JOURNAL OF BACTERIOLOGY, Apr., 1965
Vol. 89, No. 4

Conversion of D-Biotin to Biotin Vitamers by *Lactobacillus arabinosus*

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Received for publication 27 November 1964

ABSTRACT

BIRNBAUM, JEROME (University of Cincinnati College of Medicine, Cincinnati, Ohio), AND HERMAN C. LICHSTEIN. Conversion of D-biotin to biotin vitamers by *Lactobacillus arabinosus*. J. Bacteriol. **89**:1035–1040. 1965.—*Saccharomyces cerevisiae* utilizes D-biotin, biotin sulfoxide, and several vitamers of biotin, whereas *Lactobacillus arabinosus* responds to biotin and biotin sulfoxide of the naturally occurring forms of the vitamin. The use of these organisms for differential assay permitted investigation into possible conversion of D-biotin to vitamers of biotin by cells of *L. arabinosus*. Cells were grown in modified Wright-Skeggs medium containing several levels of biotin. No differences were detected between the assays for free intracellular or bound biotin up to 48 hr of growth. However, at 15 to 16 hr, in media containing an excess of biotin, the lactobacillus assay value for menstruum biotin dropped markedly, whereas the yeast assay showed no change. This suggested that biotin was converted to vitamers not active for *L. arabinosus*. The biotin-converting system appears to have characteristics of an enzyme system, i.e., a temperature optimum at 37 C, a broad *p*H optimum of 4.3 to 6.5, and requirements for Mg and Mn ions. Experiments suggest that increasing hydrogen ion concentration is a major physiological mechanism controlling the formation of this system. The vitamers were separated chromatographically and were found to have R_F values of 0.44 and 0.94, and are combinable and uncombinable with avidin, respectively. The physiological role of biotin conversion is discussed in relation to control of cell populations in cultures of *L. arabinosus*.

Burk and Winzler (1943) demonstrated the presence of several vitamers of biotin, uncombinable with avidin, in the urine of many mammals. These vitamers, which they identified as miotin, tiotin, and a dicarboxylic acid derivative of biotin, were shown to be converted by growing cells of *Saccharomyces cerevisiae* into forms combinable with avidin which were indistinguishable from biotin. More recently, Dhyse and Hertz (1958) detected a vitamer, uncombinable with avidin, in culture fluids of *Escherichia coli* K-12, which was capable of replacing biotin for the growth of *S. cerevisiae*, but ineffective for *Lactobacillus arabinosus*. Employing the same assay system, Eisenberg (1963) demonstrated that an unknown vitamer, synthesized by cells of *Phycomyces blakesleeanus*, promoted growth of the yeast but not of the lactobacillus. Thus, vitamers of biotin may appear frequently in the medium of organisms capable of synthesizing biotin, and are detected by means of a differential microbiological assay.

While studying the metabolism of biotin in *L. arabinosus*, a fastidious organism requiring several B vitamins in addition to biotin, it was observed that the recovery of the vitamin was low when assayed with the lactic organism. This finding suggested the possibility that biotin was converted by *L. arabinosus* to other forms of the vitamin which were not utilizable for its own growth. In the present study, the yeast-lactobacillus differential assay was employed to investigate further this unexpected phenomenon.

MATERIALS AND METHODS

Culture methods. L. arabinosus strain 17-5 (ATCC 8014) was maintained on APT agar (Case Labs, Chicago, Ill.) as a stab culture. For experimental work, the organism was grown in the medium of Wright and Skeggs (1944) containing 0.1 μg of biotin per ml and modified as follows: cysteine substituted for cystine, and folic acid added at 0.5 mg per liter to improve growth. The medium was adjusted to *p*H 6.8 and autoclaved at 121 C for 15 min, after which glucose was added aseptically. A 100-ml amount of medium in 250-ml Erlenmeyer flasks was inoculated with a washed-cell suspension of *L. arabinosus* equivalent to 0.4 mg (dry weight) and incubated at 30 C under stationary conditions.

