Biotin Production and Utilization in a Sewage Treatment Lagoon

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Received for publication 4 February 1971

Biotin, in a sewage oxidation lagoon also receiving potato processing wastes, was observed to increase two logs during the summer period of waste stabilization and then to decline to near earlier concentrations. Three organisms, *Aerobacter aerogenes*, *Chlorella vulgaris*, and *Thiocapsa floridana*, were at least partially responsible for these fluctuations; the latter two organisms were associated with biotin utilization and the former with biotin production. Since copious quantities of biotin are produced in these waste treatment facilities, the vitamin may act as a useful indicator of microbial action on certain organic molecules, especially in domestic and industrial wastes such as those from municipalities and potato and sugar beet processing plants. Furthermore, the presence of biotin in rivers and streams may be indicative of the discharge of incompletely stabilized wastes from these sources.

Previous studies of biotin concentration in natural waters are few. Hutchinson (5) in 1943 and Hutchinson and Setlow (6) in 1946 reported biotin in Linsley Pond and Bantam Lake in Connecticut ranging from 0.1 to 4.3 ng/liter. More recently biotin concentration in seawater has been reported by several workers. Antia (1) reported no detectable biotin at a station 43°N, 141°W in the north-eastern Pacific Ocean at depths from 5 to 3,000 m. Litchfield and Hood (8) and Carlucci and Silbernagel (2) reported biotin levels of from 0 to about 4.6 n/liter in samples from the Gulf of Mexico and north Pacific Ocean, respectively. Natarajan (9) showed detectable amounts of biotin in 38% of 21 samples in amounts ranging to 3.1 ng/liter in surface waters off southeast Alaska.

In contrast to these levels, a study by Neujahr and Hartwig (10) in Sweden indicated that samples of various fractions from a conventional sewage treatment plant yielded biotin concentrations as high as 50,000 ng/liter.

A sewage oxidation lagoon located at Grafton, N.D., yielded samples with biotin ranging from 55 to 10,800 ng/liter. This lagoon receives, in addition to domestic sewage, large quantities of potato processing wastes resulting in winter and spring anaerobic conditions followed during summer treatment by a purple sulfur bacterial phase in the primary and occasionally in the secondary cell (4). The purple sulfur phase generally gives way to the algal phase during the late summer. Concomitant stabilization of the mixture of domestic and potato processing wastes occurs.

Previous work in our laboratory has established an apparent relationship between the purple sulfur populations and acetate (4). Utilization of acetate occurs during the peaking of the purple sulfur populations. Additionally, it was suggested that formate utilization may be due to methane bacteria.

This study investigated the occurrence of the vitamin biotin in a sewage oxidation lagoon at Grafton, N.D., and in certain other aquatic environments. The production and utilization of biotin were established for several organisms isolated from the lagoon. The study included determinations on wastes from the Grafton lagoon, several other municipal and industrial oxidation lagoons in the Red River Valley of the North, the Red River which receives effluents from these municipal and industrial lagoons, and several other sources.

MATERIALS AND METHODS

Aquatic system. The two-celled sewage oxidation lagoon at Grafton, N.D., was the principal environment studied. The primary and secondary cells of this lagoon each have an area of 70 acres and were designed to be operated at liquid depths from 3 to 5 ft. Continuous flow-through from the primary to the secondary lagoon occurs. Discharge from the second-

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1 Presented at the 70th Annual Meeting of the American Society for Microbiology, Boston, Mass., 26 April-1 May, 1970.
ary lagoon is usually limited to the fall of the year after stabilization of the waste occurs (hydraulic loading may at times dictate earlier discharges). The primary cell is loaded at about 225 lb of biochemical oxygen demand (BOD) per acre per day during the processing season (September through May). Summer loadings on the primary cell are about 15 lb of BOD per acre per day [an acceptable areal loading for oxidation lagoons at this location north latitude for all seasons of the year (3)]. Lagoon samples were taken from the primary and secondary cells of this lagoon at weekly intervals during the summer of 1969.

