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Production of Hyaluronic Acid by a Streptococcal Strain in Batch Culture

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A simple method for the production and preparation of hyaluronic acid (HA) is described. Three media were tested, which all supported relatively good yields of cells, but only in one of them were appreciable amounts of HA formed. In this medium, HA was produced during the logarithmic growth phase and showed a molecular weight of 7.3×10^6. HA prepared from 24-hr cultures of the same organism had a molecular weight of only 2.6×10^6. The kinetics of HA production in batch culture were studied, as well as of cell production and glucose consumption.

Hyaluronic acid (HA), the cementing substance in connective tissue, can be extracted from mammalian organs, particularly the vitreous body of the eye and the umbilical cord, which are both rich in this polysaccharide. The purification procedures reported are laborious and the product obtained is usually contaminated with other mucopolysaccharides. To overcome these disadvantages, the preparation of HA from bacterial cultures is preferable, especially when large quantities are desired. When HA is to be specifically N- or C-labeled, this is the only practical procedure.

HA from group A hemolytic streptococci was first isolated by Kendall, Heidelberger, and Dawson (10), who obtained about 100 mg of HA/liter of culture liquid. Other methods have been reported by Seastone (13), Roseman et al. (12), and Cifonelli and Mayeda (3), with yields of 250 to 400 mg/liter. Thonard, Migliore, and Blustein (14) described a method including an ammonium sulfate precipitation, by which they obtained 0.5 to 1.0 g/liter, depending on the streptococcal strain used.

In this paper, a description is given of a simple and reproducible method for the cultivation of a streptococcal strain and for extraction of the HA from the culture liquid with yields of 300 to 400 mg/liter.

MATERIALS AND METHODS

Organism. Strain 32369, Coburn R 18, a hemolytic streptococcus, Lancefield group A, was used throughout this investigation. The strain was obtained from the New York State Department of Health.

Media. The production of HA in three different media was studied: (i) VIB-broth composed of 25 g of Difco Veal Infusion Broth per liter of distilled water; (ii) T-H-broth which contained 36.4 g of Oxoid Todd Hewitt Broth per liter of distilled water; and (iii) modified Christensen medium (2), which has been described elsewhere (7).

For the stock cultures VIB-agar plates were used, consisting of VIB-broth with 1.5% Difco agar added.

Glucose was added to the media to give the concentration indicated in each experiment.

Cultural conditions. Preliminary attempts to cultivate the organism in a stirred fermentor (7) often failed, the growth ceasing when continuous stirring of the culture was started. Reproducible results were obtained when the following procedure for the cultivation was adopted.

The stock culture was transferred weekly to new plates. A test tube containing 4 ml of VIB-broth with 1 mg of glucose/ml was inoculated from such an agar plate. After 5 hr of incubation at 37 C, one drop of this culture was transferred to 30 ml of the same medium in a 100-ml E flask. This was incubated for 10 hr without agitation, and the entire culture was then transferred to 750 ml of VIB-broth at 37 C containing 1 mg of glucose ml. After another 2 hr, more glucose was added as a 50% solution to the desired final concentration. Phenol red, 10 mg/liter, was added to the medium as a *p*H indicator, and, by addition of 2 N NaOH manually four to six times per hour, the *p*H could easily be maintained in the range 6.9 to 7.1.

The low concentration of glucose in the precultures limited growth and the production of lactic acid. Most probably as a consequence of the latter, most of the cells in the inoculum were actively growing on transfer to the main culture, minimizing the lag phase. Also, a low concentration of glucose was maintained in the main culture to prevent osmotic shock on inoculation. After growth for 2 hr, glucose could be added to the

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TABLE 1. *Production of hyaluronic acid by a group A hemolytic streptococcus on different media*

Medium[a]	Yield of cells (g/liter)	Yield of hyaluronic acid (mg/liter)
Todd-Hewitt...	0.35	20–60
Christensen....	0.7	<25
VIB-broth.....	0.5	250–400

[a] At the beginning of the logarithmic growth phase, 1.5% glucose was added to the three parallel cultures.

desired final concentration without affecting the growth rate.

Assays. For dry-weight determinations, 25 ml of culture was centrifuged; the cells were then washed with distilled water and dried at 105 C overnight.

The glucose concentration in the culture fluid was determined with *o*-toluidine by the method of Hultman (8).