Treatment of samples for biotin analysis. After incubation, the cultures were centrifuged in the cold for 10 min, and the supernatant medium was recovered and frozen for analysis of biotin at a

later time (menstruum biotin). The centrifuge tube was rinsed quickly with cold saline to remove any biotin remaining on the walls, and the pellet was suspended in distilled water. The tube was then placed in a boiling-water bath for 10 min, centrifuged, and the supernatant fluid was assayed for free biotin. The pellet was washed twice, suspended in 6 N H_2SO_4, and autoclaved for 60 min at 121 C. The autoclaved suspension was neutralized with 6 N NaOH and filtered through Whatman no 1. paper to remove cell debris. The final filtrate represented the bound biotin fraction. Thus, free biotin is defined as that amount which is extracted from the cells by boiling, and bound biotin is that amount which is released from the cells only after the acid treatment.

Microbiological assays. L. arabinosus was employed both for the assay of biotin and as the experimental organism. The general assay procedure of Wright and Skeggs (1944) was utilized, except that the modified medium was employed and bacterial growth was determined turbidimetrically in a Klett-Summerson photoelectric colorimeter at 660 mμ. The term true biotin will refer to those vitamers capable of supporting the growth of *L. arabinosus*, and includes biotin and biotin D- and L-sulfoxides. *S. cerevisiae*, Fleischman strain 139 (ATCC 9896), was employed also for the assay of biotin, according to the general procedures of Hertz (1943). In this case, growth was measured at 420 mμ. The yeast utilizes a much greater spectrum of vitamers than does the lactobacillus. For example, *S. cerevisiae* utilizes biotin, desthiobiotin, biocytin, biotin D- and L-sulfoxides, diaminobiotin, diaminopelargonic acid, and a number of unknown vitamers (Eisenberg, 1963). Therefore, the values obtained with the yeast assay are representative of total biotin. Any significant difference between total and true biotin values was considered indicative of the presence of vitamers utilizable by the yeast but not by the lactobacillus.

Chromatography. Descending paper chromatography employed a solvent system composed of *n*-butanol-water-acetic acid (4:5:1) and Whatman no. 1 filter paper. The solvent front was permitted to travel about 30 cm (10 to 11 hr) at room temperature. After drying, the chromatograms were subjected to a bioautographic technique, with *S. cerevisiae* and *L. arabinosus* as the test organisms. The paper strips were cut into 1-cm sections and placed in separate tubes, where they were eluted with distilled water. The eluates were autoclaved, cooled, and mixed with an equal volume of double-strength assay medium seeded with the respective organisms; incubation was overnight at 30 C. The tubes were then agitated to suspend the cells, and the paper sections were carefully removed before turbidity measurements were made. When the turbidity was plotted as a function of the distance from the origin, a smooth curve connecting the points permitted localization of the R_F by interpolation to within 0.02 units. The R_F values obtained by this procedure compared favorably with those reported in the literature (Wright, Cresson, and Driscoll, 1954; Waite and Wakil, 1963; Eisenberg, 1963).

RESULTS

Conversion of biotin by growing cells. Initial experiments were designed to follow the distribution of biotin (1,000 × 10^{-4} μg/ml of medium) during the growth of *L. arabinosus*. It is clear that the per cent recovery of total biotin at both 24 and 48 hr was low when assayed with the lactic organism (Table 1). An inspection of the values for menstruum biotin reveals that the variance between the assays for this fraction was, for the most part, responsible for the differences in the recovery. The lactobacillus assay value for menstruum biotin was about 18% lower at both growth periods than the values obtained with the yeast assay. For 24-hr cells, there was good agreement between the assays for free intracellular and bound biotin; however, at 48 hr, the values with the lactobacillus assay were significantly lower than those of the yeast assay.

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 during growth. If

TABLE 1. *Distribution of biotin during growth of Lactobacillus arabinosus*

Assay organism	Hr	Biotin fraction			Total biotin recovery
		Free intracellular (× 10^{-4} μg/mg)	Bound cellular (× 10^{-4} μg/mg)	Menstruum (× 10^{-4} μg/ml)	
					%
Lactobacillus arabinosus	24	12.7	25.5	760	79.7
	48	10.8	18.3	770	81.2
Saccharomyces cerevisiae	24	14.3	25.5	925	96.4
	48	15.6	25.5	925	96.9

such vitamers were biologically less active or totally inactive for *L. arabinosus*, while still utilizable by *S. cerevisiae*, differences would appear between the assays. On the basis of this assumption, the data of Table 1 suggest that conversion of biotin to vitamers occurred before 24 hr of growth, and that the vitamers accumulated in the growth medium. The vitamers did not appear intracellularly at 24 hr, as evidenced by the close similarity of free and bound biotin with both assays. However, they did appear within the cells at 48 hr, as shown by the significantly higher values for free and bound biotin when assayed with the yeast.

