an internal Geiger–Müller flow counter (Radiation Counter Laboratories, Inc., Mark 12, Model No. 1, helium-isobutane gas). The total activity (C^{14} and tritium) of dilutions of the above solution, or on dilutions to which 100 parts of carrier cytidine had been added, was $2.2 \pm 0.2 \times 10^6$ c.p.m. per μmole. Cytosine isolated after $HClO_4$ hydrolysis of the sample with carrier added had a C^{14} activity of 3080 c.p.m. per μmole. The ratio of C^{14} to total disintegrations was 1:7. Thus the C^{14} activity of the synthetic material was *ca.* 0.31×10^6 c.p.m. per μmole.

Detection of Impurities.—The above product was chromatographed on paper, descending, with 1-butanol–NH_4OH–H_2O (86:5:9) for 36 hours, with the solvent front running off the paper and the cytidine moving about 3 inches. When scanned with an automatic recording windowless paper scanning device (Radiological Service Co.) radioactivity was found not only in the main peak at the cytidine spot but also in a major, non-ultraviolet absorbing, spot at the origin. With a liquid scintillation spectrometer[18] the C^{14}/H^3 ratio of the cytidine recovered from chromatograms was approximately 1.6. That of the material applied was 1.04, and of the material at the origin was 0.009.

Similar chromatography of the tetraacetylribofuranose in butanol–H_2O (86:14) showed a similar contaminant at the origin, with tetraacetylribofuranose near the solvent front.

(18) A Packard "Tri-Carb" Scintillation Spectrometer Model 314 was used. The readings were taken at voltage tap 7 with the discriminators set at 10 v., 50 v., and 100 v. Relative ratios of C^{14}/H^3 were determined by calibrating the instrument with crotonside-2-C^{14} and adenosine-*t*. The ratios are arbitrary; they are dependent upon the efficiency of the instruments for each isotope at the settings used, and bear no relation to those found by measurement in a Geiger–Müller apparatus. We wish to express appreciation to Dr. Leon Bradlow for helpful advice and willing coöperation.

Column Chromatographic Purification of Cytidine.—A column (33 × 7 cm.) was gravity-packed with a suspension of cellulose powder (Whatman Standard Grade Ashless) in 1-butanol–NH_4OH–H_2O (86:9:5), and washed until the effluent was colorless. A solution of 382 mg. of cytidine sulfate in 15 ml. of butanol–NH_4OH–H_2O (10:2:3.5) was applied to the top of the column. It was developed with butanol–NH_4OH–H_2O (86:9:5) at the rate of 8 ml. per 3 min. The first 300 ml. was discarded, the 300 fractions of 8 ml. each were collected. Fractions 187 to 268, containing the ultraviolet absorbing material, were combined. The residue from a 1-ml. aliquot was dissolved in 0.01 *N* HCl. Its spectrum was identical with that of cytidine, and it was calculated that the recovery, as cytidine, was 264.2 mg. (80%). The main solution was largely evaporated *in vacuo* and finally lyophilized.

This purified cytidine was chromatographed in butanol–NH_4OH–H_2O, in water at *p*H 10, and in 2-propanol–HCl–H_2O. Eluates of 2-cm. segments of the papers were evaporated on planchets and assayed. Not over 1 to 3% of the total activities on the strips were found outside the cytidine areas. In the scintillation spectrometer this cytidine gave C^{14}/H^3 ratios of 1.6 to 1.7, which are of the same order as that obtained upon elution of the original material from a paper chromatogram.

Acknowledgments.—The authors wish to express their deep appreciation to Dr Jack J. Fox and Dr. Aaron Bendich for many helpful discussions and valuable suggestions. We are grateful to Mr. H. K. Vanderhoeff of Kay–Fries Chemicals, for a generous contribution of β-ethoxyacrylonitrile.

NEW YORK 21, N. Y.

