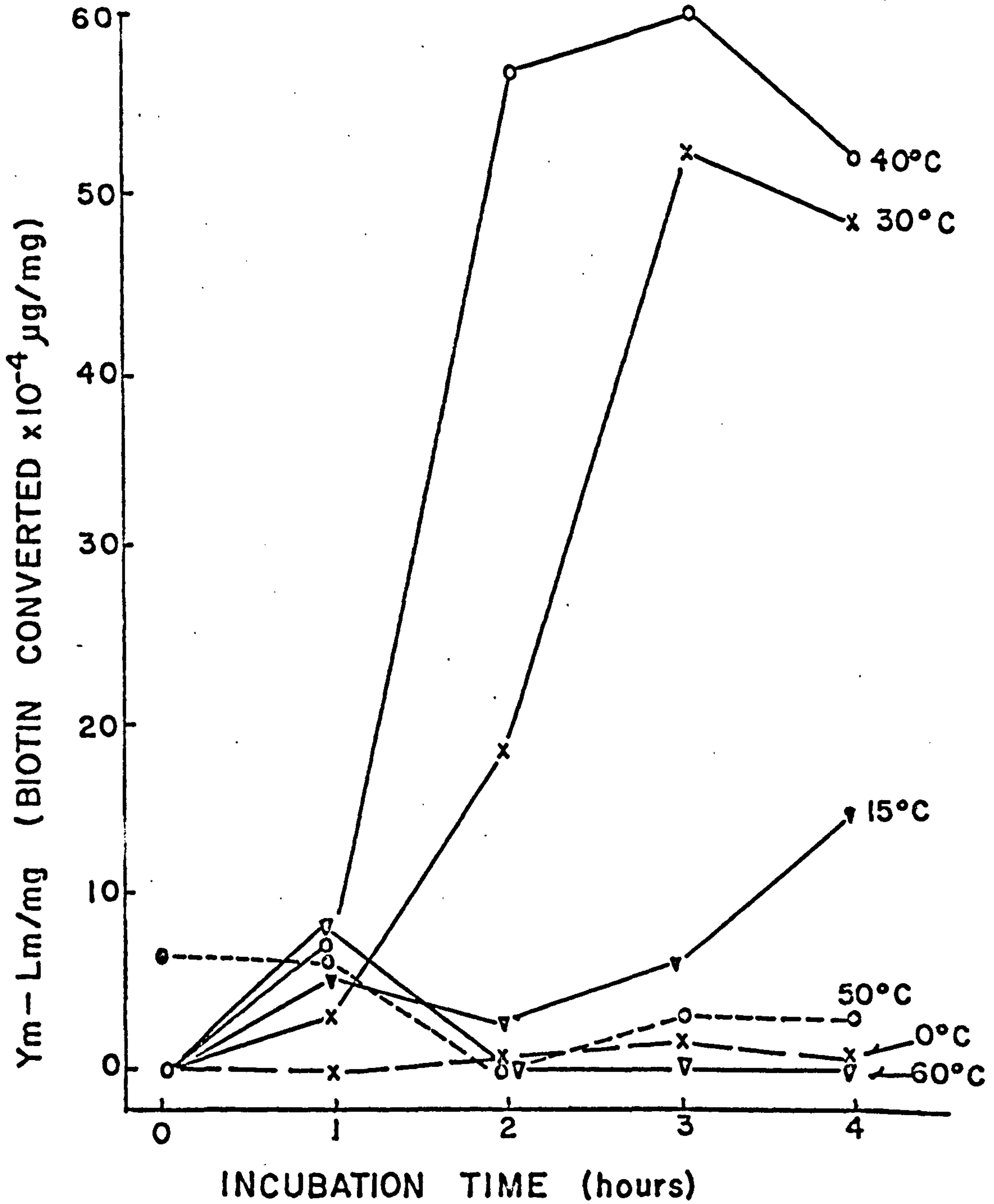

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time of incubation at the various temperatures. Little or no converting activity was noted until after one hour of incubation. The rate of conversion was high between 30 C and 40 C with a maximum at the latter temperature. However, the total amount of conversion at these two temperatures was approximately the same. Little or no conversion occurred at 0, 50 or 60 C. The total units of biotin converted at 15 C was approximately 65 per cent lower than the 40 C value at 4 hours. The temperature dependence of the biotin converting system is illustrated more clearly in figure 13 which shows an optimum at approximately 37 C with the converting activity dropping sharply on either side of this point.

The possibility that quick frozen cells may suffer some damage in their ability to accumulate and/or convert biotin was dispelled by the finding that washed, unfrozen cell suspensions produced the same results with respect to temperature effects.

2. Effect of glucose.

Glucose had been added previously to the reaction menstruum, presupposing a requirement for an energy source in the d-biotin converting process. Experiments were now designed to test the requirement for glucose in this system.

Twenty four hour cells were saturated with free biotin by a 1-hour incubation in phosphate buffer (pH 7.5) with glucose and biotin. The cells were then washed at least once in ice cold saline and placed

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Figure 13. The temperature dependence of the biotin converting system in *Lactobacillus arabinosus.*

(4 hour data of figure 12)

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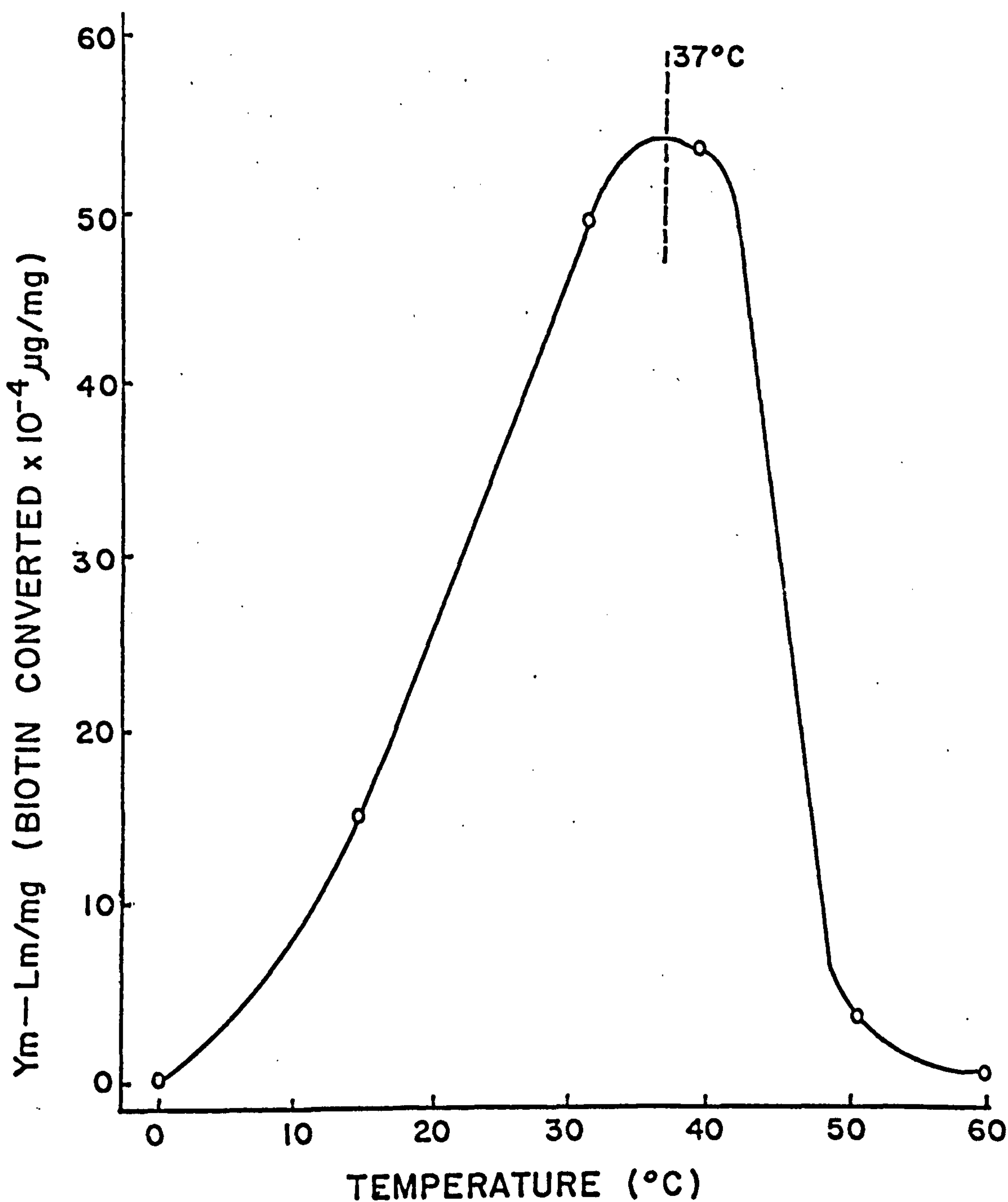