**Biotin assay.** Biotin determinations were facilitated by the microbiological assay with *Lactobacillus plantarum* ATCC 8014 as the test organism with the addition of Tween 80 to the basal medium by the method of Waller (14).

**Biotin production.** The biotin-synthesizing ability of a variety of lagoon heterotrophs isolated in pure culture from the lagoon was determined. Pure cultures were isolated on MacConkey, eosin methylene blue, endo, nutrient, and tryptone glucose extract agar (Difco) and incubated both aerobically and anaerobically at 30°C for 24 and 48 hr. Modified Wright-Skeggs medium (15, 16) was inoculated with the heterotrophic isolates and incubated at 30°C in static culture (ca. 10^6 organisms were added to 1.5 ml tubes filled with 15 ml of medium). Samples were taken from this medium at intervals of 44 and 212 hr for biotin determinations. Uninoculated controls of medium only were also assayed for their biotin content.

**Biotin utilization.** Filtrate from lagoon samples obtained by using a type HA filter (0.45 μm; Millipore Corp., Bedford, Mass.) was adjusted to pH 7.0 by the addition of 1.0 N sulfuric acid. The ability of several organisms to utilize biotin in lagoon filtrate was determined by adding 125 ml of filtrate to each of six 125-ml Erlenmeyer flasks and incubating at 30°C as follows: in the dark, in the light (three 60-w incandescent bulbs at a distance of 18 inches (45.7 cm) from the sample), in the dark with the addition of about 10^5 *Chlorella vulgaris* (per 125 ml of filtrate), in the light with the addition of about 10^6 *C. vulgaris* (per 125 ml of filtrate), in the dark with the addition of about 10^6 *Thiocapsa floridana* (per 125 ml of filtrate), and in the light with the addition of about 10^6 *T. floridana* (per 125 ml of filtrate). An additional control consisted of an unfiltered lagoon sample incubated in the dark and light at 30°C.

**Algae and purple sulfur cultures.** The cultures of

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**Fig. 1.** Biotin, 5-day biochemical oxygen demand (BOD₅), pH, and coliform changes occurring in the Grafton primary lagoon during the summer of 1969. The predominant microbial phase is also indicated.
Fig. 2. Biotin, 5-day biochemical oxygen demand (BOD₅), pH, and coliform changes occurring in the Grafton secondary lagoon during the summer of 1969. The predominant microbial phase is also indicated.

C. vulgaris were maintained on Chlorella agar (Difco). T. floridana [previously isolated from the Grafton lagoon (4)] cultures were maintained in Pfennig's medium with a trace element supplement (12). Numbers of the algal or purple sulfur organisms were determined by microscopic direct counts utilizing a Neubauer hemocytometer and phase microscope.

**BOD₅ and coliform organisms.** The methods for determining pH, 5-day BOD (BOD₅), and coliform organisms by the membrane filter technique were used as described in Standard Methods for the Examination of Water and Waste Water (11).

**RESULTS**

**Biotin production.** Biotin production in the primary cell of the Grafton lagoon increased about 100-fold during the anaerobic and purple sulfur phases of growth. [About 10⁶ to 10⁸ purple sulfur organisms/ml are present during the purple sulfur phase (4).] Figures 1 and 2 depict the pH, BOD₅, biotin, coliform organisms, and predominant phase present in the lagoons during the summer of 1969. Reduction in BOD₅ during the period of summer treatment in the primary cell was about 95%. Biotin in the primary cell increased from 100 to about 10,000 ng/liter during the anaerobic and purple sulfur phases of growth. During the terminal aspects of purple sulfur growth (4), biotin removal became apparent with 99% of the biotin present being utilized. Similarly, the levels of biotin in the secondary cell, which contained less organic material (as determined by BOD₅ values), also approached 10,000 ng/liter during the anaerobic and purple sulfur phases of growth and remained high until just before emergence of the algal phase [ca. 10⁶ algae/ml are present during this phase of lagoon treatment (4)], when utilization of about 99% of the biotin present was realized. The BOD₅ reduction in the secondary cell was only about 70% during the summer treatment period.