To ascertain more precisely the time at which conversion of biotin occurred, the course of free, bound, and menstruum biotin was followed, beginning at 12 hr of growth. No differences were detected between the two assays for free or bound cellular biotin for any period between 12 and 23 hr, which suggested the presence of only D-biotin in these fractions. However, marked differences were noted in menstruum biotin, as revealed by the disparity in assay values obtained by the two methods. Whereas the yeast-assayable biotin content of the menstruum remained constant, the lactobacillus-assayable biotin dropped sharply after 16 hr (Fig. 1). It appeared, therefore, that the conversion of biotin began at about 16 hr and continued up to about 21 hr, which corresponds to the late exponential and early stationary phases of growth.

Conversion was quantitated by subtracting the *L. arabinosus* assay values for menstruum biotin (true biotin) from the *S. cerevisiae* values (total biotin), i.e., biotin converted ($\times 10^{-4}$ μg/ml) equals total biotin minus true biotin. The difference in values between the assays is, therefore, an expression of the growth-promoting ability of the vitamer(s) for the yeast.

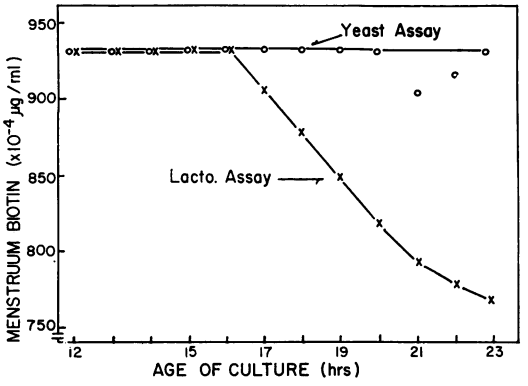

FIG. 1. *Recovery of menstruum biotin during growth of Lactobacillus arabinosus. Initial biotin concentration 1,000 $\times$ 10^{-4} μg/ml.*

Conversion of biotin by nonproliferating cells. Attempts to prepare nonproliferating (resting) cell suspensions active in the conversion of biotin employed first 0.1 M acetate buffer (*p*H 4.5 to 5.0) containing 200 $\times$ 10^{-4} μg of biotin per ml as substrate and 0.1% glucose. Incubation was at 37 C for 6 hr. Acetate was chosen because it proved to be nontoxic to the cells and was found normally in Wright-Skeggs medium. The acid *p*H was selected since the conversion of biotin appeared in the late exponential phase when the *p*H of growth had dropped from 6.8 to about 4.5. Glucose was included as an energy source. However, cells suspended in this menstruum failed to convert biotin. Subsequent experiments employed the same reaction mixture with the addition of 10^{-3} M KH_2PO_4 to satisfy the requirements of the biotin-transport system (Lichstein and Waller, 1961). Though accumulation of the vitamin was improved by the addition of phosphate, no vitamers of biotin were produced. This suggested that some component(s) of the growth medium was required for activity of the converting system. The addition of Mg and Mn (10^{-3} M) did satisfy finally the requirements for the conversion of biotin. Figure 2 shows that vitamer formation was linear for 30 to 45 min, with a reaction rate of approximately 10^{-4} μg of

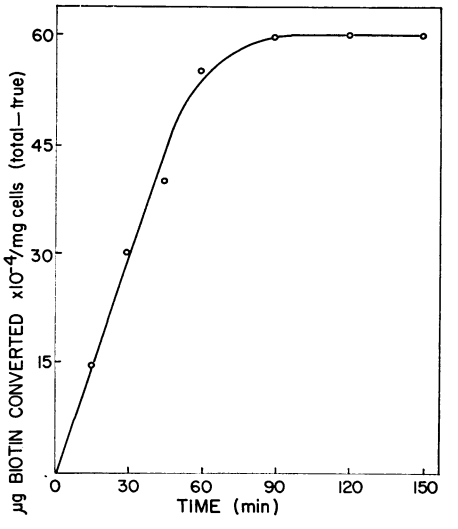

FIG. 2. *Conversion of biotin by nonproliferating cell suspensions of Lactobacillus arabinosus. Reaction mixture contains 0.1 M acetate (pH 5.0); 0.1% glucose; 10^{-3} M KH_2PO_4, $MgSO_4$, $MnSO_4$; 200 $\times$ 10^{-4} μg of biotin/ml; 17-hr cells equivalent to 1 mg (dry weight)/ml; temperature, 37 C.*