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in the modified Wright-Skeggs medium at 37 C with various concentrations of glucose. The course of biotin conversion was followed over a period of 4 hours and the results plotted in figure 14. Only a small endogenous level of biotin conversion was apparent in the absence of added glucose. Active conversion increased as the concentration of glucose rose. Small quantities of glucose are required to affect conversion (0.1 per cent) as compared to the amount required for maximum growth (approximately 1 per cent).

3. Effect of pH.

Enzymes, being proteins, are greatly affected by hydrogen ion concentration. Enzyme systems will generally exhibit catalytic properties in a limited pH range with lesser activity on either side of this range. Knowledge of the pH optimum is helpful in characterizing the enzyme being studied, and aids also in understanding the cellular physiology related to the enzyme system. Experiments were designed to study the effect of various hydrogen ion concentrations upon the biotin converting system.

For these studies, 24 hour cells were placed in a phosphate buffered biotin solution (pH 7.5) with 1 per cent glucose, and allowed to accumulate biotin for one hour. The cells were then placed in a reaction menstruum composed of the modified Wright-Skeggs medium which was changed further as follows: The concentration of phosphates was increased to 0.2 M, and 0.1 per cent glucose was used in place of the

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Figure 14. The effect of glucose concentration upon the biotin converting activity in cells of *Lactobacillus arabinosus.*

(Conditions as for figure 12, but with varying glucose concentrations.)

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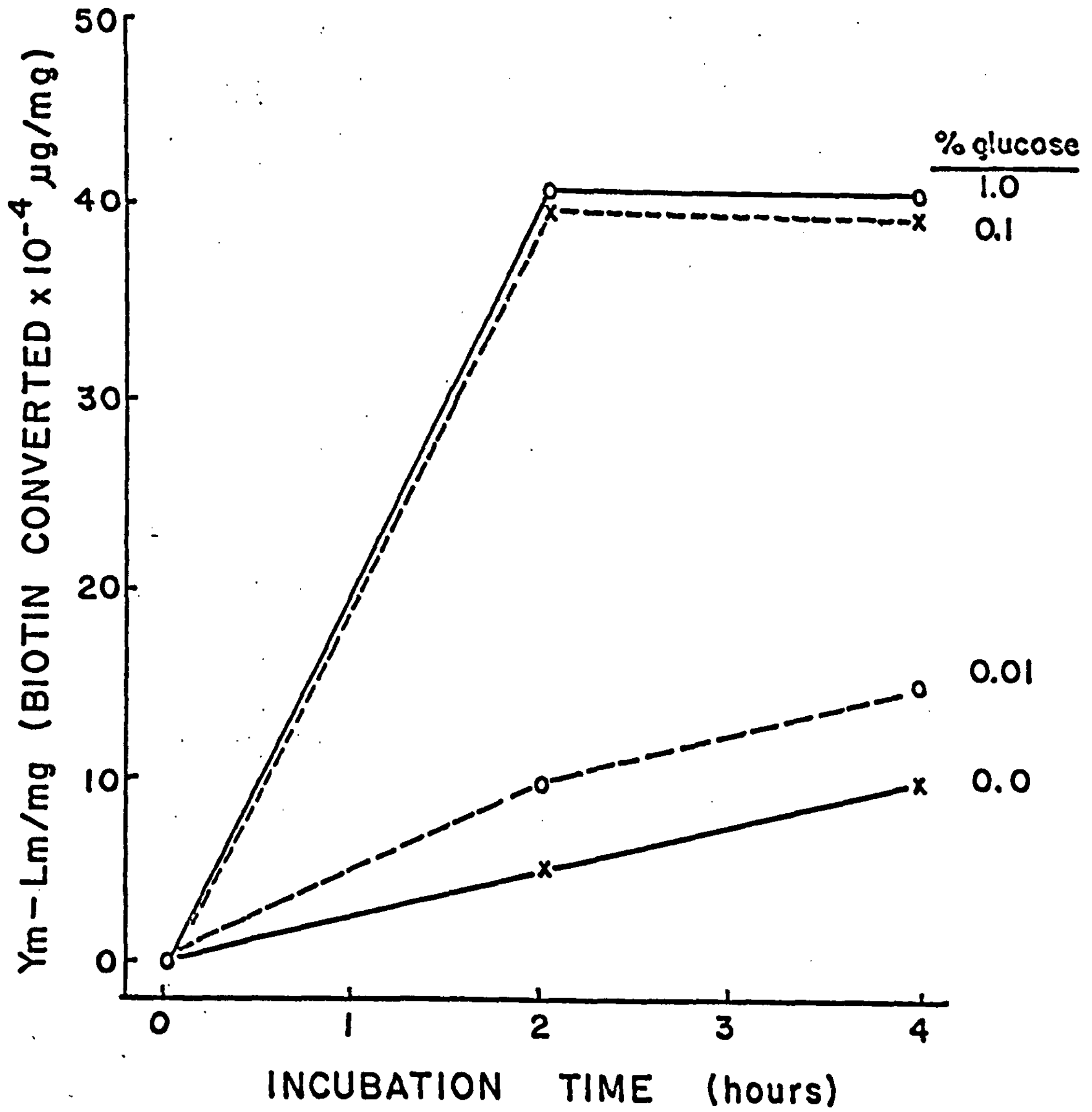

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higher concentration. The lower concentration of glucose was preferred because it was sufficient for full converting activity (figure 14) and would not lead to the formation of amounts of lactic acid sufficient to cause large changes in the pH of the menstruum. Moreover, doubling of the concentration of phosphates ensured increased buffering of the menstruum. Phosphate buffers were employed over a pH range of 3.5 to 9.5, because previous results indicated that phosphate ion is required for active transport of biotin into cells of L. arabinosus.

The results of these experiments are presented graphically in figures 15 and 16. In figure 15, the units of d-biotin converted per mg of cells are plotted against the time of incubation. The rate and magnitude of conversion at pH 4.5 through 6.5 are maximum. A 50 per cent reduction in total conversion was observed for pH 7.5 and a 75 per cent reduction for pH 3.5 and 8.5 when compared to the maximum. No significant conversion was observed at pH 9.5. A broad pH optimum ranging from pH 4.5 to 6.5 was observed, with biotin converting activity falling rapidly on either side of the plateau (figure 16). These findings agree with previous results, in that the converting system appears first at a time when the growth menstruum is in the acid range (figure 7, 15 hours).