Biotin production by lagoon heterotrophs was shown by at least three isolates (Table 1). Two *Aerobacter* species and an *Enterococcus* species produced the greatest amounts of biotin after 44 hr of incubation in modified Wright-Skeggs medium (15, 16). Another organism (a *Salmonella*
species) showed production of biotin after 212 hr of growth. The principal biotin producer isolated from the lagoon was identified as *A. aerogenes*, although it seems probable that other producers of biotin are also present and are at least as numerous.

**Biotin utilization.** Unfiltered lagoon samples incubated in the dark at 30°C showed no biotin depletion. However, samples incubated in the light showed substantial utilization of biotin with concomitant algal growth (Table 2). At least 90% of the available biotin was removed after a 5-day incubation in the light. *Chlorella* numbers increased during this period with at least one doubling of the population occurring.

After inoculation of lagoon filtrate with pure cultures of *C. vulgaris* and incubation at 30°C with illumination by artificial light, biotin removal again was observed to be about 90% of the original value (Table 3). No significant biotin utilization by *C. vulgaris* was detected when lagoon filtrate was inoculated with *C. vulgaris* and incubated at 30°C in the dark. Uninoculated filtrates incubated at 30°C with and without illumination showed little or no depletion of biotin over a 4-day period.

Membrane-filtered (Millipore Corp.) samples of lagoon wastes inoculated with pure cultures of the purple sulfur bacterium *T. floridana* also showed utilization of biotin. Incubation at 30°C either with or without illumination showed removal of about 50% of the biotin present in the tested sample (Table 4). Uninoculated controls showed no loss of biotin during the same period of incubation.

**Biotin concentrations in receiving streams.**

| TABLE 1. Biotin production by pure cultures isolated from the Grafton sewage oxidation lagoon |
|-----------------------------------------------|
| **Isolates (no.)** | **Incubation for 44 hr (ng/liter)** | **Incubation for 212 hr (ng/liter)** |
|---------------------|--------------------------------------|--------------------------------------|
| 4                   | 21                                   | 19                                   |
| 16 (Salmonella sp.) | 41                                   | 356                                  |
| 19                  | 23                                   | 35                                   |
| 20 (A. aerogenes)   | 734                                  | 2,213                                 |
| 21 (Aerobacter sp.)| 434                                  | 663                                  |
| 22                  | 32                                   | 44                                   |
| 24 (Enterococcus)  | 144                                  | 191                                  |
| Control (no inoculum) | 14                                   | 13                                   |

**TABLE 2. Biotin utilization in unfiltered lagoon sample by a mixed population of algae**

| Incubation time (hr) | Experimental | Control* |
|----------------------|--------------|----------|
| Biotin (ng/liter)    | Algae (per ml) | Biotin (ng/liter) | Algae (per ml) |
| 0                    | 283          | 1.1 × 10^6 | 283          | 1.1 × 10^6 |
| 25                   | 150          | 1.5 × 10^4 | —           | 1.4 × 10^6 |
| 48                   | 190          | 2.0 × 10^5 | —           | 1.4 × 10^6 |
| 67                   | 170          | 2.4 × 10^6 | —           | 1.4 × 10^6 |
| 96                   | 62           | 2.6 × 10^4 | 270         | 1.4 × 10^6 |
| 120                  | 32           | 2.3 × 10^6 | —           | —          |
| 138                  | 26           | 1.6 × 10^6 | —           | —          |
| 168                  | 21           | 1.6 × 10^6 | 280         | 1.3 × 10^6 |
| 193                  | 20           | 1.3 × 10^6 | —           | —          |
| 216                  | 19           | 1.2 × 10^6 | —           | —          |
| 264                  | 13           | 1.2 × 10^6 | 280         | 1.3 × 10^6 |

* Sample incubated in the dark.

* Sample not analyzed.