biotin converted per milligram of dry cells per minute, and that conversion began to level off at 45 to 60 min. The total amount of biotin converted (60×10^{-4} μg) represents only a small portion of the initial concentration (200×10^{-4} μg/ml). Therefore, the vitamer-forming reaction does not go to completion, but, rather, reaches an equilibrium in favor of D-biotin. Little or no increase in either turbidity or number of viable cells occurred during these experiments.

Conditions for conversion. The effects of temperature, glucose, and *p*H on the converting reaction in nonproliferating cells were studied next. Cells (17-hr) were incubated at temperatures ranging from 0 to 50 C for 2 hr, after which time the reaction menstruums were tested with the differential assay for the presence of vitamers of biotin. The system exhibited a sharp temperature optimum at 37 C, with activity falling off rapidly on both sides of this point (Fig. 3A). A Q_{10} of 2.3 was calculated between 20 and 30 C. Experiments designed to test the necessity for glucose demonstrated that vitamers were formed by 17-hr cells in the absence of carbohydrate, but maximal activity required the addition of at least 0.1% glucose. Studied also was the effect of *p*H on the converting reaction. Acetate and phosphate buffers (0.1 M) were employed. A broad *p*H optimum ranging from 4.3 to 6.5 was observed, with activity falling off rapidly on either side of this range (Fig. 3B).

Physiological control of biotin conversion during growth. The findings that conversion of biotin occurred in growing cells at a time when the *p*H of the medium had fallen to about 4.5, and that the *p*H optimum for conversion in nonproliferating cells was between 4.3 and 6.5 suggested that the formation of the 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 modified Wright-Skeggs medium was inoculated with cells of *L. arabinosus*. The cultures differed only in the initial *p*H; culture A was adjusted to *p*H 6.8 and culture B to 5.8 before inoculation. Beginning at 12 hr, and for hourly intervals thereafter, measurements were made of turbidity, *p*H, and biotin conversion (Fig. 4). A *p*H differential was maintained between the two cultures over the entire incubation period. It is manifest that converting activity was detectable first in culture B at 13 hr, at which time the *p*H was 4.52. No conversion appeared in culture A until 15 hr, when the *p*H decreased to 4.70. Other experiments demonstrated that buffered cultures, which did not reach the critical *p*H of 4.5 to 4.7, failed to show converting activity, whereas the unbuffered controls exhibited active conversion of biotin once the *p*H had dropped to this level. These studies suggest that the initiation of biotin-converting activity is controlled in some manner by the *p*H of the growth menstruum.

Studies on the effect of initial biotin concentration on vitamer formation during growth revealed conversion of biotin in cultures containing 100×10^{-4} μg of biotin per ml or higher (Table 2). Whereas the absolute amount of vitamin converted increased with the concentration of biotin in the growth medium, the

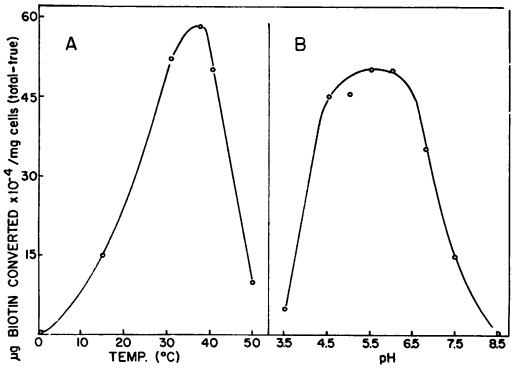

FIG. 3. *Temperature and pH dependence of biotin conversion by nonproliferating cell suspensions of Lactobacillus arabinosus. A: conditions as for Fig. 2; B: 0.1 M acetate buffers (pH 3.5 to 5.5), 0.1 M phosphate buffers (pH 6.0 to 8.5), other conditions as for Fig. 2. Reaction time, 2 hr.*

