- Cecil, R. & Ogston, A. G. (1951). *J. sci. Instrum.* **28**, 253.
- Christiansen, J. A. & Jensen, C. E. (1952). *Acta chem. scand.* **5**, 849.
- Day, T. D. (1950). *Nature, Lond.*, **166**, 785.
- Fessler, J. H. (1956). D.Phil. Thesis: Oxford University.
- Fessler, J. H. (1957). *Nature, Lond.*, **179**, 426.
- Fessler, J. H., Ogston, A. G. & Stanier, J. E. (1954). *Biochem. J.* **58**, 656.
- Gross, J. (1958). *J. biol. Chem.* **233**, 355.
- Gross, J., Highberger, J. H. & Schmitt, F. O. (1955). *Proc. nat. Acad. Sci., Wash.*, **45**, 1.
- Gross, J., Maltoltsy, A. G. & Cohen, C. (1955). *J. biophys. biochem. Cytol.* **1**, 215.
- Jackson, D. S. & Fessler, J. H. (1955). *Nature, Lond.*, **176**, 69.
- Jensen, C. E. & Djurtoft, K. (1954). *Acta chem. scand.* **8**, 1659.
- Johnston, J. P. (1955). *Biochem. J.* **59**, 620.
- Laurent, T. C. (1957). *Ark. Kemi*, **11**, 487.
- Laurent, T. C. & Gergely, J. (1955). *J. biol. Chem.* **212**, 325.
- Meyer, K. (1947). *Physiol. Rev.* **27**, 335.
- Ogston, A. G. (1953). *Trans. Faraday Soc.* **49**, 1481.
- Ogston, A. G. (1958). *Trans. Faraday Soc.* **54**, 1.
- Ogston, A. G. & Stanier, J. E. (1950). *Biochem. J.* **46**, 364.
- Ogston, A. G. & Stanier, J. E. (1952). *Biochem. J.* **52**, 149.
- Pirie, A. (1949). *Brit. J. Ophthalm.* **33**, 271.
- Simha, R. (1940). *J. phys. Chem.* **44**, 25.

Biochem. J. (1960) **76**, 132

Mode of Action of Testicular Hyaluronidase

By J. H. FESSLER

Department of Biochemistry, University of Oxford

(Received 13 October 1959)

Hyaluronidase facilitates the movement of aqueous solutions along a pressure gradient through connective tissue (Duran-Reynals, 1929). Although chemical studies have shown the enzyme to act on certain polysaccharides found in connective tissue (Meyer & Rapport, 1952), it is not clear how the movement of water is thereby facilitated. *In vitro*, hyaluronidase causes a marked reduction in the viscosity of hyaluronic acid solutions. When water is moved through connective tissue along a pressure gradient, at least two frictional interactions are likely to occur: (1) between water and mucopolysaccharide of the tissue, and (2) between mucopolysaccharide and the confining structures of the tissue. Hyaluronidase could facilitate the movement of fluid by altering either or both of these interactions and the following experiment was devised to elucidate this problem. A measure of the first interaction, between water and mucopolysaccharide, can be obtained *in vitro* by observing the movement of mucopolysaccharide through water (buffer) in the analytical ultracentrifuge. A measure of the second interaction, between mucopolysaccharide solution and confining structures, is obtained *in vitro* from the relative viscosity of such solutions. The mucopolysaccharide for these experiments was prepared from bovine synovial fluid by Ogston & Stanier's (1950) method of ultrafiltration; the material had been defined by them and called hyaluronic acid complex or ultrafiltrate residue (U.F.R.). It was degraded stepwise with hyaluronidase and the sedimentation and viscosity properties of the products were measured at

different concentrations. It was concluded that the initial action of hyaluronidase reduces the interaction of mucopolysaccharide solution with the confining structures, and that the interaction of water with mucopolysaccharide is affected only when considerable degradation has occurred. This suggests that when hyaluronidase is used as a spreading agent it causes hyaluronic acid to be moved through the tissues with the injected fluid, rather than facilitating the movement of fluid through the hyaluronic acid. The action of trypsin on the hyaluronic acid complex of bovine synovial fluid was also studied with the ultracentrifuge.