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Figure 15. The effect of pH upon the biotin converting activity in cells of *Lactobacillus arabinosus.*

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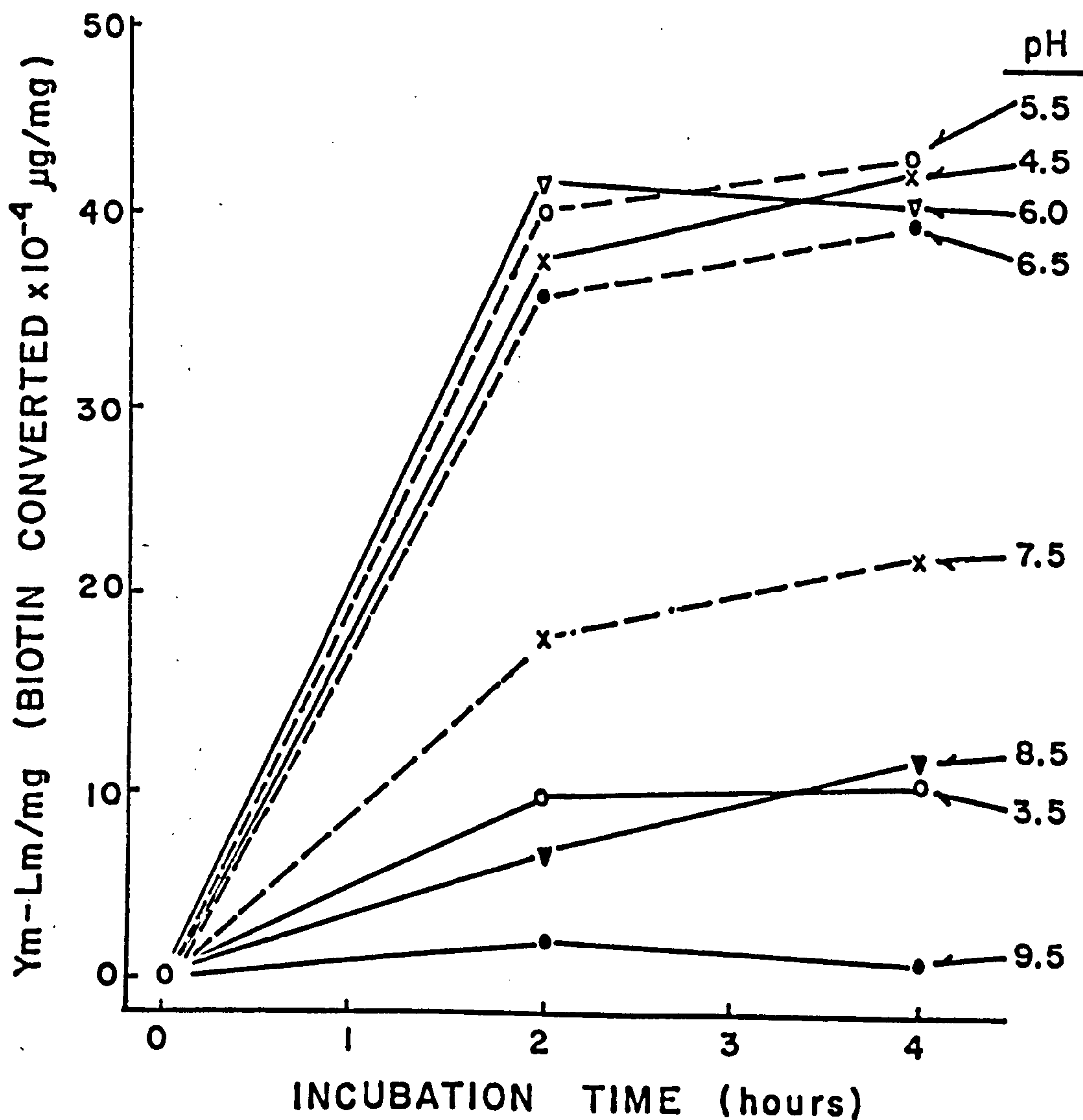

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Figure 16. The pH dependence of biotin conversion in *Lactobacillus arabinosus*.

(Conditions as for figure 15.)

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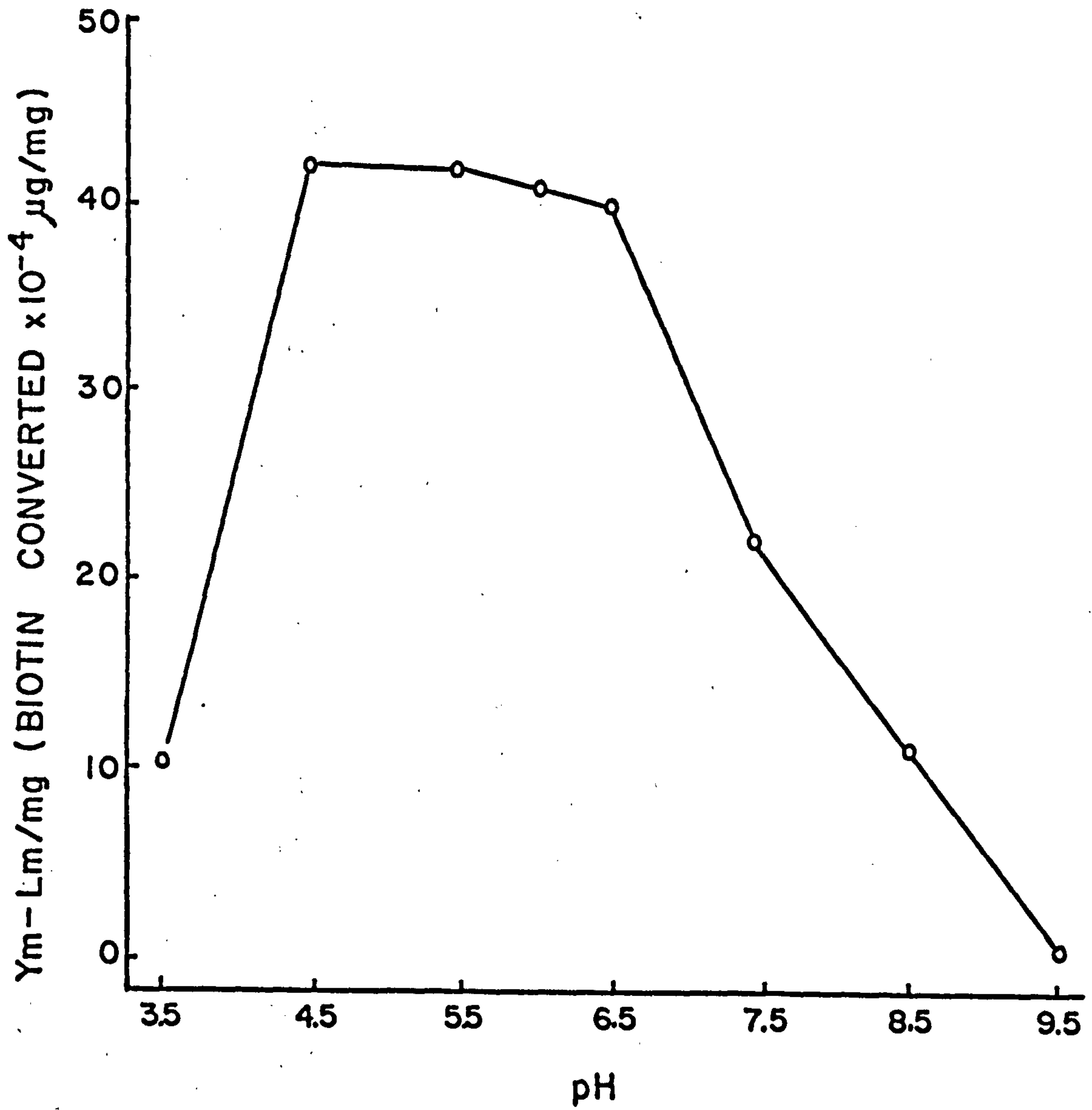

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D. Physiological Control of d-Biotin Conversion

Although the data accumulated thus far establishes the existence of a biotin converting system in *L. arabinosus*, it was nevertheless somewhat unexpected to find that this organism would convert biotin to vitamers not utilizable for its own growth. It seemed appropriate therefore to seek an explanation for the apparent control of biotin conversion in this organism. Since the converting system appears at about 15 hours of incubation (figure 7) and since the stationary phase of growth does not occur until approximately 20 hours (figure 6) it is reasonable to assume that physiological conditions for growth and metabolism are still appropriate. Biotin conversion was shown to have a broad pH optimum in the acid range, and becomes active when the growth menstruum is approximately at pH 4.5. These findings suggested the possibility that the formation and/or activation of the d-biotin converting system was controlled by the hydrogen ion concentration of the growth medium. Experiments were designed to test this hypothesis.

