Physicochemical Properties of Hyaluronic Acid formed by *Streptococcus haemolyticus*

BY B. S. BLUMBERG* AND A. G. OGSTON
Department of Biochemistry, University of Oxford

AND D. A. LOWTHER† AND H. J. ROGERS
National Institute for Medical Research, Mill Hill, London, N.W. 7

(Received 17 January 1958)

Examination of the sedimentation of a sample of protein-free hyaluronic acid, isolated from suspensions of *Streptococcus haemolyticus* by Lowther & Rogers (1956), suggested that it might have been partly degraded by hyaluronidase at some stage of its preparation. Accordingly the presence of hyaluronidase in the suspensions was looked for, and found. A preparation of hyaluronic acid was finally made under the same conditions, but with the addition of the hyaluronidase inhibitor 53 D_K (Rogers & Spensley, 1954, 1955); this proved to be much more highly polymerized than the first material.

The opportunity was taken of determining the specific refractive increment of protein-free hyaluronic acid.

EXPERIMENTAL

Culture of the organisms. A strain (T₁₃) of *Streptococcus haemolyticus* (Lancefield Group A) was grown in Hedley Wright broth (Wright, 1933), containing 1% (w/v) of glucose, 2.5% (w/v) of sodium β -glycerophosphate and 10% (v/v) of horse serum, and bacterial suspensions were prepared as described by Lowther & Rogers (1956). From two such cultures (*A* and *B*) suspensions of washed organisms were prepared, each containing 90 mg. of bacterial nitrogen, at a concentration of 0.5 mg. of bacterial N/ml. of suspending medium, and incubated at 35° for 2 hr. The fluid of the final suspension contained: 80 mM-sodium β -glycerophosphate; 25 mM-phosphate buffer, pH 7.0 (Sørensen); 20 mM-MgSO₄; 28 mM-glucose; 5 mM-glutamine. To one of the suspensions (*B*) was added 100 μ g./ml. of the hyaluronidase inhibitor 53 D_K (Rogers & Spensley, 1954, 1955).

Separation of protein-free hyaluronic acid. After incubation the suspensions were cooled to 0° and the cells removed by centrifuging. The supernatant was dialysed under pressure for 4 days against flowing distilled water, and freeze-dried. These preparations were used without further treatment for physicochemical measurements. The preparation (*B*) which contained 53 D_K was further purified

before chemical analysis: the hyaluronate was precipitated with cetylpyridinium chloride (Scott, 1955) and redissolved in 0.2M-Na₂SO₄; the cetylpyridinium chloride was removed by treatment with fuller's earth; the hyaluronate was dialysed against water, de-ionized by passage through a short column of Zeo-Karb 225 (acid form) and the resulting hyaluronic acid was dried at 105°.

Analysis of hyaluronic acid. Glucosamine was determined by the method of Elson & Morgan (1933). Glucuronic acid in the sample *A* made without 53 D_K was determined by the method of Ogston & Stanier (1951); because of the small amount available, glucuronic acid in the sample *B* made with 53 D_K was determined by the colorimetric method of Dische (1947).

Estimation of concentration for physicochemical measurements. A representative sample of the freeze-dried preparation *A* was dissolved in water (3.24 mg./g. of solution); the material dissolved completely, to a clear solution. A weighed sample of this solution was dried to constant weight at 105°, giving 2.74 mg. dry wt./g. of solution; this showed that the preparation contained 84.5% of dry material. Subsequent solutions were made up by weight in buffer, with use of this value to obtain their contents of dry material. The value of the specific refractive increment obtained with *A* (see below) was used to determine the concentration of hyaluronic acid in *B*, by measurement of the area of its boundary diagram in the ultracentrifuge. Of the total refractive increment of *B*, 80% was due to hyaluronic acid and 20% to the accompanying 53 D_K.

Refractive increment. This was determined with the refractometer of Cecil & Ogston (1951) at 546 m μ .

Specific refractive increment. This was determined from the sedimentation diagram of a solution of *A*, which contained 0.344 g. of dry material/100 ml. of buffer. The area of the sedimentation boundary above the base-line (determined in a separate, parallel run with buffer alone) was determined by trapezoidal integration, corrected for dilution and converted into units of refractive index by use of the optical constants of the system (Baldwin, 1957).

Sedimentation. This was observed in a Svedberg oil-turbine ultracentrifuge at 900 rev./sec. and at 26–28°, by the method of Cecil & Ogston (1948).

Viscosity. The Couette viscometer of Ogston & Stanier (1953) was used to show that the solutions possessed no non-Newtonian viscosity. Subsequent measurements were therefore made with Ostwald viscometers of water-flow times 48, 108 and 200 sec.