EXPERIMENTAL

Synovial fluid. This was collected and stored as described by Fessler, Ogston & Stanier (1954) from cattle at the slaughterhouse of the Oxford Co-operative Society. Merthiolate [sodium ethyl mercurithiosalicylate solution no. 45 (0.1%, w/v), monoethanolamine (0.1%, w/v) Boots Pure Drug Co. Ltd., Nottingham] was added to a final concentration 1:34 000 (w/v).

Buffer. This was 0.2 M-NaCl-7.7 mM- Na_2HPO_4 -2.3 mM- KH_2PO_4 , pH 7.3.

Enzymes. Hyalase was obtained from Benger Ltd. and Trypsin (reagent grade) from British Drug Houses Ltd.

Preparation of hyaluronic acid complex by ultrafiltration. This was carried out by the method of Ogston & Stanier (1950). Dilute hyaluronic acid complex (U.F.R.) solution was clarified by ultracentrifuging (Spinco rotor no. 40, 39 000 rev./min., 40 min.), concentrated by further ultrafiltration and the viscous remnant dialysed against standard buffer. A marble was enclosed in the dialysis bag for stirring.

Incubation of concentrated hyaluronic acid complex with hyaluronidase. Conc. U.F.R. (1 ml.) was placed in an Ostwald viscometer at 37° (flow time for 1 ml. of water = 30.6 sec.). Hyalase (0.03 Benger unit) was added in 0.01 ml. of buffer and the solutions were thoroughly mixed. For complete degradation 20 Benger units of enzyme were used. Flow times were determined during incubation and when, after 3–24 hr., these had reached the required values, the viscometer was cooled in ice and dilutions with buffer were rapidly made by weight and kept in ice. Analytical ultracentrifuge runs were made within a few hours.

Incubation of hyaluronic acid complex with trypsin. The same procedures were used as with Hyalase, but with 0.03 ml. of approx. 1% trypsin solution in place of the Hyalase solution and incubating for 8 hr. at 37°.

Hyaluronic acid complex concentration. This was measured, before degradation, by the method of mucin-clot weight (Ogston & Stanier, 1950) after adding excess of protein solution. Conc. protein solution was obtained from the first filtrate of synovial fluid through a sintered-glass filter (Ogston & Stanier, 1950) by further ultrafiltration in a dialysis (Visking) sac.

Ultracentrifuging. For preparations a Spinco model L ultracentrifuge was used and for analyses the Svedberg oil-turbine ultracentrifuge of the Department of Biochemistry, Oxford; operation, calculation of sedimentation coefficients and their correction to water at 20° (S_{20}) were carried out by the methods of Cecil & Ogston (1948).

RESULTS

In Fig. 1 are summarized the results obtained after the action of hyaluronidase. As shown in the histogram, the relative viscosity (η_R) was reduced from approx. 470 to 63, 20, 7 and 1.1. Each of these samples was then diluted and analysed in the ultracentrifuge at four, or more, different concentrations. For all but the most degraded sample, no difference of sedimentation coefficient of hyaluronic acid was found at any one concentration. This is shown in the upper curve of Fig. 1, the small vertical bars indicating the widest variations, which occurred randomly among the samples. The most degraded material (η_R 1.1) gave the relation of sedimentation coefficient to concentration shown in the lower curve of Fig. 1. In the schlieren diagrams obtained from all except the most degraded sample the hyaluronic acid peak was the usual very sharp spike.

Trypsin digestion did not alter the sedimentation behaviour of hyaluronic acid, the sedimentation results being included in the upper curve of Fig. 1. This investigation was not concerned with the action of proteolytic enzymes on the viscosity of hyaluronic acid-complex solutions. Although η_R changed, during trypsin incubation, from approx. 470 to approx. 180, no attempt was made to ensure that this action was not due to some other agent than trypsin.

Fig. 1. Effect of hyaluronidase on the sedimentation coefficients of hyaluronic acid complex. Concentrated hyaluronic acid complex was degraded with hyaluronidase to different extent, as measured by the relative viscosity (η_R) given in the inset histogram. The sedimentation coefficient (S_{20}) of each degraded sample was measured at different concentrations. Results for the most degraded sample are shown in the bottom curve (Δ). Sedimentation coefficients of all other samples fell on the common, upper curve; the range of variation of S_{20} among samples on this upper curve at a given concentration is indicated by the height of the symbols.