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

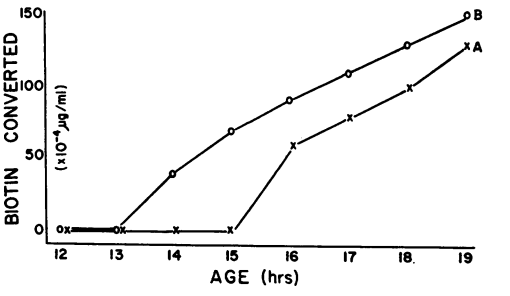

FIG. 4. *Effect of pH on time of appearance of biotin conversion in cultures of Lactobacillus arabinosus. Wright-Skeggs medium as modified in text, temperature, 30 C; initial biotin concentration, 1,000 × 10⁻⁴ μg/ml; initial pH, 6.8 (culture A) and 5.8 (culture B).*

percentage converted was highest at 500 × 10^{-4} μg/ml and decreased thereafter. It appeared, therefore, that biotin conversion begins when the vitamin is present at approximately 20-fold the minimal concentration required for maximal growth (5 × 10^{-4} μg/ml), and is most active at 100- to 200-fold this concentration. Table 3 shows the biotin-converting activity of nonproliferating cells harvested from media containing several concentrations of biotin. No activity was exhibited by cells grown at the two lowest concentrations; intermediate converting activity was obtained with cells grown at 100 × 10^{-4} μg of biotin per ml, and maximal vitamer formation was observed with cells grown at 500 to 10,000 × 10^{-4} μg/ml. These findings were, in the main, similar to those using growing cells, except that no inhibitory effect was noted at the highest concentrations.

Nature of the vitamers produced. Paper chromatography of biotin yielded two peaks of activity with both the lactobacillus and yeast assays, with R_F values of 0.57 and 0.82. The lower peak corresponded to biotin-D-sulfoxide, which is formed by the oxidation of biotin on the paper (Wright, Cresson, and Driscoll, 1954). Chromatograms of 12-hr culture supernatant fluids of *L. arabinosus* produced two peaks of activity corresponding to biotin and the sulfoxide form (Fig. 5A). Cultures (24-hr) showed two peaks at R_F 0.44 and 0.94, in addition to those for biotin and biotin sulfoxide (Fig. 5B). Thus, the presence of the vitamers in the growth medium can be detected on the chromatograms, and the negative result at 12 hr is consistent with that found in earlier experiments (Fig. 1 and 4).

Combination with avidin has been used to characterize vitamers of biotin (Sebrell and Harris, 1954). The combinability with avidin of the two unknown vitamers was tested by adding sterile avidin to each assay tube after elution of the 1-cm paper sections. The tubes then received double-strength assay medium inoculated previously with the assay organism, and were incubated at 30 C. A growth response was found only in those tubes which contained vitamers uncombinable with avidin. It is clear (Fig. 5C) that the peaks for biotin and the sulfoxide form, as well as that at R_F 0.44, had disappeared

TABLE 2. *Influence of biotin concentration in growth medium on conversion to vitamers by Lactobacillus arabinosus*

Initial biotin concn in medium (× 10^{-4} μg/ml)	Biotin converted in growth menstruum* (× 10^{-4} μg/ml)	Initial biotin converted
		%
10,000	1,300	13
1,000	160	16
500	100	20
100	10	10
10	0	0
5	0	0

* Biotin converted = lactobacillus assayable value (true biotin) subtracted from yeast assayable value (total biotin). Growth time, 19 hr.

TABLE 3. *Effect of initial biotin concentration in growth medium on biotin conversion by nonproliferating cells of Lactobacillus arabinosus*

Initial biotin concn in medium (× 10^{-4} μg/ml)	Biotin converted by nonproliferating cells* (× 10^{-4} μg/mg)
10,000	60
1,000	65
500	60
100	35
10	0
5	0

* Cultures, 19-hr; incubation time, 2 hr; other conditions as for Fig. 2.