For the hyaluronate determination, cell-free samples were concentrated by vacuum evaporation to one-tenth of their original volume. Three volumes of ethyl alcohol was added to one volume of concentrate. The precipitate was dissolved in distilled water. After centrifugation, a 1% solution of cetyl pyridinium chloride (CPC) was added to the clear supernatant fluid (11), whereupon HA precipitated as cetyl pyridinium hyaluronate, and this again was dissolved in 0.5 M NaCl containing 4% ethyl alcohol (to dissolve excess of CPC). The ethyl alcohol precipitation was repeated, and after centrifugation the sediment was dissolved in distilled water. Glucuronic acid in this solution was determined according to Dische (4). By multiplying this concentration by a factor of 1.9, the hyaluronate concentration was obtained.

This procedure eliminated most of the substances interfering in the assay. For the production of HA, the procedure was extended as follows. CPC precipitation was repeated and after centrifugation the sediment was dissolved in 0.5 M NaCl. To remove protein impurities Micro-Cel E (Johns Manville Co., 3.5 g/100 ml) was added, and the suspension was stirred for 2 hr. Centrifugation for 90 min at 10,000 × *g* at room temperature gave a clear supernatant fluid containing the HA. To minimize losses, the Micro-Cel E sediment was dissolved in an equal amount of 0.5 M NaCl and was centrifuged. The supernatant fluids were combined and HA was precipitated by addition of 2 volumes of ethyl alcohol. Finally, the HA was dissolved in distilled water and lyophilized.

The viscosity of the liquid was determined by means of an Oswald viscosimeter with outflow times of 15 sec for water. In the experiments illustrated in Fig. 2 and 3, the viscosity of the culture fluid was not determined, but measurements were made on partially purified HA dissolved in half the original volume.

Limiting viscosity numbers were determined according to Laurent, Ryan, and Pietruszkiewics (11) and the relation $(\eta) = 0.036 \times M^{0.78}$ was used to calculate the molecular weight of the HA.

The following equations and symbols were adopted from Fencl (6) and Aiba, Humphrey, and Millis (9), and were used to calculate the kinetic evaluation of hyaluronic acid formation and growth.

Specific growth rate, μ hr^{-1}:

$$\mu = \frac{(\log X_2 - \log X_1) \cdot 2.3}{t_2 - t_1} = \frac{dX}{dt} \cdot \frac{1}{X_a}$$

Specific rate of HA formation, k_{HA} hr^{-1}:

$$k_{HA} = \frac{\Delta HA}{\Delta X} \cdot \mu = \frac{d(HA)}{dt} \cdot \frac{1}{X_a}$$

Specific rate of glucose consumption, k_G hr^{-1}:

$$k_G = \frac{-\Delta G}{\Delta X} \cdot \mu = \frac{-dG}{dt} \cdot \frac{1}{X_a}$$

where X_1 and X_2 = dry weight of cells (mg/ml) at times t_1 and t_2, respectively; X_a = mean value of the dry weight in the time interval t_a; HA = concentration of hyaluronic acid (mg/ml); G = glucose concentration (mg/ml); ΔX, ΔHA, and ΔG = changes in the values obtained during a time interval Δt.

The curves presented in Fig. 3 are calculated from the experimental results plotted in Fig. 2.

RESULTS

The three media tested all gave a relatively good yield of cells (Table 1). However, in two of them, the production of HA was rather poor (20 to 60 mg/ml or less), whereas in the third medium, VIB-broth, concentrations of 250 to 400 mg/liter were obtained. This medium was chosen for further studies.

For the production of labeled HA, it was of interest to know at which glucose concentration the most economical production of HA was achieved. Cultivations were performed at different glucose concentrations in the range from 2.5 to 55 μmoles/ml (Fig. 1). At the lower concentration level, 11% of the glucose was converted to HA, but with increasing glucose concentration the conversion efficiency decreased to a value of 4% at 30 μmoles/ml. A maximal HA concentration of 400 mg/liter in the culture was achieved with 55 μmoles/ml, corresponding to a conversion efficiency of 4%. In one experiment with ^{14}C-glucose in the medium, as much as 8% of the radioactivity was recovered in HA.

