ELECTRON MICROSCOPIC STUDIES OF HYALURONIC ACID–PROTEIN GELS

FREDERICK A. BETTELHEIM AND DELBERT E. PHILPOTT
Chemistry Department, Adelphi College, Garden City, New York, and the Marine Biological Laboratory, Woods Hole, Mass. (U.S.A.)

(Received September 21st, 1958)

SUMMARY

Electron micrographs were taken of the hyaluronic acid gel and its complexes with proteins occurring in connective tissues. The hyaluronic acid formed anisotropic aggregates which were sandwiched between protein layers. The forces directing aggregate formations were discussed. Complexes of hyaluronic acid with blood albumin were produced at different pH's and different hyaluronic acid/protein ratios. The effect of the hyaluronic acid aggregates upon the structure formation of the complexes was discussed.

INTRODUCTION

Hyaluronic acid in its native state is always associated with proteins. Many investigators tried to elucidate the nature of the complexing. MEYER[1] believed that this association is of polar nature between the free amino groups of protein and the carboxylic groups of hyaluronate since the complex dissociates in alkali or salt solutions. OGSTON AND STANIER[2–4] were of the opinion that part of the protein (27 %) is more firmly bound to hyaluronic acid than a reversible polar association would indicate. It was speculated that such an interaction may have biological importance in fibrillogenesis, or in general, in orientation and organization of structural elements[1,5,6]. If hyaluronic acid produces an orientation in complexing it has to have an oriented macromolecular or aggregate-super-structure. Previous investigations[7,8] indicated that hyaluronic acid does not possess crystalline structure although it is birefringent. Recently, one of us (F.A.B.) found that sodium hyaluronate, when completely free of protein, is highly crystalline[9]. Hence, structural organization on the molecular level exists.

Electron microscopic studies can give information on the super-structure of the hyaluronic acid. GROSS[10], JENSEN AND CARLSEN[11] and ROWEN, BRUNISH AND BISHOP[12] reported that when hyaluronic acid is dried from dilute solution one obtains in part amorphous lakes and in part fibrous structure in the electron micrographs depending on the mode of preparation. These observations were confirmed by us in a preliminary investigation. The purpose of the present studies was to determine whether hyaluronic acid has an aggregate orientation in the gel form and to investigate the effect of hyaluronic acid (HA) upon the special structure formations in complexing with proteins.

References p. 134/135.

METHODS AND MATERIALS

The electron micrographs were taken with a RCA EMU 2B electron microscope under conditions described previously[13].

HA was isolated from umbilical cords by the method of JEANLOZ AND FORCHIELLI[14]. Pepsin and trypsin were used as proteolytic enzymes in the first 2 steps. The remaining proteins were denatured by Sevag's procedure and discarded after the extraction. Chondroitin sulfate impurities were eliminated by extraction with ammonium sulfate solution and pyridine mixture. The HA was also purified from the remaining protein impurities by using Lloyd's reagent. The resulting material was homogeneous as viewed in the ultracentrifuge and had an average mol. wt. of 77,000. Acid hydrolysis with 4 *N* HCl and subsequent paper chromatography showed only D-glucuronic acid and D-glucosamine spots. No amino acids were present in the