Each of two flasks containing Wright-Skeggs broth was inoculated with 0.4 mg cells of *L. arabinosus* per 100 ml of medium. The cultures differed only in the following way: Culture "A" was adjusted to pH 6.8 before inoculation and culture "B" was adjusted to pH 5.8. The flasks were incubated at 30 C under stationary conditions. Beginning at 12 hours, and for hourly intervals thereafter, turbidity

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and pH measurements were made. At each interval a small aliquot of the cell suspension was removed and assayed for biotin conversion. The changes in turbidity and pH for cultures A and B from 12 to 19 hours of growth are given in table 2. Growth initiation in culture B was slightly more rapid as evidenced by the higher turbidity reading at 12 hours. However, growth in both cultures was approximately the same thereafter. A pH differential was maintained between the two cultures over the entire period of incubation. If the appearance of biotin converting activity is dependent upon the pH of the growth menstruum, the culture which approaches the appropriate hydrogen ion concentration most rapidly will show biotin conversion first.

It can be seen from figure 17 that converting activity was detectable first in culture B, i.e., 13 hours, pH 4.52. No conversion appeared in culture A until 15 hours when the pH decreased to 4.70. Though the time of appearance of conversion differed by 2 hours, the rates of the reactions once initiated were approximately the same. This study suggests that the initiation of converting activity is dependent upon the pH of the growth menstruum, and by manipulating the hydrogen ion concentration it is possible to control the time of appearance of this system.

Inasmuch as *L. arabinosus* is a homolactic organism, the acidity produced from glucose fermentation is almost entirely lactic acid.

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TABLE II

Turbidity and pH Changes at 12 to 19 Hours of Growth in Cultures of Lactobacillus arabinosus

Hours of Growth	Culture A Initial pH, 6.8 Turb.[1]	pH	Culture B Initial pH, 5.8 Turb.	pH
12	255	5.65	275	4.70
13	285	5.35	290	4.52
14	300	4.80	305	4.35
15	312	4.70	312	4.20
16	330	4.50	327	4.05
17	335	4.45	335	4.05
18	340	4.25	340	4.00
19	345	4.10	345	4.00

[1] Klett reading at 660mμ

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Figure 17. The effect of pH upon the time of appearance of biotin conversion in *Lactobacillus arabinosus*.

(A, Wright-Skeggs medium, initial pH 6.8;
B, Wright-Skeggs medium, initial pH 5.8.)

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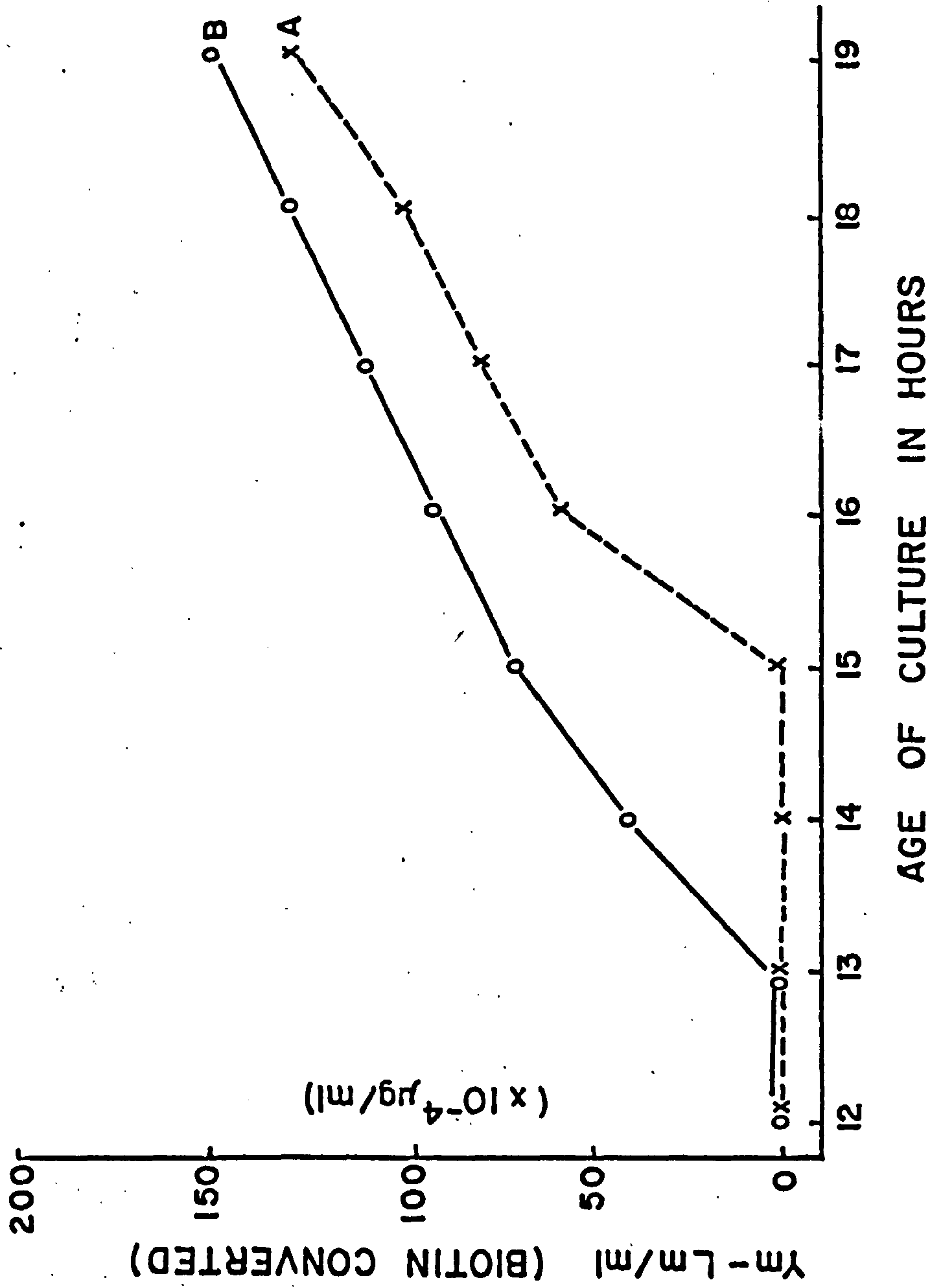
Hour	12	13	14	15	16	17	18	19
pH (A)	5.65	5.35	4.80	4.70	4.50	4.45	4.25	4.10
pH (B)	4.70	4.52	4.35	4.20	4.05	4.05	4.00	4.00



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The amount of lactic acid produced by this organism could be controlled by simply limiting the supply of glucose in the growth medium. After glucose is utilized, lactic acid production should cease and the pH of the menstruum would become stabilized. The results of such an experiment are given in table 3. It is clear from the data that growth reached a maximum at 14-15 hours which was coincident with the minimum pH of the culture. No further change in pH was therefore noted unless purposely altered as in culture B. An inspection of figure 18 reveals that the converting system appeared between 16 and 17 hours in culture B (after purposeful adjustment of the pH to 4.25) but failed to appear at all in culture A (minimum pH 4.65). Since no further growth was evident after 14 to 15 hours the cells were considered to be in a resting state. These results suggest further that decreasing pH in cultures of L. arabinosus is directly responsible for the appearance of the biotin converting system.