Effects of streptococcal suspensions on hyaluronic acid. Suspensions of washed *S. haemolyticus* were incubated with

* Fellow of the Arthritis and Rheumatism Foundation, U.S.A.; present address: National Institutes of Health, Bethesda, Maryland, U.S.A.

† Present address: Department of Chemical Pathology, St Mary's Hospital, London, W. 2.

solutions of (a) hyaluronic acid complex made from ox synovial fluid (Ogston & Stanier, 1950), (b) a protein-free preparation of hyaluronic acid from umbilical cord, and (c) streptococcal hyaluronic acid, in the normal suspending medium. Control experiments were performed with suspensions of streptococci which had been first heated to 70°. Estimations were also made of testicular hyaluronid-

ase (Rhondase, Evans Medical Supplies Ltd.) in the presence of heated cells in the suspending medium. The suspensions were assayed for hyaluronidase activity both viscometrically (McLean & Hale, 1941) and by the turbidimetric method of Tolksdorf, McCready, McCullough & Schwenk (1949).

Buffers. Physicochemical measurements were made in 0.2M-NaCl, 0.0077M- Na_2HPO_4 , 0.0023M- KH_2PO_4 , pH 7.3.

Table 1. *Glucosamine and glucuronic acid contents of hyaluronic acids from Streptococcus haemolyticus*

The values were obtained on material dried at 105° and have not been corrected for ash content.

Sample	Glucosamine (g./100 g.)	Glucuronic acid (g./100 g.)
A	36.1	42*
B	38.8	43†

* By decarboxylation (Ogston & Stanier, 1951).

† Colorimetric (Dische, 1947).

Fig. 1. Ultracentrifuge diagrams of hyaluronic acids from *S. haemolyticus*. Speed 900 rev./sec.; temperature 26–28°. a, Sample A 0.344 g./100 ml. of buffer 112 min. after reaching full speed; b, sample B 0.47 g./100 ml. 33 min. after full speed. The hyaluronic acid boundary is sharp compared with that of A. The small diffuse boundary due to the 53 D_K is seen ahead of the hyaluronic acid boundary, and all the light-absorbing material (546 mμ) is associated with this boundary; c, sample B 0.12 g./100 ml. 55 min. after full speed. The hyaluronic acid boundary is less sharp than in b, and the boundary of 53 D_K is not observable. Sedimentation is occurring from right to left. m, meniscus; h, boundary of hyaluronic acid; d, boundary of 53 D_K.

RESULTS

Composition. The percentages of glucosamine and of glucuronic acid for the two samples are given in Table 1.

Sedimentation. The results are given in Table 2 and illustrated in Figs. 1 and 2. Sample A gave

Table 2. *Measurements of sedimentation and viscosity made on hyaluronic acid from Streptococcus haemolyticus, and derived particle dimensions*

	Sample	
	A	B
$S_{20,w}^0$ (s)	2.5	6.5
$dS_{20,w}^0/dc$ at $c=0$ [s ⁻¹ (g./100 ml.) ⁻¹]	0.75	1.9
$[\eta]_c$ (g./100 ml.) ⁻¹	2.3	6.0
10^{-3} (average particle wt.)	115	930
Hydrodynamic specific volume (ml./g.)	21	200
Axial ratio a/b	9	2

Fig. 2. Variation of $1/S_{20,w}$ (s⁻¹) against concentration, for samples of hyaluronic acid from *S. haemolyticus*. ○, Sample A formed in the absence of hyaluronidase inhibitor; ●, sample B formed in the presence of hyaluronidase inhibitor.

a diffuse boundary even at higher concentration, a low value of the sedimentation coefficient at zero concentration ($S_{20,w}^0$) and a low and linear rate of variation of sedimentation coefficient with concentration (dS^{-1}/dc); all these are typical of a relatively low degree of polymerization and probably of considerable polydispersity. The sharp boundary given by sample *B*, its high values of $S_{20,w}^0$ and of dS^{-1}/dc , and the variation of the last with concentration (Ogston & Woods, 1954) are all typical of a much higher degree of polymerization. At lower concentrations the boundary of *B* became diffuse, suggesting that it also is considerably polydisperse.

The pigmented material, 53 D_K, present in sample *B*, sedimented with a very diffuse boundary, well ahead of the hyaluronic acid (Fig. 1).

Viscosity. The intrinsic viscosities $[\eta]_c$ were obtained by linear extrapolation of $(\eta_{rel} - 1)/c$ to zero concentration. The values are given in Table 2.

Particle dimensions. These were calculated, approximately, by the method of Ogston (1953) from the values of S^0 , dS^{-1}/dc and $[\eta]_c$. The results, given in Table 2, confirm that *B* is much more highly polymerized than is *A*.