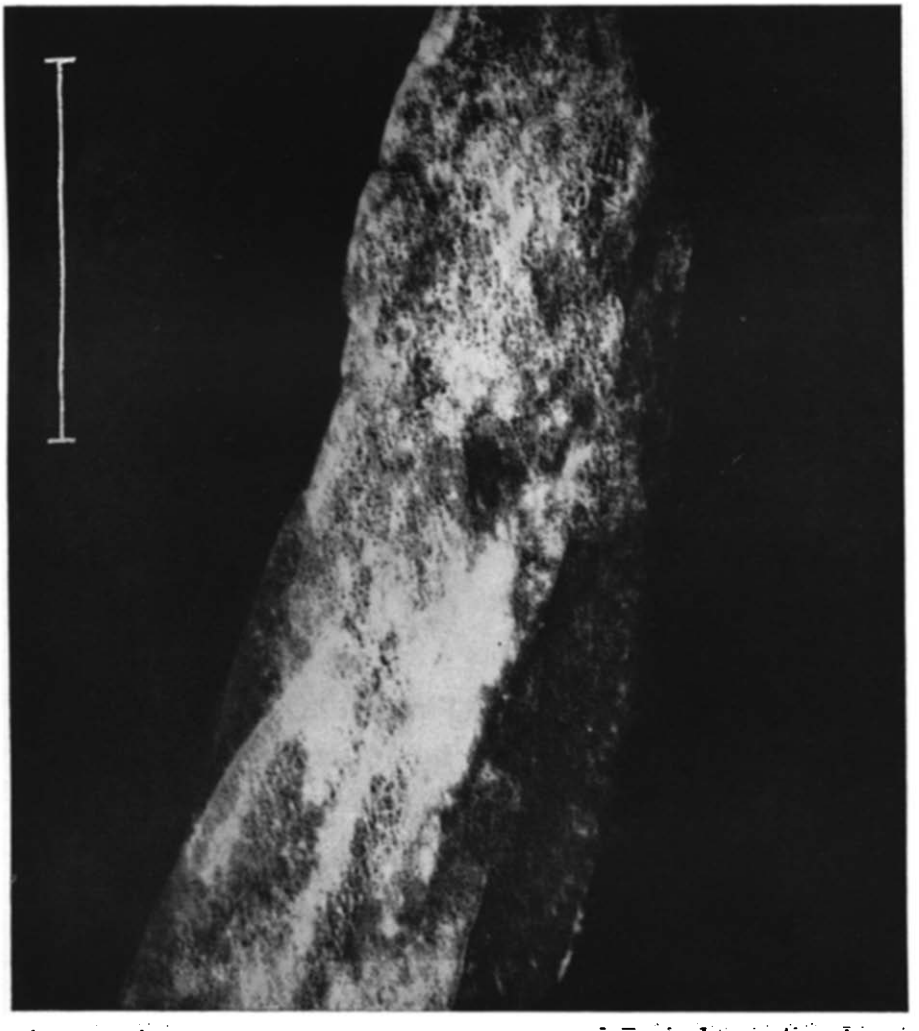
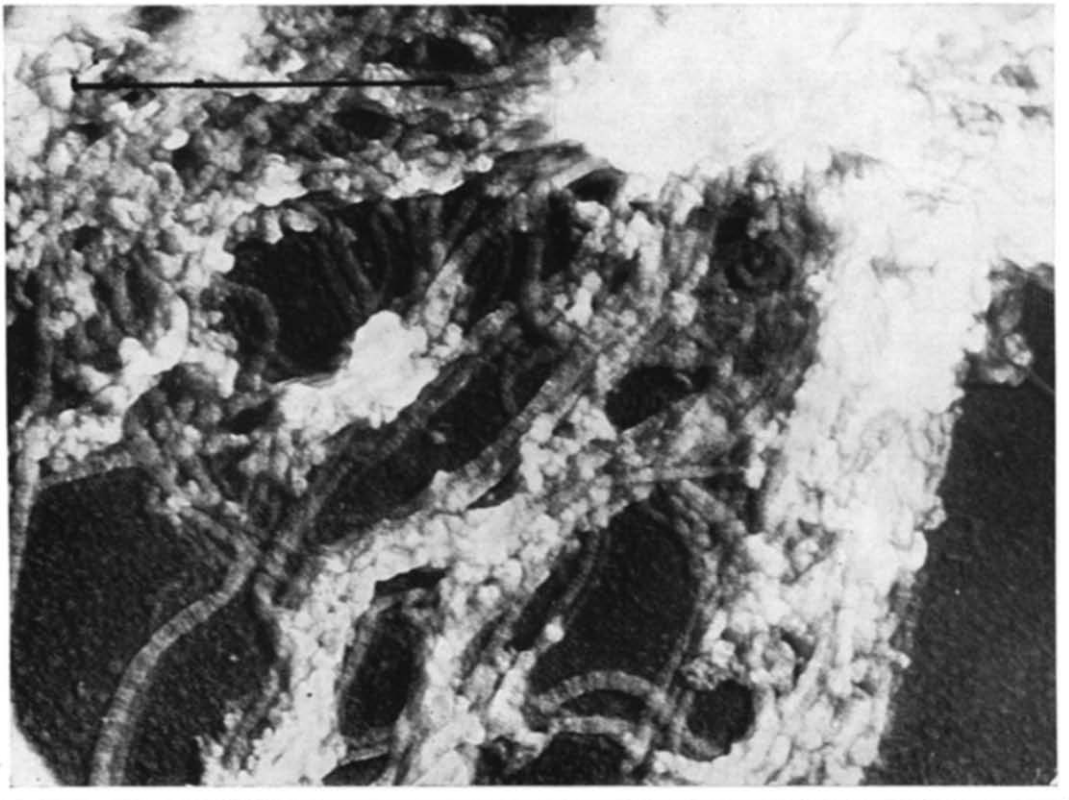