A small, fast-moving boundary is often observed during the ultracentrifuging of U.F.R. (Johnston, 1955), and was denoted as 'fast component'. Fast component was not affected by hyaluronidase, and in Fig. 2 are shown sedimentation coefficients of fast component at different concentrations obtained from various hyaluronidase digests. No fast component was seen in the schlieren diagrams of trypsin digests of U.F.R.

The action of the enzyme had to be stopped, or at least greatly reduced, by a method which would neither modify the polysaccharide nor interfere in sedimentation experiments. After several inhibitors had been found unsuitable, conditions were chosen which greatly reduced enzymic activity by dilution and cooling. With 0.03 Benger unit/ml. of U.F.R., 3½ hr. was required at 37° to reduce η_R from approx. 470 to 63. The rate of decrease of η_R with time itself rapidly decreases, and the reaction rate of testicular hyaluronidase is in addition at least halved by the reduction in temperature of 10° (Dorfman, 1948), which is

Fig. 2. Sedimentation coefficients of 'fast component' of ultrafiltrate residue at different concentrations and after treatment with hyaluronidase. Native ultrafiltrate residue (○) of relative viscosity (η_R) 470 was degraded with hyaluronidase, to products of decreasing η_R at the same concentration. The values of η_R are: □, 63; Δ, 20; *, 7.0; x, 1.1.

required for ultracentrifuge runs at approx. 27°. At 0° ten times the strength of enzyme used reduced η_R from 11 to 8 in 17 hr., whereas at 37° only very weak action was found by one-tenth of the used enzyme concentration. Some reduction of relative viscosity must have occurred during the sedimentation experiments, but as S_{20} was found to be independent of η_R over the applicable range studied, such change did not matter.

DISCUSSION

Sedimentation coefficient of degraded hyaluronic acid complex. From the results, it is concluded that hyaluronidase does not affect the rate of movement in the ultracentrifuge of the very sharp boundary associated with hyaluronic acid complex, unless severe degradation has occurred. The sedimenting material is probably heterogeneous, but as

long as it sediments as a single boundary (due to particle-particle interactions), the apparent single sedimentation coefficient is a measure of the rate at which frictional work is done in moving mucopolysaccharide relative to buffer. The following treatment does not apply to the most degraded sample (η_R 1.1) in which more than one boundary was indicated by the schlieren diagram.

The rate at which frictional work is done during sedimentation can either be expressed in terms of the applied force or of a frictional coefficient f' between particles and solvent, characteristic of the system, i.e.

$$[Nm(1 - \bar{V}\rho)\omega^2x] \frac{dx}{dt} = \left(f' \frac{dx}{dt}\right) \frac{dx}{dt},$$

where N is the number of particles of mass m and partial specific volume $\bar{V}$, in solvent density ρ , under centrifugal acceleration ω^2x . It is unlikely that the initial action of hyaluronidase alters $\bar{V}$, and so we can write

$$f' = K'/S,$$

where $K' = Nm(1 - \bar{V}\rho) = \text{constant}$
and

$$S = \omega^2x / \frac{dx}{dt} = \text{sedimentation coefficient.}$$

Now S was experimentally found to be constant, therefore

$$f' = \text{constant.}$$

Hence, it is concluded that hyaluronidase does not alter the frictional interaction between buffer and U.F.R.

Mode of action of hyaluronidase. Whereas hyaluronidase did not decrease the frictional interaction between buffer and U.F.R., it did greatly reduce the interaction between solution and containing wall, i.e. the relative viscosity from 470 to 7. From this it is concluded that, at least under these conditions, hyaluronidase admixed with a fluid and infused, extravascularly, into the animal body would change polysaccharide so that it would be pushed through the tissue spaces with the fluid, rather than that the enzyme would enable the fluid to permeate the carbohydrate more readily. How well do the conditions *in vitro* represent those that can be presumed to exist *in vivo*? Interstitial polysaccharide will include sulphur-containing carbohydrate, which is not found in synovial-fluid U.F.R. Little precise evidence exists about the range of action of different commercially prepared samples of testicular hyaluronidase. It is known that testicular hyaluronidase acts on both hyaluronic acid and chondroitin sulphates A and C (Meyer & Rapport, 1952), but not on chondroitin sulphate B, kerato-sulphate or heparin. Bacterial hyaluronidases only