[CONTRIBUTION FROM THE DEPARTMENTS OF BIOCHEMISTRY AND MEDICINE, AND THE STUDY GROUP ON RHEUMATIC DISEASES, NEW YORK UNIVERSITY COLLEGE OF MEDICINE]

Electrophoretic Behavior of Chondromucoprotein[1]

BY ROBERT C. WARNER AND MAXWELL SCHUBERT

RECEIVED APRIL 11, 1958

An electrophoretic study has been made of the chondromucoprotein of bovine nasal cartilage and of its polysaccharide component, chondroitin sulfate. The mobility–*p*H curves over the range 2 to 11, while close, are distinct and have parallel trends with the chondroitin sulfate always a little faster than the chondromucoprotein. At *p*H 12.5 the chondromucoprotein appears to be dissociated completely into two components of which one has the same mobility as chondroitin sulfate while the other is slower. Bringing the *p*H of such a solution to 3.3 does not reverse this dissociation.

The chondromucoprotein of beef nasal cartilage appears to be a compound of protein and chondroitin sulfate in which all the carboxyl and ester sulfate groups of the polysaccharide component occur as free anionic groups. In native cartilage these anionic groups are presumably associated with sodium cations and account for the high sodium content of cartilage. In the preparation which has been described these anionic groups are associated with potassium cations.[2] The nature of the linkage between the protein and the polysaccharide components is not known but it is broken in 20 hr. at 25° in 0.1 *M* NaOH.[3] A study of the *p*H range over which the combination of protein and polysaccharide is stable might be helpful in a study of this linkage. The electrophoretic behavior of the chondromucoprotein has, therefore, been examined over the *p*H range 2 to 12 and compared with that of chondroitin sulfate.

Methods

The electrophoretic experiments were carried out at 0° as previously described.[4] Solutions were prepared by dissolving the dry material, chondromucoprotein or chondroitin sulfate as their potassium salts, in the buffer to be used and dialyzing about 16 hr. against two changes of buffer. Solutions prepared at extreme *p*H's were kept at 0 to 2° during all preparative operations. In the *p*H range from 3 to 5 the solutions were cloudy. Similar patterns were obtained whether or not such solutions were centrifuged to remove small amounts of insoluble material. In all experiments a total concentration of 0.33 g. of polyelectrolyte per 100 ml. was used.

Methods for the preparation of chondromucoprotein and of chondroitin sulfate, both as potassium salts, have been described elsewhere.[2,3]

Results

The patterns obtained with the mucoprotein and with chondroitin sulfate showed in general a single boundary. Some of these are illustrated in Fig. 1

(1) This investigation was supported by research grants H1642 and A-28(C) from the National Heart Institute and the National Institute of Arthritis and Metabolic Diseases, respectively, United States Public Health Service.

(2) J. Shatton and M. Schubert, *J. Biol. Chem.*, **211**, 565 (1954).

(3) I. Malawista and M. Schubert, *ibid.*, **230**, 535 (1958).

(4) S. Ehrenpreis and R. C. Warner, *Arch. Biochem. Biophys.*, **61**, 38 (1956).