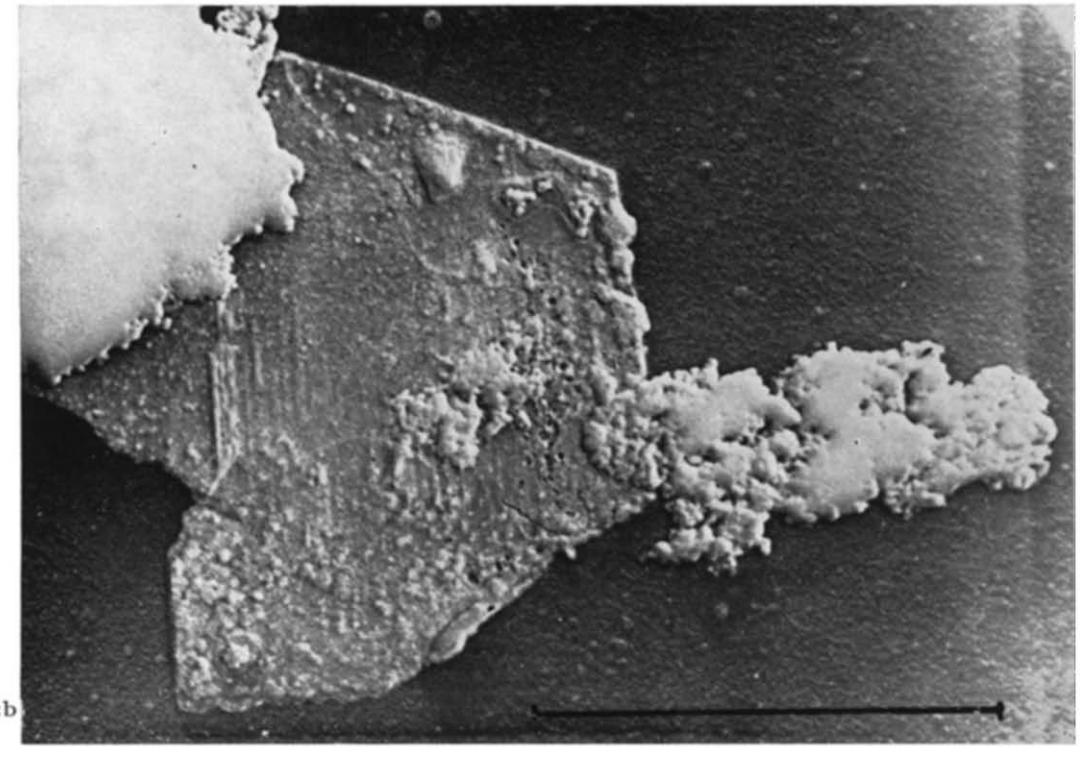