**TABLE 3. Biotin utilization by Chlorella vulgaris in filtrate from the Grafton sewage oxidation lagoon**

| Incubation time (hr) | Biotin (ng/liter) | C. vulgaris (organisms/ml) |
|----------------------|------------------|----------------------------|
|                       | **Experimental** | **Control**               |
|                       | Light           | Dark                      | Light           | Dark                      |
| 0                    | 6,600           | 6,600                     | 7.0 × 10^6      | 7.0 × 10^6 |
| 8                    | 4,900           | 6,600                     | 1.4 × 10^4      | 1.2 × 10^4 |
| 19                   | 4,200           | 6,600                     | 2.7 × 10^4      | 1.5 × 10^4 |
| 24                   | 4,200           | 6,600                     | 3.6 × 10^4      | 1.5 × 10^4 |
| 33                   | 2,400           | 6,700                     | 5.6 × 10^4      | —                        |
| 45                   | 1,400           | —                         | 9.4 × 10^4      | 1.5 × 10^4 |
| 69                   | 1,200           | —                         | 2.6 × 10^4      | —                        |
| 93                   | 500             | 6,400                     | 6.3 × 10^4      | 1.5 × 10^4 |

* Experimental flasks (membrane-filtered lagoon effluent) were inoculated with 7 × 10^4 *C. vulgaris* per ml of medium and incubated at 30°C in the light and dark. See text for light source. Controls consisted of membrane-filtered lagoon effluent incubated likewise at 30°C in the light and dark.

* Sample not analyzed.
Table 4. Biotin utilization by Thiocapsa floridana in filtrate from the Grafion sewage oxidation lagoon

| Incubation time (hr) | Biotin (ng/liter) |       |       |       |       |
|----------------------|-------------------|-------|-------|-------|-------|
|                      | Experimental      | Control|       |       |       |
|                      | Light             | Dark  | Light | Dark  |       |
| 0                    | 8,500             | 8,500 | 8,500 | 8,500 |       |
| 53                   | 4,600             | 3,600 | 8,500 | 8,500 |       |
| 192                  | —b                | 2,800 | 8,500 | 8,500 |       |

a Experimental flasks (membrane-filtered lagoon effluent) were inoculated with T. floridana (ca. 10⁶ organisms per 125 ml of filtrate) and incubated at 30°C in the light and dark. See text for light source. Controls consisted of uninoculated membrane-filtered lagoon effluent incubated likewise at 30°C in the light and dark.

b Sample not analyzed.

tin levels in several other domestic and industrial wastes varied from 10 to 4,400 ng/liter, respectively (Table 5). The synthesis of biotin in these wastes appears to be related to the amount of organic materials present in the waste, since concomitant determinations for BOD₅ show the highest level of biotin in those wastes of highest BOD₅ strength.

The results of BOD₅ and biotin determinations on the Red River of the North from a point above waste effluents (0 miles) and several points below waste effluents (miles 22 through 188) are presented in Table 6. Several municipalities and occasionally a sugar processing plant discharge their effluents into the Red River just beyond mile 0 (13). The results of spring “break-up” appear in the April samplings and display the effects of additional effluents reaching the Red River.

DISCUSSION

Ecological relationships between organic and microbial species in aquatic environments must be described before a complete understanding of the desired effects of treating wastes in oxidation lagoons can be realized. Additionally the parameters for the detection of water pollution (or more specifically stream biology) must again relate to these organic and microbial species interactions.

The vitamin biotin appears useful as an indicator of microbial action in waste lagoons receiving municipal and food processing wastes. It has been demonstrated in this paper and by others (10) that large amounts of biotin are synthesized in domestic and industrial wastes. Although many organisms may be responsible for production and utilization of the vitamin, the results presented here suggest that the enteric organisms (as coliforms) are among the producers of biotin in the aquatic system examined.

Biotin production by A. aerogenes, as reported here, was not a new finding in that Landy and Dicken (7) noted some 30 years ago that their strain of A. aerogenes could produce up to 3,400 ng of the vitamin per liter. Other studies (2) have indicated biotin to be required by certain marine algae.