Since these cells were grown in a limited supply of glucose, it is probable that little or no sugar was present in the culture medium after cessation of growth. However, maximum converting activity was shown to require glucose in small quantities. A possible explanation for biotin conversion in the absence of glucose, is that the cells have stored energy in the form of labile high energy compounds such as adenosine triphosphate (ATP). A second explanation may be that an

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TABLE III

Turbidity and pH Changes at 12 to 19 Hours of Growth in Cultures of *Lactobacillus arabinosus* Containing 0.5 Per Cent Glucose

Hours of Growth	Culture A Turb.[↓]	Culture A pH	Culture B Turb.	Culture B pH
12	172	5.10	175	5.00
13	212	4.82	226	4.85
14	256	4.80	255	4.75
15	252	4.65	260	4.75
	pH not adjusted		pH adjusted to 4.25	
16	252	4.65	260	4.25
17	254	4.65	258	4.25
18	254	4.65	259	4.25
19	256	4.65	258	4.25

[↓] Klett reading at 660mμ

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Figure 18. The effect of pH upon the time of appearance of biotin conversion in cultures of *Lactobacillus arabinosus* containing 0.5 per cent glucose.

(Wright-Skeggs medium, initial pH 6.8; 0.5 per cent glucose.)

Culture A, pH not adjusted; minimal pH 4.65 at 15 hrs.
Culture B, pH adjusted to 4.25 at 15 hrs.

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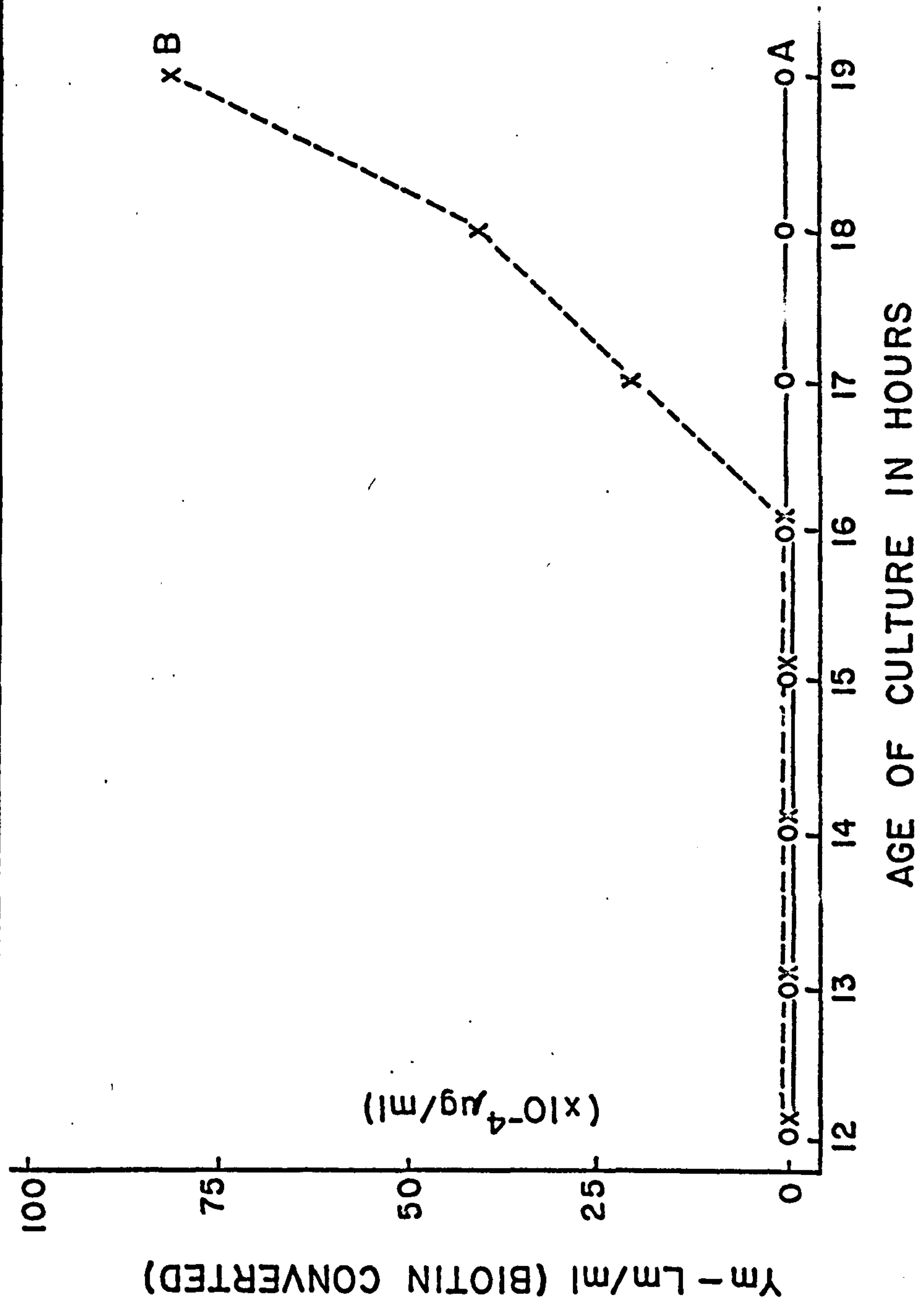
Hours	12	13	14	15	16	17	18	19
pH (A)	5.10	4.82	4.80	4.65	4.65	4.65	4.65	4.65
pH (B)	5.00	4.85	4.75	4.75	4.25	4.25	4.25	4.25



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accumulated end product of glucose metabolism is required rather than glucose itself, and that such a product may be present in sufficient quantities even after exhaustion of the carbohydrate.

If decreasing pH is a major physiological mechanism responsible for control of the appearance of the biotin converting system, it should be possible to prevent its formation by simply using strongly buffered cultures. To this end one flask of Wright-Skeggs medium containing the normal phosphate concentration (0.006 M) at pH 6.8 and a second culture containing 0.1 M phosphate buffer at the same pH were inoculated with *L. arabinosus* and incubated at 30 C. Growth initiation in the buffered culture lagged behind the control culture as can be seen by the considerably lower turbidity at 12 hours (table 4). The high concentration of phosphate is probably responsible for this lag. However, growth in culture B surpassed that in culture A between 16 and 17 hours and continued to be higher throughout the experiment. A pH differential was maintained between the two cultures. At 19 hours, the pH in culture B was 4.7 which is at the threshold of the pH required for the appearance of the conversion system. In the unbuffered culture (A) the normal decrease in pH was seen, and the critical hydrogen ion concentration (about 4.5) was reached at 14 hours. As can be seen from figure 19, the strongly buffered culture showed no biotin conversion, whereas the control culture exhibited excellent biotin converting activity beginning at 14 hours.

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TABLE IV

Turbidity and pH Changes at 12 to 19 Hours of Growth in Phosphate Buffered Cultures of <u>Lactobacillus arabinosus</u>

Hours of Growth	Culture A[1] 0.006M Phosphate		Culture B[1] 0.1M Phosphate	
	Turb.[2]	pH	Turb.[2]	pH
12	170	4.90	79	6.40
13	220	4.70	117	6.30
14	257	4.55	166	6.10
15	293	4.40	242	5.85
16	318	4.31	304	5.40
17	336	4.20	355	5.00
18	348	4.15	380	4.80
19	351	4.10	399	4.70

[1] Wright-Skeggs medium, initial ph, 6.8.

[2] Klett reading at 660mμ.

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Figure 19. The effect of phosphate buffer upon the time of appearance of biotin conversion in *Lactobacillus arabinosus*.

(Culture A, Wright-Skeggs medium, 0.006 M phosphate buffer, initial pH 6.8;
Culture B, Wright-Skeggs medium, 0.1 M phosphate buffer, initial pH 6.8.)