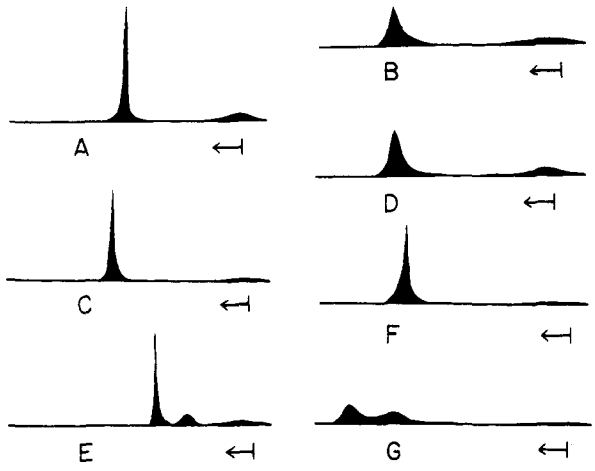


Fig. 1.—Ascending electrophoretic patterns of chondromucoprotein (MP) and chondroitin sulfate (ChS): A, MP at *p*H 9.7, 105 min. at 2.82 v./cm.; B, ChS at *p*H 9.7, 116 min. at 3.31 v./cm.; C, MP at *p*H 1.7, 238 min. at 2.08 v./cm.; D, ChS at *p*H 1.7, 229 min. at 2.01 v./cm.; E, MP at *p*H 12.5, 270 min. at 0.91 v./cm.; F, MP at *p*H 3.3, 212 min. at 2.38 v./cm.; G, MP at *p*H 3.3, 251 min. at 2.51 v./cm. after exposure to *p*H 12.5 at 0° for 16 hr.

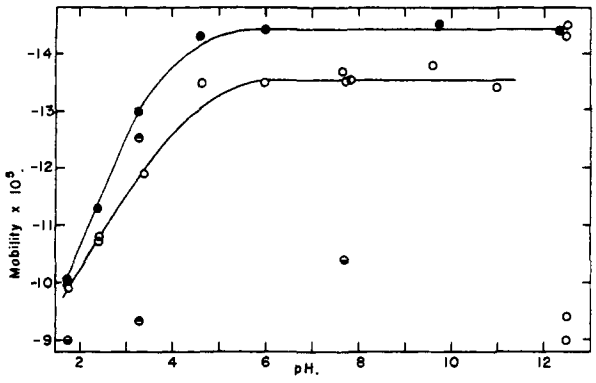


Fig. 2.—Mobilities (cm.^{2}volts^{-1}sec.$^{-1}$) of chondromucoprotein (○) and chondroitin sulfate (●) as a function of *p*H in 0.1 ionic strength buffers at 0°. The buffers employed were: *p*H 1.7, HCl, NaCl; *p*H 2.4, glycine, HCl; *p*H 3.3, formate; *p*H 4.6, acetate; *p*H 6, cacodylate; *p*H 7.8, barbital, NaCl; *p*H 9.7 and 11, glycine, NaOH; *p*H 12.5, NaOH. The mobilities of the two peaks in the reversal expt. in which the mucoprotein was exposed to *p*H 12.5 and run at *p*H 3.3 are indicated by ◓. Expts. with buffers containing 0.01 *M* $CaCl_2$ are indicated by ◒.

Deviations from enantiography were more marked with the mucoprotein than with chondroitin sulfate. For this reason and because the patterns were not appreciably changed by raising the ionic strength to 0.2, the deviations cannot be attributed to large conductance gradients resulting from the high charge on the polyelectrolytes. Some of the mucoprotein patterns (Fig. 1) showed the presence of a poorly resolved shoulder which may indicate partial dissociation of the components. This was most evident in acetate buffer at *p*H 4.6. In barbital buffer large deviations from enantiography were observed. In all cases the mobility was calculated from the bisecting ordinate of the descending pattern. The mobilities are plotted against *p*H in Fig. 2. When the *p*H was raised to 11 a very small component appeared with a lower mobility. Essentially complete dissociation took place at *p*H 12.5 where the slower component represented about 30% of the pattern area and the mobility of the fast component was increased to that of chondroitin sulfate. This dissociation was not reversed by lowering the *p*H. An experiment which shows this is included in Figs. 1 and 2. A solution which had been exposed to *p*H 12.5 was subsequently run at *p*H 3.3. Two components were still evident, the faster of which approached the mobility of chondroitin sulfate. By contrast, the mucroprotein at *p*H 3.3 showed a single boundary of intermediate mobility. The more slowly moving product of the dissociation at *p*H 12.5 appears to have about the same mobility at *p*H 3.3 as at 12.5. The high negative mobility of this component is consistent with its being protein still containing some anionic polysaccharide.

Figure 2 shows that the mucoprotein and chondroitin sulfate have substantially parallel *p*H–mobility relations. Both have constant mobilities above *p*H 5 and both fall off below this value, the polysaccharide somewhat more steeply. The drop in mobility at low *p*H may be correlated with protonation of the glucuronate carboxylate groups. No estimate of the pK can be made from these data because there is no indication of leveling off of the curve at still lower *p*H.