Our experiments verified these observations in that pure culture studies revealed at least three microorganisms potentially responsible for fluc-

Table 5. Average biotin and 5-day biochemical oxygen demand (BOD₅) levels in industrial and domestic wastes

| Source                              | Biotin (ng/liter) | BOD₅ (μg/ml) |
|-------------------------------------|-------------------|--------------|
| Holding pond, protein water         | 1,226             | 12,700       |
| waste potato starch manufacturing   |                   |              |
| Holding pond, sugar manufacturing   | 3,660             | 542          |
| waste                                |                   |              |
| Aerated lagoon, domestic            | 77                | 50           |
| waste                               |                   |              |
| Facultative lagoon, domestic        | 122               | 25           |
| waste                               |                   |              |

Table 6. Biochemical oxygen demand (BOD₅) and biotin relationships in a river receiving domestic wastes

| Sample period (1969) | BOD₅ (μg/ml)/biotin (ng/liter) |
|----------------------|--------------------------------|
|                      | 0b    | 21   | 46   | 59   | 87   | 127  | 162  | 188  |
| January              | 2/3c  | 8/20 | 4/20 | 3/...d| 2/-  | 3/3  | 2/4  | 2/5  |
| February             | 2/2   | 8/24 | 3/19 | 3/19 | 2/10 | 2/6  | 2/4  | 2/3  |
| April                | 8/28  | 11/30| 12/35| 13/- | 11/- | 14/45| 16/38| 15/30|

a Industrial and domestic waste discharged after 0 miles.
b River mile.
c Values less than 2 are listed as 2.
d Determination for biotin not made.
tations in biotin levels in an overloaded oxidation lagoon. *A. aerogenes* (producing under artificial conditions about 10-fold more biotin than the enterococci isolated and about 7-fold more biotin than the *Salmonella* species isolated) was one of the biotin producers in the lagoon studied. Two photosynthetic organisms, *C. vulgaris* and *T. floridana*, were observed to be biotin utilizers. A previous study in our laboratory (4) on this lagoon showed that *Chlorella* was one of the major algal photosynthetic species in the lagoon during the algal phase and *Thiocapsa* was the predominant purple sulfur organism during the purple phase.

Biotin levels in wastes from other oxidation lagoons with very low BOD$_3$ loadings (ca. 20 lb of BOD per acre per day) and in lagoons with very high BOD$_3$ loadings (ca. 100 lb of BOD per acre per day) ranged from 10 to 4,400 ng/liter, respectively. Lagoons which were heavily loaded with sugar beet processing wastes (actually these are holding ponds and remain anaerobic throughout the summer) show biotin levels in the range of 1,200 to 3,000 ng/liter or higher. Biotin levels in the sugar-processing waste lagoons (in which algae did not become predominant and which remained anaerobic) remain high during the summer holding period. Conversely, as indicated previously, in those lagoons in which photosynthetic species predominate, biotin utilization occurs.

Samples of Red River water taken before and after treatment by a conventional water treatment plant in Grand Forks were also assayed for biotin. No reduction of the vitamin was observed by water treatment. It appears probable that other soluble organic molecules also are passing through this water treatment plant.

Biotin levels in 70 samples from rivers and lakes in northeastern North Dakota and northwestern Minnesota ranged from 1.4 to 11.4 ng/liter, with a mean value of 4.6 ng/liter. Downstream from known effluents (domestic or food processing industries), the mean biotin level from 48 river samples was 23.5 ng/liter. During summer months, utilization of the vitamin occurred normally 10 to 15 miles downstream from effluents since mean values of 4 ng/liter were again observed. Algal growth apparently was responsible for this diminution in the vitamin.

**ACKNOWLEDGMENTS**

This investigation was supported by the North Dakota Water Resources Research Institute with funds provided by the U.S. Department of Interior, Office of Water Resources Research under PL 88-379. The senior author (G.M.F.) is a National Defense Education Act Fellow.

The technical assistance of Janice Granum is acknowledged. We thank J. R. Waller for his continuing interest.

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