Specific refractive increment. The value obtained for sample *A* was $1.44 \pm 0.01 \times 10^{-3}$.

Action of streptococci on hyaluronic acids. The properties of the hyaluronic acid produced in the absence of inhibitor were consistent with a partial breakdown of the mucopolysaccharide having occurred during preparation. Accordingly the presence of hyaluronidase was sought in washed suspensions of the organisms. Incubation at 37° of 1.5 ml. of a solution of hyaluronic acid complex (Ogston & Stanier, 1950) with cells equivalent to 1.0 mg. of bacterial nitrogen reduced the relative viscosity of the solution from 3.4 to 2.4 in 2 hr. This decrease would correspond with the presence of 0.16 Viscosity Reducing Unit (McClellan & Hale, 1941) of hyaluronidase, and did not occur if the cell suspension had first been heated to 70°. Testicular hyaluronidase (Rhondase), diluted in suspending medium containing heated streptococcal cells, when incubated with hyaluronate solutions showed only about 6% of the activity of dilutions made in buffer containing gelatin and pyrophosphate (Rogers & Spensley, 1954). It appears probable therefore that considerably more hyaluronidase was in fact present in the streptococcal suspensions than had been detected, the enzyme not being fully active under the conditions in which the hyaluronic acid was produced. When isolated streptococcal hyaluronate was incubated with 0.16 Viscosity Reducing Unit of testicular hyaluronidase for 2 hr. at 37° there was no decrease in the turbidity given by adding acidified serum under the conditions of Tolksdorf *et al.* (1949).

DISCUSSION

The hyaluronic acid formed in a washed streptococcal suspension under the conditions used by Lowther & Rogers (1956) is shown to have undergone degradation by hyaluronidase present in, or secreted by, the cells. Faber & Rosendal (1954) and MacLennan (1956*a, b*) have shown that many strains of streptococci which produce hyaluronic acid also produce hyaluronidase. The presence of the compound 53 D_K which inhibits both the action and liberation of hyaluronidase leads to the formation of a much more highly polymerized product; however, since it is difficult to prove that the enzyme was completely inhibited, the cells may be capable of forming still more highly polymerized hyaluronic acid.

The value of 1.44×10^{-3} obtained for the specific refractive increment of protein-free hyaluronate at 546 m μ agrees well with the value obtained less directly by Ogston & Stanier (1950). It is of similar magnitude to values reported for other saccharides, for example: dextran 1.51×10^{-3} at 589 m μ (Snyder, Isbell, Dryden & Holt, 1954), 1.49×10^{-3} at 546 m μ (Ogston & Woods, 1954); fructose 1.41×10^{-3} at 589 m μ (Jackson & Mathews, 1932). However, it disagrees markedly with the values obtained by Blumberg & Oster (1954) (1.8×10^{-3} at 546 m μ) and by Laurent & Gergely (1955) (1.7×10^{-3} at 436 m μ). These differences should be well outside the experimental error of the methods used. A low value would be obtained if the material were not completely dry; but to explain the discrepancies our material would have to contain 15% of water even after drying to constant weight at 105°.

SUMMARY

1. The physicochemical properties of hyaluronic acid formed by a group A strain of *Streptococcus haemolyticus* in the presence and absence of the hyaluronidase inhibitor 53 D_K have been measured. The material formed in the presence of the inhibitor is much more highly polymerized.

2. Washed cells of *S. haemolyticus* have been shown to exert a hyaluronidase action; this is abolished by heating the cells to 70°.

3. A measurement has been made of the specific refractive increment of protein-free hyaluronic acid. The value obtained, 1.44×10^{-3} , is believed to be accurate.

REFERENCES

- Baldwin, R. L. (1957). *Biochem. J.* **65**, 490.
- Blumberg, B. S. & Oster, G. (1954). *Science*, **120**, 432.
- Cecil, R. & Ogston, A. G. (1948). *Biochem. J.* **43**, 592.
- Cecil, R. & Ogston, A. G. (1951). *J. sci. Instrum.* **28**, 253.

- Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
- Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
- Faber, V. & Rosendal, K. (1954). *Acta path. microbiol. scand.* **35**, 159.
- Jackson, R. F. & Mathews, J. A. (1932). *J. Res. nat. Bur. Stand.* **8**, 403.
- Laurent, T. C. & Gergely, J. (1955). *J. biol. Chem.* **212**, 325.
- Lowther, D. A. & Rogers, H. J. (1956). *Biochem. J.* **62**, 304.
- MacLennan, A. P. (1956*a*). *J. gen. Microbiol.* **14**, 134.
- MacLennan, A. P. (1956*b*). *J. gen. Microbiol.* **14**, 143.
- McLean, D. & Hale, C. W. (1941). *Biochem. J.* **35**, 159.
- Ogston, A. G. (1953). *Trans. Faraday Soc.* **49**, 1481.
- Ogston, A. G. & Stanier, J. E. (1950). *Biochem. J.* **46**, 364.
- Ogston, A. G. & Stanier, J. E. (1951). *Biochem. J.* **49**, 591.
- Ogston, A. G. & Stanier, J. E. (1953). *Biochem. J.* **53**, 4.
- Ogston, A. G. & Woods, E. F. (1954). *Trans. Faraday Soc.* **50**, 635.
- Rogers, H. J. & Spensley, P. C. (1954). *Biochim. biophys. Acta*, **13**, 293.
- Rogers, H. J. & Spensley, P. C. (1955). *Biochem. J.* **60**, 635.
- Scott, J. E. (1955). *Chem. & Ind.* p. 168.
- Snyder, C. F., Isbell, H. S., Dryden, N. R. & Holt, N. B. (1954). *J. Res. nat. Bur. Stand.* **53**, 131.
- Tolksdorf, S., McCready, B. A., McCullough, D. R. & Schwenk, B. (1949). *J. Lab. clin. Med.* **34**, 74.
- Wright, H. D. (1933). *J. Path. Bact.* **37**, 257.

Initial Stages in the Biosynthesis of Porphyrins

1. THE FORMATION OF δ -AMINOLAEVULIC ACID BY PARTICLES OBTAINED FROM CHICKEN ERYTHROCYTES

BY W. G. LAVER,* A. NEUBERGER AND S. UDENFRIEND†

Department of Chemical Pathology, St Mary's Hospital Medical School, London, W. 2

(Received 28 January 1958)

The biosynthesis of porphyrins is thought to occur by the initial condensation of glycine with an unsymmetrical derivative of succinic acid (for review see Shemin, 1955) to form α -amino- β -oxoadipate, the latter being decarboxylated to δ -aminolaevulinic acid, which is then converted through porphobilinogen into porphyrins. Observations indicating the formation of δ -aminolaevulinic acid as an intermediate have been made with animal tissues (Shemin & Russell, 1953; Shemin, Russell & Abramsky, 1955; Neuberger & Scott, 1953; Berlin, Neuberger & Scott, 1956*a, b*; Dresel & Falk, 1953, 1956*a, b*) and in micro-organisms by Lascelles (1956, 1957) and Pawelkiewicz & Zodrow (1956). The evidence provided by these investigations for the formation of δ -aminolaevulinic acid is quite strong, but somewhat indirect, being based largely on results obtained from isotope-dilution experiments, on a demonstration that this acid was much more active *in vitro* than glycine as a precursor of haem, and on the fact that δ -aminolaevulinic acid is converted into porphobilinogen or porphyrins in a wide variety of systems (Gibson, Neuberger & Scott, 1955; Granick, 1954). A net synthesis of δ -aminolaevulinic acid has not yet been demonstrated in systems *in vitro* and little evidence as to

the nature of the enzymic mechanisms involved has been obtained.

The difficulty of demonstrating a net synthesis of the acid *in vitro* is mainly due to two causes. In the first place all the animal tissues used so far in studies in biogenesis of porphyrins, such as mammalian reticulocytes, red cells from non-anaemic birds, liver and bone marrow, produce on incubation *in vitro* so little δ -aminolaevulinic acid that it cannot easily be measured by available methods without the use of isotopes. Secondly, the tissues mentioned metabolize the acid fairly rapidly, so that it does not accumulate to any appreciable extent. Both these difficulties have been overcome in the present work. It was found that erythrocytes obtained from anaemic chickens synthesize δ -aminolaevulinic acid from glycine and succinate or α -oxoglutarate at a fairly high rate. Moreover, a particulate fraction was obtained from the lysates of such cells which retains the capacity to synthesize δ -aminolaevulinic acid if suitably fortified with cofactors, but is free of enzymes concerned with the further metabolism of this acid. Chemical work on α -amino- β -oxoadipic acid (W. G. Laver, A. Neuberger & J. J. Scott, unpublished work) showed that this material loses carbon dioxide rapidly under physiological conditions, and in the present work we have therefore concentrated on demonstrating the formation of δ -aminolaevulinic acid. For a preliminary account see Laver & Neuberger (1957).

* Holder of Studentship of the Australian Commonwealth Scientific and Industrial Research Organization.

† U.S. Public Health Research Fellow. Present address: Laboratory of Clinical Biochemistry, National Heart Institute, National Institutes of Health, Bethesda, Maryland, U.S.A.