The addition of 0.01 *M* calcium ion at a constant ionic strength reduced the mobility by about 24% at *p*H 7.7 and by 10% at *p*H 1.7. From the fact that the decrease is greater at the higher *p*H it may be inferred that the calcium ion is at least in part bound by the glucuronate carboxylate groups which are largely protonated at the lower *p*H.

Discussion

Blix[5] extracted bovine nasal septa with water, clarified and concentrated the extract, dialyzed it against buffer (*p*H 8.0, ionic strength 0.1) and on electrophoresis found two components with mobilities 17.0 and 7.9 × 10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$. Analysis of the solution of the fast component showed that it had 4.7 atoms N per mole of hexosamine. The corresponding value for the chondromucoprotein used in the present work was 4.0. Since his solution gave a negative biuret test Blix disregarded his N analysis and preferred to believe the fast component he observed represented almost pure chondroitin sulfate. Partridge[6] extracted cartilage at 60° with a salt and buffer solution and on electrophoresis found two or three components of which the fastest had a mobility of 10.5 to 11.7 × 10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$ over the *p*H range 5.2 to 9.0. The method of extraction, analytical results and behavior of the preparations make it seem likely they contained degraded collagen. Mathews and Dorfman[7] used electrophoretic studies to judge the purity of their chondroitin sulfate preparations. Their best preparation at *p*H 7.5 and ionic strength 0.2 showed a single component with mobility 12.7 ×

(5) G. Blix, *Acta physiol. Scand.*, **1**, 29 (1940).
(6) S. M. Partridge, *Biochem. J.*, **43**, 387 (1948).
(7) M. B. Mathews and A. Dorfman, *Arch. Biochem. and Biophys.*, **42**, 41 (1953).

10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$. The present results show that over the *p*H range 5 to 11 both chondromucoprotein and chondroitin sulfate migrate essentially as single components with mobilities constant at 13.6 and 14.4 $\times$ 10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$, respectively, a difference of only about 6%.

The drop in mobility as the *p*H falls from 5 to 2 is similar for both chondromucoprotein and chondroitin sulfate. Over this range carboxylate groups would be protonated and the charge of both chondromucoprotein and chondroitin sulfate might be expected to be cut to about half. Yet the mobilities drop less than 30%. This behavior is consistent with the finding of extensive association of counterion with polyelectrolytes in solution.[8] The amount of cation associated with the polyion may be of the order of 50 to 70% of the number of negative sites available on the polyanion. The theory of association of such counterion predicts that as the number of sites is reduced, as by protonation of the carboxyls, the number of associated counterions is more than proportionately reduced so the reduction in net charge is less than proportional to the amount of protonation. The lowered mobility of the chondromucoprotein in the presence of calcium ions may be interpreted similarly as an effect of the higher degree of association of calcium ion over sodium ion with the polyanion thus reducing its net charge.[9]

The dissociation of chondromucoprotein at *p*H 12.5 into two components and the irreversibility of this dissociation on acidification is the basis of the method of alkaline extraction of cartilage to prepare chondroitin sulfate used since the time of Krukenberg.[10] The data presented here suggest that this is not simply a dissociation of the chondromucoprotein into chondroitin sulfate and protein but that the protein component still contains anionic polysaccharide.

Acknowledgments.—We are indebted to Francis Chen and John Perz for valuable technical assistance during the course of this work.

(8) J. R. Huizenga, P. F. Grieger and F. T. Wall, THIS JOURNAL, **72**, 2636 (1950); F. T. Wall and R. H. Doremus, *ibid.*, **76**, 1557 (1954).

(9) S. J. Farber and M. Schubert, *J. Clin. Invest.*, **36**, 1715 (1957).

(10) C. F. W. Krukenberg, *Z. Biol.*, **20**, 307 (1884).

NEW YORK, N. Y.

[CONTRIBUTION FROM THE MERCK SHARP & DOHME RESEARCH LABORATORIES, DIVISION OF MERCK & CO., INC.]

