[8] J. KENNER AND G. N. RICHARDS, *J. Chem. Soc.*, (1957) 3019.
[9] E. F. L. T. ANET, *J. Am. Chem. Soc.*, 82 (1960) 1502; *Chem. Ind. London*, (1962) 262.
[10] N. V. SIDGWICK, *The Organic Chemistry of Nitrogen*, Oxford University Press, 1937, p. 484.
[11] T. WHITE, *J. Chem. Soc.*, (1940) 428.
[12] D. H. LEABACK AND P. G. WALKER, *Chem. Ind. London*, (1957) 1012.
[13] D. H. LEABACK AND P. G. WALKER, *J. Chem. Soc.*, (1957) 4754.
[14] C. G. GREIG AND D. H. LEABACK, *J. Chem. Soc.*, in the press.
[15] L. A. ELSON AND W. T. J. MORGAN, *Biochem. J.*, 27 (1933) 1824.
[16] G. A. LEVVY AND A. MCALLAN, *Biochem. J.*, 73 (1954) 127.
[17] J. V. SCUDI, G. E. BOXER AND V. C. JELINEK, *Science*, 104 (1946) 486.
[18] K. HEYNS, H. BREUER AND H. PAULSEN, *Chem. Ber.*, 90 (1957) 1374.
[19] G. P. ELLIS, *Advan. Carbohydrate Chem.*, 14 (1959) 70.
[20] N. F. BOAS, *J. Biol. Chem.*, 205 (1953) 553.

Received December 28th, 1962

SC 2230

Periodate oxidation of hyaluronic acid

Studies by both chemical and enzymic methods have accumulated a body of evidence that supports a structure for hyaluronic acid composed of sequences of 2-acetamido-2-deoxy-3-*O*-[β-D-glucopyranosyluronic acid]-β-D-glucopyranosyl units linked to each other through position 4 of the D-glucuronic acid residues. The details of these studies have been reviewed recently[1,2]. Such a structure should be open to oxidation of the acid residues by periodate but studies of this reaction have been interpreted to indicate uronic units linked through position 3 (see refs. 3, 4) or equally through positions 3 or 4 (see ref. 5). The discrepancy between the results of periodate oxidation and other studies is untenable.

In the present studies the preparation of hyaluronic acid from umbilical cords followed essentially the procedures previously described. These involved digestion with papain[6], elimination of protein by Sevag's method, dialysis, and fractional precipitation as the cetylpyridinium complex[6]. The hyaluronic acid, isolated as the magnesium salt, showed $[\alpha]_D^{23}$ −80° in water[5] (*c* 0.23) and hexosamine, hexuronic acid, and *N*-acetyl in the mole ratios 1.00:1.00:1.05. The material gave very viscous aqueous solutions and contained no ester sulfate groups. Chromatographic analysis on Dowex 1 × 2 (formate form) by the procedure of SCHILLER *et al.*[7] showed only hyaluronic acid.

The general procedure for the oxidation of magnesium hyaluronate with periodate is illustrated by the following particular example. To magnesium hyaluronate (304 mg, by hexuronic acid and hexosamine determination) in water (220 ml) at 5° was added cold 0.35 M sodium metaperiodate (25 ml) and the solution diluted to 250 ml. A blank solution was similarly prepared and both were kept in the dark at 5°. At zero time and periodically thereafter, aliquots of the solution were analyzed for periodate consumption[8,9]. Aliquots (5 ml) were also added to 5% aq. ethylene glycol (1 ml) and after about 30 min a portion (5 ml) of this solution was placed in a well-washed dialysis casing. The solution was dialyzed overnight at 5° against distilled water and then electrodialyzed until free of iodate. The solution was quantitatively washed from the dialysis bag into a volumetric flask to which was added $NaBH_4$ (20 mg). The reduction proceeded overnight and the solution was then acidified with glacial

acetic acid to decompose the excess borohydride. The final solution, diluted to volume, was analyzed for hexuronic acid[10,11] and hexosamine[12] as previously described.

The recovery of hyaluronate from mixtures with sodium iodate by the procedure described above was found in several experiments to be quantitative within the errors (± 3 %) of the colorimetric methods.

The duplicate results of the periodate oxidation of magnesium hyaluronate are summarized in Fig. 1. In the early stages of the reaction the consumption of periodate followed reasonably closely the rate described previously[3,5]. However, since other studies[13,14] with similar mucopolysaccharides had shown that the consumption of periodate cannot be interpreted simply, the rate of cleavage of hexuronic acid and *N*-acetylhexosamine in the hyaluronate was followed concomitantly. It is seen that the rate of oxidation of hyaluronic acid is relatively slow but occurs with the destruction of approx. 90 % of the hexuronic acid and 10 % of the hexosamine residues. The earlier workers[3–5] had seemingly interpreted the slow oxidation as "over-oxidation" and extrapolated a primary rate to zero time.