Fig 1. An electron micrograph of pure sodium hyaluronate gel. Pt shadow casting. Line indicates 1 μ length.

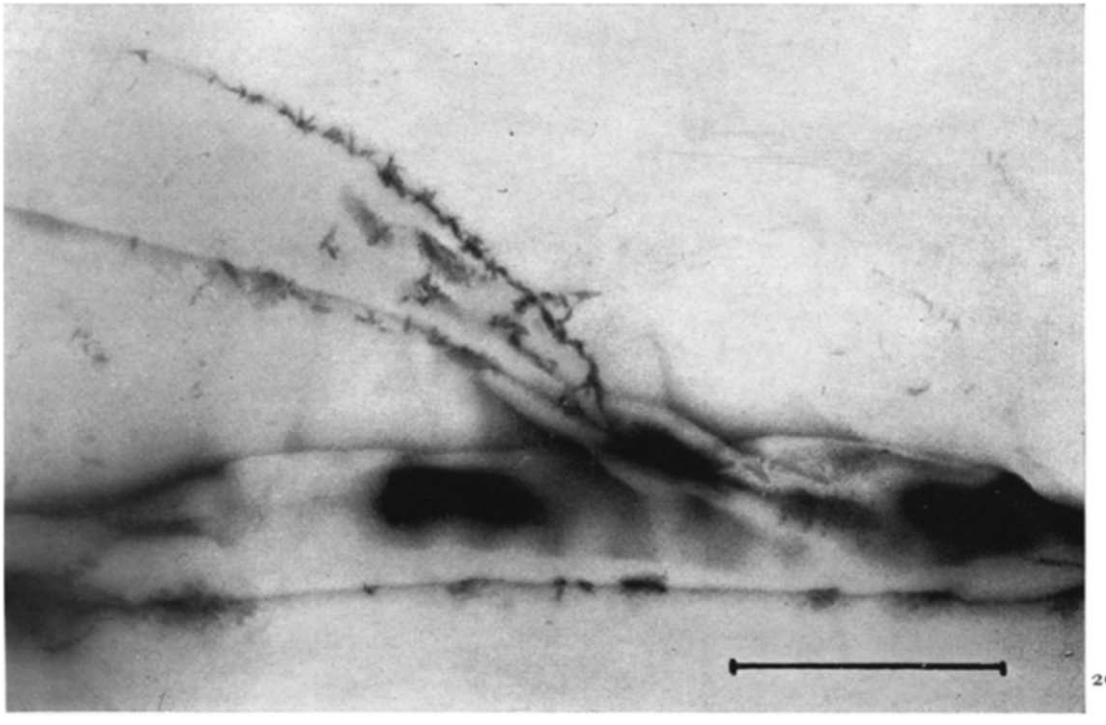
References p. 134/135.