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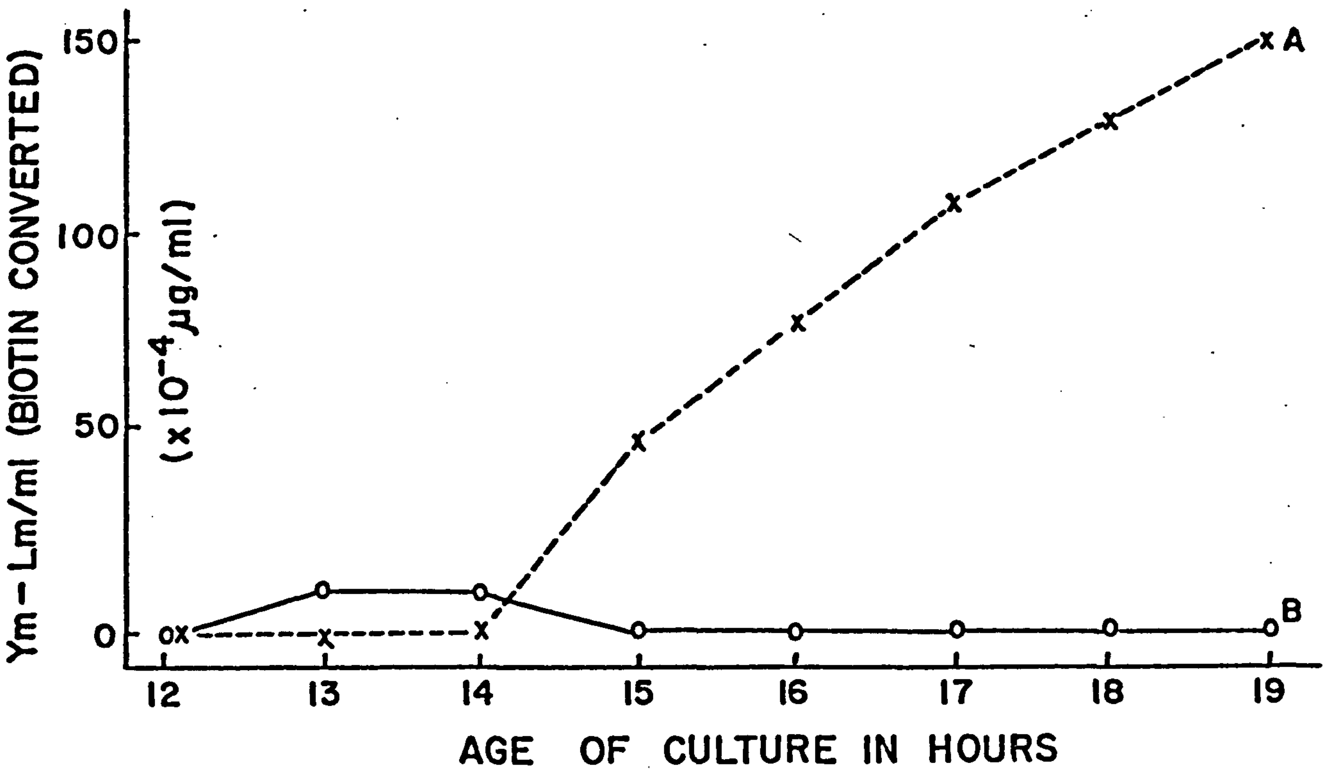
Hour	12	13	14	15	16	17	18	19
pH (A)	4.90	4.70	4.55	4.40	4.31	4.20	4.15	4.10
pH (B)	6.40	6.30	6.10	5.85	5.40	5.00	4.80	4.70



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The studies show quite conclusively that increasing hydrogen ion concentration is a major factor in the physiological control of d-biotin conversion, as well as in the regulation of cell populations in *L. arabinosus*.

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DISCUSSION

The experimental evidence presented here clearly shows that biotin is converted by cells of _L. arabinosus_ (17-5) to a vitamer(s) which is not utilizable for growth by this organism. This vitamer may, however, be utilized in place of biotin by _S. cerevisiae_ (139). It is easily detected in the presence of biotin by a differential microbiological assay procedure based on the fact that _L. arabinosus_ will respond to d-biotin while the yeast will respond to both d-biotin and the vitamer(s). The difference in values between the two assays is an expression of the growth promoting ability of the vitamer(s) for _S. cerevisiae._

Nearly all of the vitamer is found in the surrounding menstruums, a small perhaps insignificant amount is in the free intracellular form, and none appears in the bound form (coenzyme) until 48 hours of growth. Studies on the biotin content in cultures of _L. arabinosus_ showed quite clearly that the conversion begins at approximately 15 hours and terminates at 22 to 23 hours. This period represents the late log or early stationary phase of the growth cycle. During this time only 15 to 20 per cent of the biotin supplied initially to the organism is converted to the vitamer, and no further conversion appears after 23 hours. Thus the conversion is maintained in an equilibrium which greatly favors

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d-biotin. Studies with non-proliferating cells indicate that the free intracellular levels of biotin must be at a maximum before active conversion ensues.

The data obtained from studies on the parameters of the biotin conversion system compare reasonably well with the general characteristics of enzymes. Conversion could be demonstrated only in Wright-Skeggs medium suggesting that some component is required for biotin conversion. Cofactor requirements either inorganic or organic or both, are characteristic of many enzyme systems.

Temperature curves similar to those exhibited by biotin conversion in _L. arabinosus_ are characteristic of enzyme reactions for two reasons, the first being the comparatively high temperature coefficient, and the second being the dramatic decrease in activity at 50 C. Enzymatic reactions behave like ordinary chemical reactions in that, as the temperature is increased, the rate increases. Enzymes however, suffer from thermal inactivation at higher temperatures due to their proteinaceous nature, and hence a decrease in reaction rate may be noted.

The requirement for glucose is suggestive also of enzymic action, since catabolic energy is often necessary. Where the reaction appears to proceed in the absence of glucose, the involvement of stored energy (ATP) is entirely possible.

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The data obtained for the pH dependence of biotin conversion are also consistent with the participation of an enzyme. A broad pH optimum was observed which is characteristic of many enzymes (*e.g.*, catalase). In a consideration of the effect of pH on the rate and magnitude of enzyme-catalyzed reactions, at least three factors must be borne in mind. The first of these is the influence of pH on the stability of the enzyme. There are many pH-dependence curves in which the drop in rate on one side of the optimum is due to enzyme inactivation. A second factor stems from the fact that enzymes, like other proteins, are multivalent dipolar ions. Their dissociation depends, therefore, on the pH of the medium. Although it is not yet possible to specify precisely the nature of the protein groups involved in the formation of enzyme-substrate-complexes it has been assumed that the steep portion of a pH-dependence curve corresponds to the dissociation of ionizing groups on the enzyme. The third factor of importance is the dissociation of the substrate which may affect its affinity for the enzyme surface. The pH dependence curve obtained for biotin conversion exhibits also the sharp decrease in activity on either side of the broad optimum.

In summary then it may be concluded that the conversion of d-biotin to one or more vitamers is an enzyme mediated reaction. This enzyme has a pH optimum of 4.5 to 6.5 and a temperature optimum of 37 C. It requires small amounts of glucose for maximal activity, and

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requires also a "cofactor" present in Wright-Skeggs medium.

Hydrogen ion concentration of the growth medium has been demonstrated in these studies, to be the major physiological mechanism controlling the appearance and activity of the biotin converting system. The production of lactic acid by L. arabinosus causes a decrease in pH from 6.8 initially to about 4.5 (late log phase). The latter pH represents the critical hydrogen ion concentration at which the converting enzyme appears. At this time, the biotin vitamer may be detected in the growth menstruum by use of the differential microbiological assay.

It is interesting to note that the optimum range for converting activity is pH 4.5 to 6.5, but the converting enzyme(s) appears only when the pH of the growth medium has dropped to about 4.5. This suggests that the pH range necessary for the formation of an enzyme need not be the same as the range in which the enzyme will exhibit maximal activity. This finding suggests further that the physiological factors affecting the activity of an enzyme are not necessarily the same as the factors controlling its formation.