act on hyaluronic acid. The concentration of material *in vivo* is unknown, indeed the meaning of the term concentration becomes ill-defined when applied to a system known to be inhomogeneous in phase and distribution of components. Over the experimental range studied, the conclusions are valid independently of the proportions of water to solids. The most concentrated material, approaching the gel state, flowed only with difficulty. The measurements do not show any trend that might indicate that the conclusions are invalid outside the range studied. Whereas prolonged action of hyaluronidase may be expected to lead to quite different conditions (e.g. sample η_R 1.1), the minimal effective action may be presumed to be the most important one clinically.

Nature of fast component and action of trypsin. Fast component is concluded to be a protein of relatively large particle size, which is somehow trapped together with hyaluronic acid complex during ultrafiltration. Thus even after severe action of hyaluronidase (sample η_R 1.1), the sedimentation behaviour of fast component was unaffected, but its boundary disappeared after the action of trypsin.

The sedimentation coefficients of fast component and their variation with concentration, found here, are in agreement with the measurements of Johnston (1955) on human synovial fluids. Johnston suggested from indirect evidence that fast components arose from hyaluronic acid, or could arise due to various interactions of hyaluronic acid. The present findings with hyaluronidase and trypsin do not support this view.

Whatever the constitution of hyaluronic acid complex may be, the trypsin experiments show that the translational hydrodynamic interaction

between hyaluronic acid and buffer does not depend on any material open to attack by trypsin.

SUMMARY

1. The action of hyaluronidase and trypsin on the sedimentation and viscosity behaviour of hyaluronic acid complex was studied.

2. It is concluded that hyaluronidase does not affect the frictional interaction involved in moving buffer relative to hyaluronic acid complex, except after prolonged action.

3. It is suggested that during the use of hyaluronidase as a spreading agent, it facilitates the movement of fluid together with polysaccharide through the tissue spaces, rather than enabling a movement of fluid through polysaccharide.

4. Trypsin did not affect the frictional interaction involved in moving buffer relative to hyaluronic acid complex.

5. Fast component of bovine-synovial-fluid hyaluronic acid complex was found to be degraded by trypsin but not by hyaluronidase.

I thank Dr A. G. Ogston for most helpful discussion, and the Medical Research Council for financial support.

REFERENCES

- Cecil, R. & Ogston, A. G. (1948). *Biochem. J.* **43**, 592.
 Dorfman, A. (1948). *J. biol. Chem.* **172**, 377.
 Duran-Reynals, F. (1929). *J. exp. Med.* **50**, 327.
 Fessler, J. H., Ogston, A. G. & Stanier, J. E. (1954). *Biochem. J.* **58**, 656.
 Johnston, J. P. (1955). *Biochem. J.* **59**, 626.
 Meyer, K. & Rapport, M. M. (1952). *Advanc. Enzymol.* **13**, 199.
 Ogston, A. G. & Stanier, J. E. (1950). *Biochem. J.* **46**, 364.

Biochem. J. (1960) **76**, 135

The Metabolism of [^{14}C]Glucose, [^{14}C]Fructose and [2- ^{14}C]Pyruvate after Limb Ischaemia in the Rat

By H. B. STONER, D. F. HEATH AND O. MYFANWY COLLINS

Toxicology Research Unit, M.R.C. Laboratories, Woodmansterne Road, Carshalton, Surrey

(Received 28 October 1959)

The general response to an injury includes numerous changes in carbohydrate metabolism (see Stoner, Threlfall & Green, 1952; Stoner & Threlfall, 1960). These changes may be important, since in small mammals after severe injury there are rapid falls in the temperature of the liver and brain which cannot, in the early part of the response, be

explained by alterations in tissue blood flow or the availability of oxygen (Stoner, 1954, 1958a). The liver mitochondria from injured rats behave normally *in vitro* (Aldridge & Stoner, 1960). This has led us to seek the cause of the temperature changes in the earlier stages of carbohydrate metabolism with [^{14}C]glucose, [^{14}C]fructose and [2- ^{14}C]pyruvate.