The yields of bacterial dry weight at different initial glucose levels are shown in Fig. 1. From these figures, the molar yield coefficient was calculated to be 18 μg of bacteria per μmole of glucose, when glucose consumption was corrected for glucose incorporated into HA. The value obtained corresponds well with the figure of 21 found by Bauchop and Elsden (1) for *Strepto-*

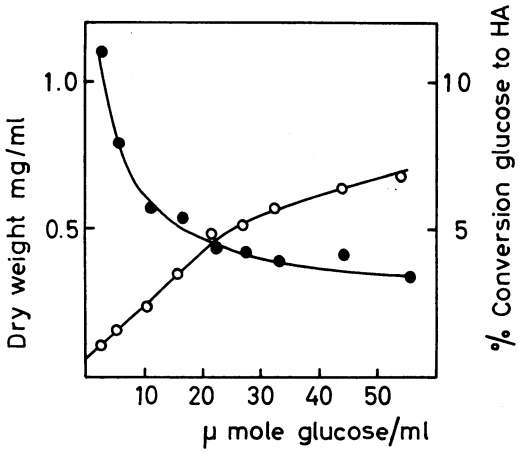

FIG. 1. *Yield of bacterial dry weight and per cent conversion of glucose to HA at different initial glucose concentration. Symbols;* ○, *dry weight of bacteria (mg/ml);* ●, *per cent conversion of glucose to HA.*

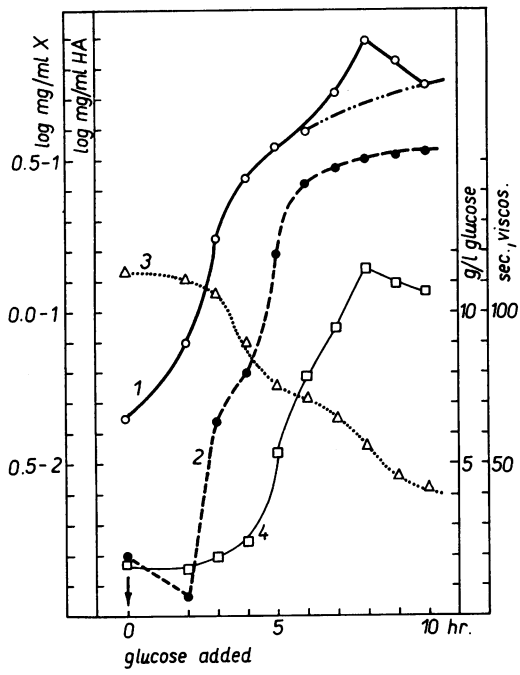

FIG. 2. *Growth curve and production of HA in the culture of a streptococcus strain. (1) Dry weight of bacteria, log mg/ml (dotted-line, assumed values of dry weight); (2) hyaluronic acid concentration, log mg/ml; (3) glucose concentration, g/liter; (4) relative viscosity, out-flow time in seconds.*

coccus faecalis, which is considered characteristic for cell yields from a homolactic fermentation of glucose.

Figure 2 shows the growth curve of a culture in VIB-broth to which 1.1% glucose was added 2 hr after inoculation. After a further 2 hr, the culture reached the logarithmic growth phase, glucose was consumed rapidly, and the dry weight of bacteria increased from the 2nd hr to the 8th hr after glucose addition, during which growth phase HA was produced (curve 2, Fig. 2). After the 10th hr, the concentration remained constant.

To describe the kinetics of HA formation, the following values were calculated: specific growth rate, μ hr^{-1}, specific rate of product formation, k_{HA}; and specific rate of glucose consumption, k_G. These data are plotted in Fig. 3.

Centrifugation of culture fluid samples taken 0 to 8 hr after glucose addition resulted in a fluffy highly viscous precipitate. In samples taken at a later stage of the cultivation, the cells sedimented as a hard pellet. This could probably be due to capsular HA surrounding the streptococci in the earlier part of the growth phase, although neither India ink staining nor electron microscopy could verify this. Furthermore, the drop in dry weight from the 8th to the 10th hr after glucose addition might also be explained by the fact that capsular material sedimented together with the cells during active production of HA but became loosened from the cells when synthesis of HA