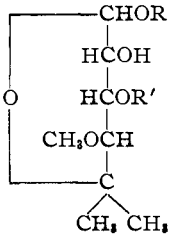
Novobiocin. VIII. The Configuration of Noviose

BY EDWARD WALTON, JOHN O. RODIN, CHARLES H. STAMMER, FREDERICK W. HOLLY AND KARL FOLKERS

RECEIVED APRIL 14, 1958

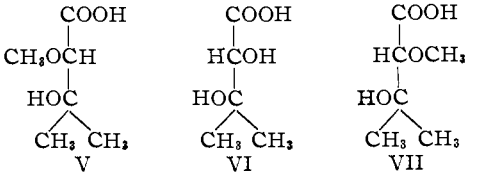
The L-lyxose configuration has been assigned to 3-O-carbamylnoviose, the aldose moiety of novobiocin. This configurational assignment which was based in part on empirical rules of optical rotation has been confirmed by the synthesis of 2,3-isopropylidene-5-O-methylnovionic acid from L-rhamnose.

The aldose moiety of novobiocin, 3-O-carbamylnoviose (I)[1,2] has been assigned the configuration of L-lyxose. This assignment, which was the subject of a preliminary report,[3] was based in part on rules of optical rotation. Synthetic confirmation of the proposed configuration now has been obtained.



I, R = H, R′ = $CONH_2$
II, R = CH_3, R′ = $CONH_2$
III, R = H, R′ = H
IV, R = CH_3, R′ = H

During the work which permitted the elucidation of the structure of 3-O-carbamylnoviose (I), (−)-α-methoxy-β-hydroxyisovaleric acid (V) was isolated as a degradation product.[1] This acid, obtained by oxidative cleavage of 1-deoxynoviose,[1] represents the aldose moiety minus carbon atoms 1 and 2. Its optical antipode, (+)-α-methoxy-β-hydroxyisovaleric acid (VII), was synthesized[1] from (−)-α,β-dihydroxyisovaleric acid (VI).[4] The



V VI VII

rotation of the dihydroxy acid VI in 1 *N* hydrochloric acid is $[\alpha]^{25}D$ − 14.7° (*c* 1.64); in 1*N* sodium hydroxide, $[\alpha]^{30}D$ + 4.8° (*c* 1.8). This positive shift in rotation in going from the acid to its ion is characteristic[5] of D-α-hydroxy acids having one asymmetric center. The C-2 hydroxy group in the dihydroxy acid VI is, therefore, on the right in the Fisher projection as is the C-2 methoxyl in the synthetic methoxy acid VII. The C-2 methoxyl in the degradation product V is then on the left. Since C-2 in the methoxy acid V corresponds to C-4 in noviose and its derivatives (I through IV), the C-4 methoxyl in these compounds is also on the left. This leads to the conclusion that noviose is an L-aldose.

Hydrolysis of methyl noviopyranoside (IV)[1,2] with 0.1 *N* hydrochloric acid followed by reaction

(1) C. H. Shunk, C. H. Stammer, E. A. Kaczka, E. Walton, C. F. Spencer, A. N. Wilson, J. W. Richter, F. W. Holly and K. Folkers, THIS JOURNAL, **78**, 1770 (1956).

(2) H. Hoeksema, E. L. Caron and J. W. Hinman, *ibid.*, **78**, 2019 (1956); J. W. Hinman, E. L. Caron and H. Hoeksema, *ibid.*, **79**, 3789 (1957).

(3) E. Walton, J. O. Rodin, C. H. Stammer, F. W. Holly and K. Folkers, *ibid.*, **78**, 5454 (1956).

(4) J. R. Sjolander, K. Folkers, E. A. Adelberg and E. L. Tatum, *ibid.*, **76**, 1085 (1954).

(5) M. Winitz, L. Block-Frankenthal, N. Izumiya, S. M. Birnbaum, C. G. Baker and J. P. Greenstein, *ibid.*, **78**, 2423 (1956).