The reasons for cessation of bacterial multiplication in ordinary batch cultures have never been fully established. Most writers (49, 50) who discuss this problem mention several or all of the following possibilities: exhaustion of a limiting nutrient, accumulation of toxic substances, lack of available oxygen, and limited "biological space" (51). However, increasing hydrogen ion concentration and increasing levels of non-

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ionizable lactic acid appear to be mainly responsible for the cessation of growth in Streptococcus lactis (52). L. arabinosus, being a homo-lactic organism, should be subject also to control by these factors. However, the formation of non-ionizable lactic acid may be eliminated as an important factor. In these studies, buffered cultures showed increased growth over non-buffered controls even though the lactic acid content in the former would be equal to or higher than the latter. It appears that increasing hydrogen ion concentration is the major factor responsible for the cessation of growth in L. arabinosus.

Just how decreasing pH affects the level of cell populations is for the most part unknown, but some speculations may be offered. It is possible that decreasing pH inactivates certain enzymes that are vital to the growth or reproductive process. Hydrogen ion concentration can affect markedly the ionization of enzyme proteins and/or substrates in such a manner as to inhibit their reactions. It is for this reason that enzymes exhibit pH-dependence curves. Another possibility is that decreasing pH may induce the formation of enzyme systems which are directly responsible for "shutting-off" growth. It is to this category that the biotin converting enzyme(s) may belong. It has been stated previously that the biotin converting enzyme is physiologically controlled by hydrogen ion concentration, and that the decreasing pH is the major factor responsible for cessation of growth. Therefore it appears that the formation of this system may be significantly correlated with the

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cessation of growth. Just how the products of the vitamer forming reaction act to inhibit further growth is unknown. The converting enzyme may be one of many systems that appear only when the pH of the growth menstruum has fallen to a critical level, and which thus function in the control of cell populations. The concept of enzymes appearing during the stationary phase is not limited to biotin conversion but has been demonstrated with other systems. For example, Nomura and Yoshikawa (53) have shown that two enzymes (amylase and protease) are formed post-logarithmically in the growth cycle of *Bacillus subtilis*.

The vitamers formed as a result of biotin conversion in the present study may be characterized by microbiological activity. It has been established that the vitamer is completely inactive in replacing biotin in the growth of *L. arabinosus*, but is active for *S. cerevisiae*. The chemical nature of this vitamer(s) is presently unknown. The possibility of oxybiotin being this vitamer may be eliminated, since it has never been found in natural materials and is only 10 - 25 per cent as active as biotin in the growth of *S. cerevisiae* and only partially active for many lactobacilli (32). Biotin sulfoxide may be eliminated also since it is as effective as biotin in the growth of *L. arabinosus* (30). Biocytin comes close to matching the microbiological activity (24, 54) of the vitamer, but may be eliminated since biocytin is found generally in the bound form. The liberation of biotin from bound forms at 48

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hours of growth (bound forms at this time contain the vitamer) by acid hydrolysis should yield the same biotin levels with both assays if biocytin is the vitamer. However, the levels were significantly higher with the yeast assay, suggesting that biocytin could not be the vitamer. Desthiobiotin exhibits the same activity as biotin for S. cerevisiae, is completely inactive for L. arabinosus, and will not act as an antibiotin in either organism (19, 21, 23, 35, 38). Though desthiobiotin has not been isolated in pure form from natural materials, it has been demonstrated chromatographically in extracts of microbes and mammalian tissue (20). Desthiobiotin is the naturally occurring vitamer of biotin that most closely resembles that detected in this study. The final solution to this aspect must await isolation and characterization of the substance produced from biotin by L. arabinosus.

At variance with the results observed in this study, are those obtained by Krueger and Peterson (23), employing Lactobacillus pentosus. L. pentosus is considered to be very similar to L. arabinosus, if not identical (55). In their study biotin was provided in the growth medium in concentrations many times higher than that required for maximal growth. The biotin was not converted to other forms but was metabolized by the growing cells possibly as a carbon source. Several differences exist between their experimental methods and those employed in this study. The growth medium used by Krueger and Peterson contained only one per cent glucose as a carbon and energy source. Glucose, when provided

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at this concentration, would be depleted from the growth medium early in the stationary phase, since the amount of glucose required to give maximum growth is only slightly less than one per cent. In their studies, samples were taken for biotin analysis at 72 hours which represents a stage of growth far beyond the beginning of the stationary phase. Therefore, it is probable that the organisms utilized other compounds to provide energy for maintenance. Biotin may well have been so utilized because it was present in such excess.

In the preset studies twice the glucose concentration (2 per cent) was supplied to the growing organism, so that the energy source would not become limiting at any time during the growth cycle. There would be no need for the cell to call upon other substances such as biotin to provide a source of either energy or carbon building blocks. Experiments in the present study that did employ the lesser concentration (one per cent) were carried out only to 19 hours of growth when the energy source was not yet limiting. Possibly the vitamers noted in the present study may have been detected by Krueger and Peterson if analyses were carried out earlier than 72 hours.

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SUMMARY

S. cerevisiae, strain 139, utilizes d-biotin and several vitamers of biotin whereas *L. arabinosus*, strain 17-5, responds to d-biotin and biotin sulfoxide of the naturally occurring forms of the vitamin. The use of these organisms for differential assay permitted investigation into the conversion of d-biotin to biotin vitamers by cells of *L. arabinosus*. The studies herein presented clearly show that biotin is converted by *L. arabinosus* to a vitamer(s) which is not utilizable for growth by this organism.

1. The conversion appears in the late log phase of the growth cycle and reaches an equilibrium in favor of d-biotin (*i.e.*, 80 - 85 per cent d-biotin to 15 per cent biotin vitamer).

2. Nearly all of the vitamer is found in the growth menstruum during the late log phase, and does not appear in the bound form until the late stationary phase.

3. Biotin conversion in non-proliferating cells could be demonstrated only in Wright-Skeggs medium, and when free intracellular biotin was at a maximum.

4. The conversion of biotin appears to have characteristics of an enzyme system, *i.e.*, a temperature optimum at 37 C and a broad pH optimum of 4.5 to 6.5. Glucose is required for maximal activity.

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5. Experiments on the effect of growth pH on the time of appearance of the converting system suggest that increasing hydrogen ion concentration may be a major physiological mechanism responsible for the control of this system.

6. A suggested physiological role for the d-biotin converting system is the control of cell populations in cultures of L. arabinosus.

7. The biotin vitamer formed as a result of the converting process was characterized on the basis of microbiological activity and was found to resemble desthiobiotin.

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BIBLIOGRAPHY

1. Wildiers, E. (1901) as cited in Beerstecher, E. and Shive, W. (1950). The Biochemistry of the B Vitamins. p. 452, Reinhold Publ. Corp. N.Y.
2. Kogl, F. and Tonnis, B. (1936). Uber das Biosproblem, Z. Physiol. Chem. 242, 43-73.
3. duVigneaud, V. (1942). The structure of biotin. Science 96, 455-461.
4. duVigneaud, V. Hofmann, K. Melville, D.B. Gyorgy, P. (1941). Isolation of biotin (vitamin H) from liver. J. Biol. Chem. 140, 643-651.
5. Hofmann, K. Dickel, D.F. and Axelrod, A.E. (1950). The preliminary characterization of two biotin containing fractions in beef liver. J. Biol. Chem. 183, 481-487.
6. Burk, D. Winzler, R.J. and duVigneaud, V. (1941). The role of biotin in fermentation and the Pasteur effect. J. Biol. Chem. 140, xxi - xxii.
7. Koser, S.A. Wright, H.M. and Dorfman, A. (1942). Aspartic acid as a partial substitute for the growth-stimulating effect of biotin in *Torula cremoris.* Proc. Soc. Exptl. Biol. Med. 51, 204-205.
8. Winzler, R.J. Burk, D. and duVigneaud, V. (1944). Biotin in fermentation, respiration, growth and nitrogen assimilation by yeast. Arch. Biochem. 5, 25-47.
9. Stokes, J.L. Larsen, A. and Gunness, M. (1947). Biotin and the synthesis of aspartic acid by microorganisms. J. Bacteriol. 54, 219-230.
10. Lichstein, H.C. and Umbreit, W.W. (1947). A function for biotin. J. Biol. Chem. 170, 329-336.