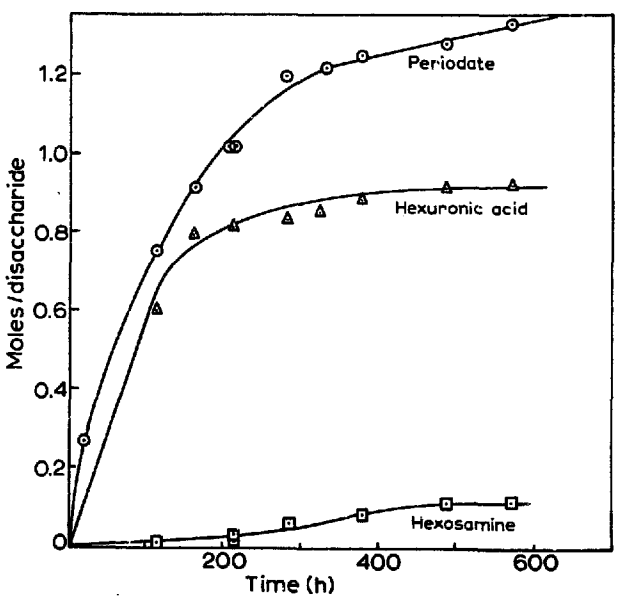

Fig. 1. Periodate oxidation of magnesium hyaluronate. ⊙—⊙, consumption of periodate; △—△, destruction of hexuronic acid; ⊡—⊡, destruction of hexosamine.

The present results support in the main the structure described above for hyaluronic acid, as does a recent abstract of some methylation studies[15]. However, approx. 10 % of the sugar residues behave differently from that anticipated for the proposed structure, which may reflect admixture with some other polysaccharide or additional types of linkages, either within a linear molecule or as branch points. The preparation of hyaluronate used in these studies was homogeneous by ion-exchange chromatography and free of ester sulfate. It is clear that the finer structural aspects of hyaluronic acid still present a challenge.

This investigation was supported by research grant A-1696 from the National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service.

Department of Biochemistry, State University of Iowa, Iowa City, Iowa (U.S.A.)

REX MONTGOMERY
SABITA NAG

[1] H. J. ROGERS, *The Biochemistry of Mucopolysaccharides of Connective Tissue, Biochem. Soc. Symp.*, No. 20, 1961, p. 55.
[2] M. STACEY AND S. A. BARKER, *The Carbohydrates of Living Tissue*, D. Van Nostrand, London, 1962, chapter 2.
[3] R. W. JEANLOZ AND E. FORCHIELLI, *J. Biol. Chem.*, 190 (1951) 537.
[4] K. H. MEYER, J. FELLIG AND E. H. FISCHER, *Helv. Chim. Acta*, 34 (1951) 939.
[5] G. BLIX, *Acta Chem. Scand.*, 5 (1951) 981.
[6] J. E. SCOTT, *Methods Biochem. Anal.*, 8 (1961) 154.
[7] S. SCHILLER, G. A. SLOVER AND A. DORFMAN, *J. Biol. Chem.*, 236 (1961) 983.
[8] P. FLEURY AND J. LANGE, *J. Pharm. Chim.*, 17 (1933) 107, 196.
[9] F. SMITH AND R. MONTGOMERY, *Methods Biochem. Anal.*, 3 (1956) 182.
[10] Z. DISCHE, *Methods Biochem. Anal.*, 2 (1955) 343.
[11] R. MONTGOMERY, *Biochim. Biophys. Acta*, 48 (1961) 591.
[12] Y. C. LEE AND R. MONTGOMERY, *Arch. Biochem. Biophys.*, 93 (1961) 292.
[13] A. K. CHATTERJEE, G. J. DURANT, H. HENDRICKSON, Y. C. LEE AND R. MONTGOMERY, *Biochem. Biophys. Res Commun*, 4 (1961) 425.
[14] G. J. DURANT, H. R. HENDRICKSON AND R. MONTGOMERY, *Arch. Biochem. Biophys.*, 99 (1962) 418.
[15] R. W. JEANLOZ AND P. J. STOFFYN, *Federation Proc.*, 21 (1962) 81.

Received December 3rd, 1962

Biochim. Biophys. Acta, 74 (1963) 300–302

SC 2246

Specific analysis of terminal reducing glucosamine and glucuronic acid groups
Hyaluronate hydrolysis by hyaluronate lyases

Specific microanalytical methods for terminal reducing glucuronic acid and glucosamine are particularly desirable in the study of hyaluronate lyases (EC 4.2.99.1, formerly known as hyaluronidase). Such procedures would not only establish the nature of the linkage undergoing cleavage but also reveal the extent of participation of the ubiquitous enzymes, β-glucuronidase (EC 3.2.1.31) and acetyl-β-glucosaminidase. The latter enzymes are capable of further degrading the mucooligosaccharide products of hyaluronate hydrolysis.

Recently, YUKI AND FISHMAN[1] applied the alkaline hypoiodite oxidation of the glucuronyl aldehydic function[2] to the differential analysis of glucuronate, glucosiduronate and hyaluronate. Such a method would be capable of measuring terminal reducing glucuronic acid groups of mucooligosaccharides in relation to the total glucuronic acid present.

In a subsequent attempt to analyze mixtures of glucosamine and hyaluronate, hypoiodite oxidation of the glucosaminyl aldehyde was first explored. It was abandoned when success was not realized in reducing the level of contaminating cations (introduced to remove iodide ions) to a value which did not interfere with the subsequently applied color reaction for glucosamine (ELSON–MORGAN[3]).

Oxidation of reducing glucosamine proved to be satisfactory and convenient with cupric ions* (pH 9.7) and, fortunately, the excess cupric ions could be removed completely by converting them to the highly insoluble cuprous oxide by glucose

* The pH of the cupric ion oxidation of the glucosaminyl aldehyde group must be regulated between 9.3 and 9.7, since above the latter pH the glucosaminidic linkages in hyaluronate are unstable (see also refs. 4 and 5).