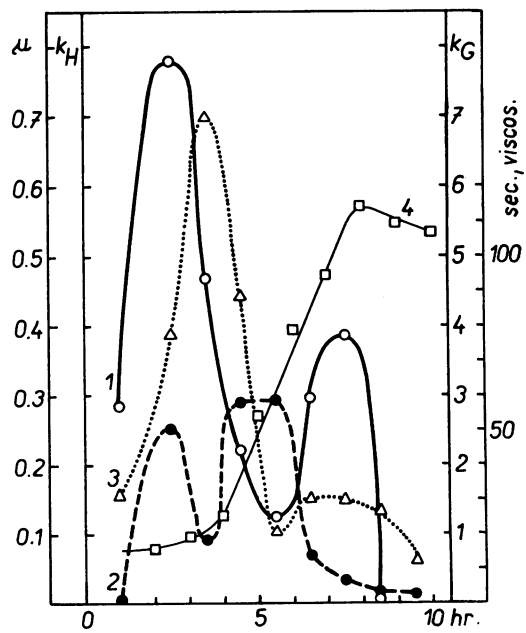

FIG. 3. *Kinetic data calculated from values plotted in Fig. 2. (1) Specific growth rate, μ hr^{-1}; (2) specific rate of HA formation, K_{HA}, hr^{-1}; (3) specific rate of glucose utilization, K_G, hr^{-1}; (4) relative viscosity, out-flow time in seconds.*

TABLE 2. *Variation in molecular weight of hyaluronic acid by age of culture*

Time after glucose addition (hr)	Limiting viscosity no. (ml/g)	Calculated molecular wt[a]
3	1,350	7.3×10^5
5	1,350	7.3×10^5
7	1,220	6.4×10^5
24	625	2.7×10^5

[a] Molecular weight calculated from limiting viscosity numbers by following equation: $(\eta) = 0.036 \times M^{0.78}$.

ceased. The hypothetical dotted-line part of curve 1 in Fig. 2 is assumed to correspond to the actual dry weight corrected for capsular HA.

The viscosity of the fractions precipitated by ethyl alcohol increased during the logarithmic growth phase and reached a maximum 8 hr after glucose addition, as can be seen in Fig. 2. During the stationary phase, there was a considerable drop in viscosity. Determination of limiting viscosity numbers on HA isolated and purified from samples taken at different stages of the cultivation showed that during the first 8 hr HA with a relatively high molecular weight was formed. When the cultivation was prolonged over 24 hr, the HA molecules previously formed were split to an average of one-third the molecular weight (Table 2). This degradation was probably not due to enzymatic activity, as no hyaluronate lyase could be determined in the culture fluid at any stage of growth with the strain used in these experiments.

DISCUSSION

Faber and Rosendal (5) have shown that the ability to produce HA is not a characteristic of specific antigenic types among the group A streptococci, but is a capability of individual strains. Willoughby, Ginsburg, and Watson (16) demonstrated that addition of glutamine to the medium could enhance the production of HA by certain strains. With the strain employed here and grown in VIB-broth, addition of glutamine had an inhibitory effect on both growth and HA production.

Although HA is produced in the logarithmic growth phase, as has been shown by Warren and Gray (15) and confirmed in this study, high yields of cells do not necessarily result in large quantities of HA. All three media tested resulted in approximately the same yield of cells, but only one of them, VIB-broth, supported extensive production of HA. If an organism grows well in one medium with little or no production of HA, this does not imply that the same organism cannot produce HA on another medium or in vivo.

The specific growth rate (curve 1, Fig. 3) had two maxima when calculated from the observed dry-weight curve (curve 1, Fig. 2). Since HA accumulated around the cells in increasing amounts until released at cessation of growth, an interpolation of the part of the curve between the 6th and 10th hr was made. This probably corresponds more closely to the true value of bacterial cell mass. When specific growth rate was calculated from the interpolated figures, curve 1 in Fig. 3 had the shape generally found with one peak corresponding to the logarithmic growth phase.

The changes in specific rate of product formation and consumption of glucose could be explained by the accumulation of HA around the cells. K_{HA} and K_G both had two maxima (Fig. 3), but were out of phase with each other. When synthesis of HA decreased after its first maximal value, glucose consumption reached its peak value. Then glucose consumption decreased and the rate of HA formation reached a second maximum. The probable explanation is that HA enveloped the cells and inhibited the diffusion of nutrients into the cells; consequently, the synthesis of HA was inhibited.

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