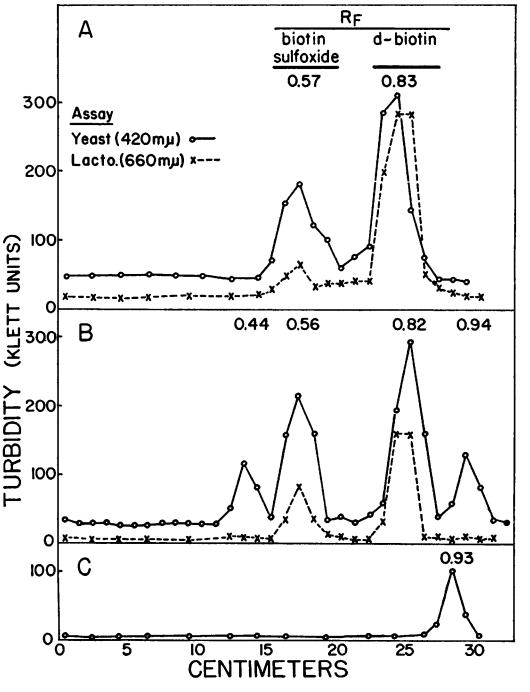

FIG. 5. *Biochromatography of culture supernatant fluids of Lactobacillus arabinosus. Growth medium contained 1,000 × 10^{-4} μg of biotin per ml; temperature, 30 C. A = 12-hr culture; B = 24-hr culture; C = 24-hr culture treated with avidin (0.02 mg per assay tube).*

when avidin was present. The second unknown vitamer having an R_F of 0.94 remained, even in the presence of avidin. These results indicate that two vitamers are formed by *L. arabinosus* when grown in excess biotin. The R_F values of these vitamers are 0.44 and 0.94; the former is combinable with avidin and the latter is uncombinable.

DISCUSSION

The data presented show that *L. arabinosus* grown in excess biotin converts a portion of this excess to two vitamers of biotin not utilizable for its growth but assayable by *S. cerevisiae*. Essentially all of the vitamers were found in the surrounding menstruum, a small, perhaps insignificant, amount intracellularly in a free form, and apparently none in the bound form until 48 hr of growth. Conversion begins at approximately 15 hr and terminates at about 21 hr, at which time only 15 to 20% of the biotin supplied initially is found as vitamers. Thus, the conversion is maintained in an equilibrium which greatly favors D-biotin. Studies with nonproliferating cells yielded results characteristic of an enzyme-mediated reaction.

Although the physiological significance of the converting reaction in *L. arabinosus* is unknown, speculations can be offered. The work of Rogers and Whittier (1928) showed that decreasing *p*H, as a result of the production of large amounts of organic acid, is a major factor responsible for the cessation of growth in cultures of lactic organisms. The appearance of the biotin-converting reaction is coincident with the cessation of bacterial growth, and is physiologically controlled by increasing hydrogen ion concentration of the growth medium. It is tempting, therefore, to speculate that this system plays some role in controlling cell populations in batch cultures of *L. arabinosus*. The converting enzymes may be one of many systems that appear only when the *p*H of the growth menstruum has fallen to a critical level. For example, Moat and Lichstein (1953) reported that the enzyme(s) converting pyruvate to acetoin in *L. arabinosus* is not formed until the growth *p*H reaches about 4.0.

Studies on the effect of initial biotin concentration on the degree of conversion (Tables 2 and 3) provide a major objection to this hypothesis. Whereas conversion was detected only when the biotin concentration in the growth medium reached 100×10^{-4} μg/ml, the extent of growth was the same at all initial levels of the vitamin in the range 5×10^{-4} to $10{,}000 \times 10^{-4}$ μg/ml. If the vitamers formed play a role in controlling growth, one might expect improved growth in the cultures not containing the vitamers. It is of interest in this regard that the natural environment for *L. arabinosus*, namely, milk and other dairy products, normally contains much more biotin than that required for maximal growth. According to Sebrell and Harris (1954), cow's milk contains from 160 to $1{,}100 \times 10^{-4}$ μg of biotin/ml, which represents a range where good converting activity was observed in batch cultures (Table 2).

ACKNOWLEDGMENT

This investigation was supported by the National Science Foundation (G-19578) and the Office of Naval Research, Department of the Navy (NR 103-555).

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