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11. Shive, W. and Rogers, L.J. (1947). Involvement of biotin in the biosynthesis of oxalacetic and alpha ketoglutamic acids. J. Biol. Chem. 169, 453-454.

12. Lynen, F. Knappe, J. and Lorch, E. (1962). Mechanism of action of the water soluble vitamins. Ciba Foundation, Study group no. 11, Little, Brown and Company.

13. Waktl, J.S. and Gibson, D.M. (1960). Studies on the mechanism of fatty acid synthesis. VIII. The participation of protein-bound biotin in the biosynthesis of fatty acids. Biochim. Biophys. Acta 41, 122-129.

14. Kaziro, Y. Leone, E. and Ochoa, S. (1960). Biotin and propionyl carboxylase. Proc. Nat. Acad. Sci. 46, 1319-1327.

15. Utter, M.F. and Keech, D.B. (1960). Formation of oxalacetate from pyruvate and CO_2. J. Biol. Chem. 235, PC 17.

16. Keech, D.B. and Utter, M.F. (1963). Pyruvate carboxylase. I. Nature of the reaction. J. Biol. Chem. 238, 2603-2608.

17. Swick, R.N. and Wood, H.G. (1960). The role of transcarboxylation in propionic acid fermentation. Proc. Nat. Acad. Sci. 46, 28-41.

18. Burk, D. Hesselbach, M.L. MacNeary, D.F. and Winzler, R.J. (1944). Section on Biology. Trans. N.Y.Acad. Sci. 6, 275-283.

19. Burk, D. and Winzler, R.J. (1943). Heat labile, avidin uncombinable species specific and other vitamers of biotin. Science 97, 57-60.

20. Eisenberg, M.A. (1963). Biotin biosynthesis. I. Biotin yield and vitamers in cultures of *Phycomyces blakesleeanus*. J. Bacteriol. 86, 673-680.

21. Lilly, V.G. and Leonian, L.H. (1944). The antibiotin effect of desthiobiotin. Science 99, 205-206.

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22. Duschinsky, R. Dolan, L.A. Flower, D. and Rubin, S.H. (1945). "O-Heterobiotin", a biologically active oxygen analog of biotin. Arch. Biochem. 6, 480-481.

23. Krueger, K.K. and Peterson, W.H. (1948). Metabolism of biotin and oxybiotin by Lactobacillus pentosus 124-2. J. Bacteriol. 55, 693-703.

24. Wright, L.D. Cresson, E.L. Skeggs, H.R. Wood, T. R. Peck, R.L. Wolf, D. E. and Folkers, K. (1950). Biocytin, a naturally occurring complex of biotin. J. Am. Chem. Soc. 72, 1048.

25. Thoma, R.W. and Peterson, W.H. (1954). The enzymatic degradation of soluble bound biotin. J. Biol. Chem. 210, 569-579.

26. Chang, W.S. and Peterson, W.H. (1951). Purification of bound forms of biotin. J. Biol. Chem. 193, 587-595.

27. Gyorgy, P. and Rose, C. S. (1942). Distribution of biotin and avidin in hens egg. Proc. Soc. Exptl. Biol. Med. 49, 294-298.

28. Dittmer, K. Melville, D. B. and duVigneaud, V. (1944). The possible synthesis of biotin from desthiobiotin by yeast and the antibiotin effect of desthiobiotin for L. casei. Science 99, 203-205.

29. Leonian, H. L. and Lilly, V.G. (1945). Conversion of desthiobiotin into biotin or biotin like substances by some microorganisms. J. Bacteriol. 49, 291-297.

30. Melville, D.B. Genghoff, D.S. and Lee, J.M. (1954). Biological properties of biotin d and l sulfoxides. J. Biol. Chem. 208, 503-512.

31. Hofmann, K. McCoy, R.H. Felton, J.R. Axelrod, A.E. and Pilgrim, F.G. (1945). The biological activity of oxybiotin for the rat and chick. Arch. Biochem. 7, 393-396.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

32. Winick, T. Hofmann, K. Pilgrim, F.J. and Axelrod, A.E. (1945). The microbiological activity of DL-oxybiotin and related compounds. J. Biol. Chem. 161, 405-410.

33. Pilgrim, F.J. Axelrod, A.E. Winnick, T. and Hofmann, K. (1945). The microbiological activity of an oxygen analog of biotin. Science 102, 35-36.

34. Rubin, S.H. Flower, D. Rosen, F. and Drekter, L. (1945). The biological activity of O-heterobiotin. Arch. Biochem. 8, 79-90.

35. Melville, D.B. Dittmer, K. Brown, G.B. and duVigneaud, V. (1943). Desthiobiotin. Science 98, 497-499.

36. Tatum, E. (1945). Desthiobiotin in the biosynthesis of biotin. J. Biol. Chem. 160, 455-459.

37. Stokes, J. L. and Gunness, M. (1945). The microbiological activity of synthetic biotin its optical isomers and related compounds. J. Biol. Chem. 157, 121-126.

38. Dittmer, K. and duVigneaud, V. (1944). Antibiotins. Science 100, 129-131.

39. Axelrod, A.E. Flinn, B.C. and Hofmann, K. (1947). The metabolism of oxybiotin in yeast. J. Biol. Chem. 169, 195-202.

40. Lichstein, H.C. (1955). The biotin requirements of the genus Propionibacterium. Arch. Biochem. Biophys. 58, 423-430.

41. McIlwain, H. and Hughes, D.E. (1944). Biochemical characterization of the actions of chemotherapeutic agents. I. Measurement of growth of streptococcal cultures through their gaseous metabolism, and the effects of pantothenate and pantoglutaurine upon the metabolism and growth. Biochem. J. 38, 97-105.

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42. Metzger, W.I. (1947). Microbic decomposition of pantothenic acid. J. Bacteriol. 54, 135-148.

43. Koser, S.A. and Baird, G.R. (1944). Bacteriol destruction of nicotinic acid. J. Inf. Dis. 75, 250-261.

44. Foster, J. W. (1944). Microbial aspects of riboflavin. J.Bacteriol. 47, 27-41.

45. Mirick, G.S. (1943). The oxidation of p-aminobenzoic acid and anthranilic acid by specifically adapted enzymes of a soil bacillus. J. Expt. Med. 78, 255-272.

46. Wright, L.D. and Skeggs, H.R. (1944). Determination of biotin with Lactobacillus arabinosus. Proc. Soc. Exptl. Biol. Med. 56, 95-98.

47. Hertz, R. (1943). Modification of the yeast growth assay method for biotin. Proc. Soc. Exptl. Biol. Med. 52, 15-17.

48. Lichstein, H.C. and Waller, J.R. (1961). Factors affecting the accumulation of biotin by Lactobacillus arabinosus. J. Bacteriol. 81, 65-69.

49. Sinclair, N. A. and Stokes, J.L. (1962). Factors which control maximal growth of bacteria. J. Bacteriol. 83, 1147-1154.

50. Freter, R. and Ozawa, A. (1963). Explanation for limitation of populations of Escherichia coli in broth cultures. J. Bacteriol. 86, 904-910.

51. Bail, O. (1929). Ergebnisse experimenteller Populations forschung. Z. Immunitaetsforsch 60, 1-22.

52. Rogers, L.A. and Whittier, E.O. (1928). Limiting factors in the lactic fermentation. J. Bacteriol. 16, 211-229.

53. Nomura, M. and Yoshikawa, H. (1959). Stimulation of amylase formation by a ribonucleic acid preparation from Bacillus subtilus. Biochim. Biophys. Acta 31, 125-134.

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54. Wright, L.D. Cresson, E.L. Liebert, K. V. and Skeggs, H. R. (1952). Biological studies of biocytin. J. Am. Chem. Soc. 74, 2004-2006.

55. Breed, R.S. Murry, C.G. and Smith, N.R. (1957). Bergey's Manual of Determinative Bacteriology. 7th ed. p. 549. Williams and Wilkins Co., Baltimore.

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