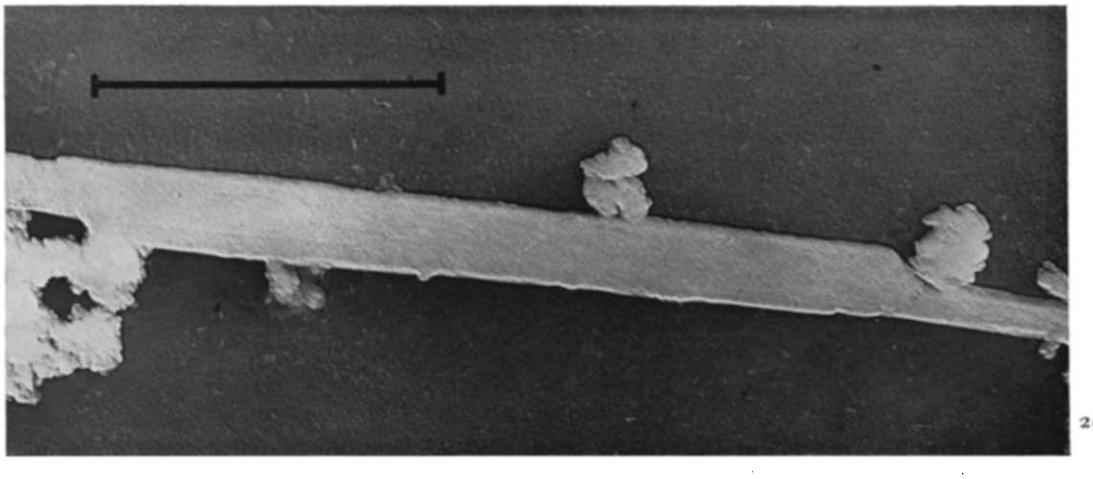
2a



2b

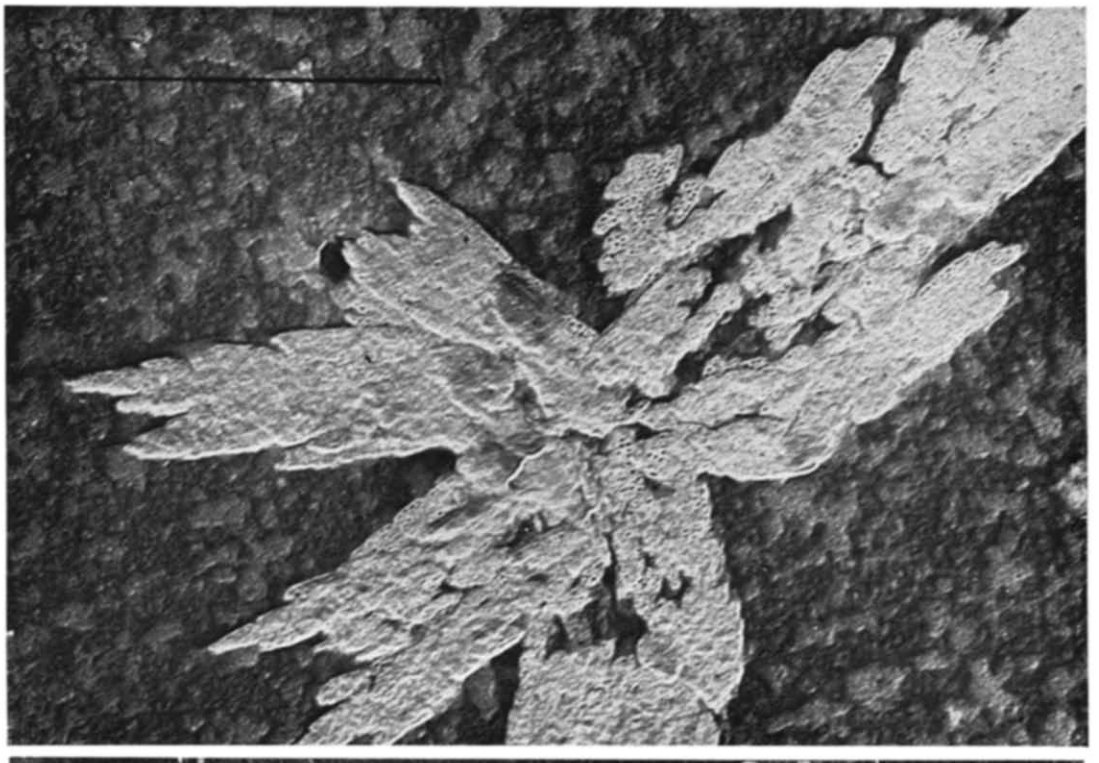


2c

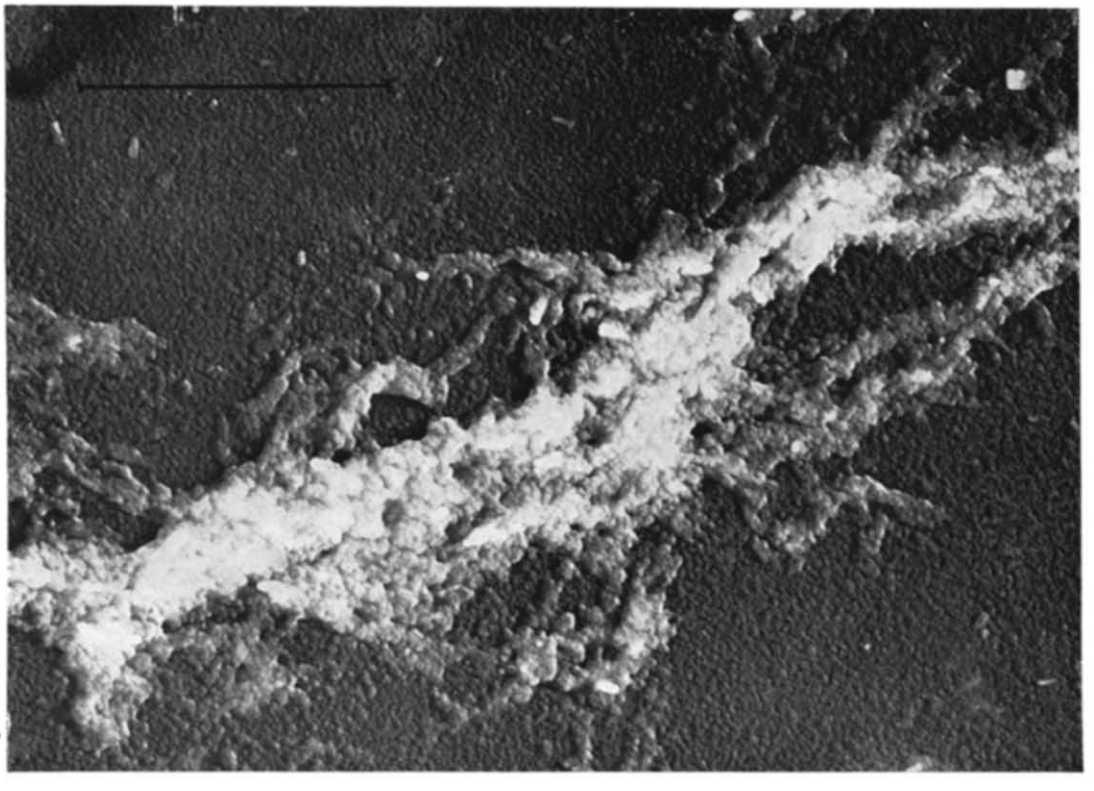


2d

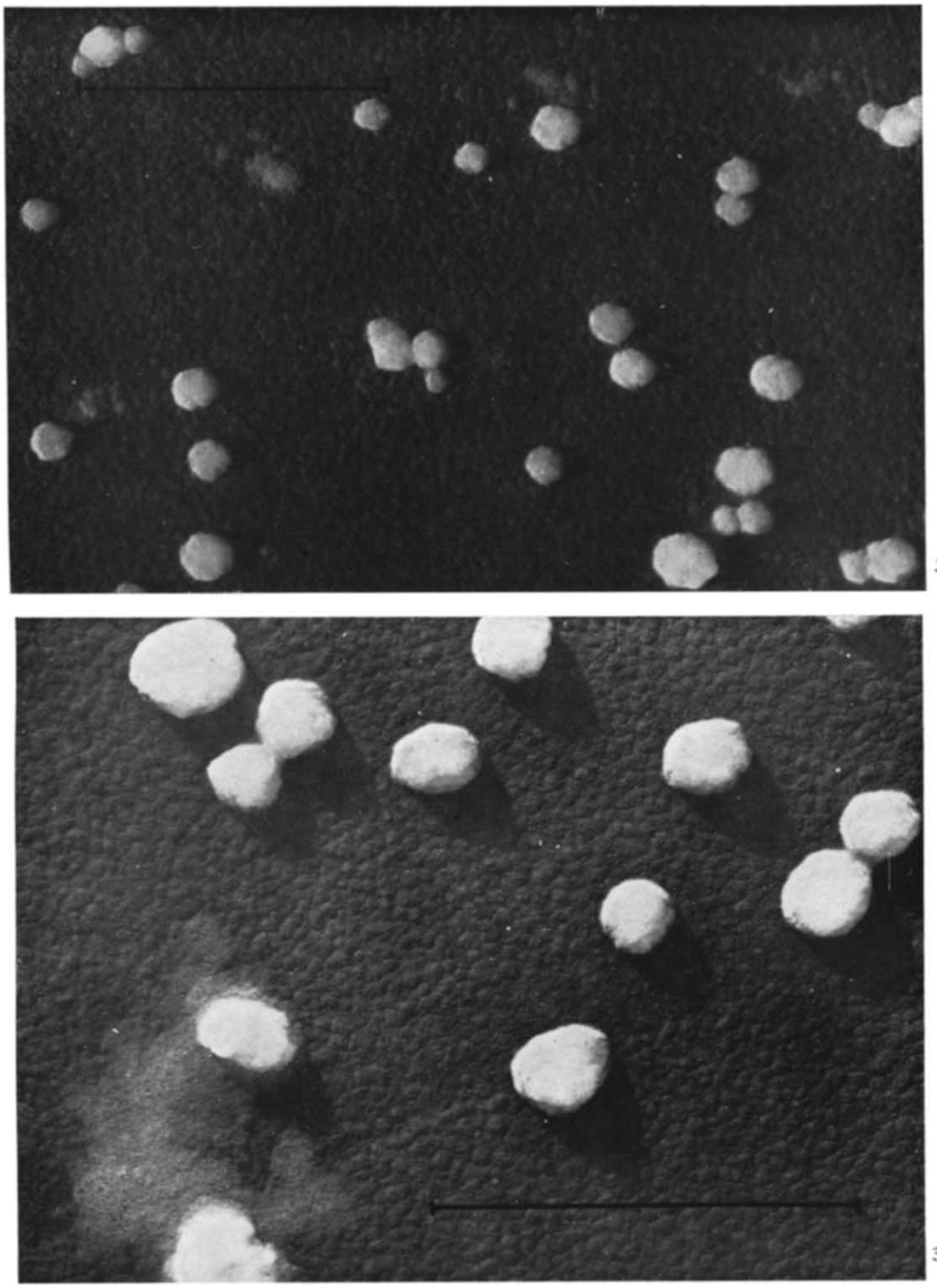
Fig. 2. Electron micrographs of hyaluronic acid–protein complexes during the different stages of HA purification. Lines indicate 1 μ length. a. Umbilical cord tissue dispersed in ethanol. Cr shadow casting. b. Tissue after digestion with pepsin and trypsin. Precipitate suspended in 66% ethanol. Cr shadow casting. c. The same as 2b. Precipitate treated with phosphotungstic acid in ethanol and embedded directly in methacrylate. Longitudinal cut, along the fiber axis. Precipitate water-soluble, difficult to section. d. HA preparation after the Sevag procedure. Precipitate suspended in 66% ethanol. Cr shadow casting.



3a



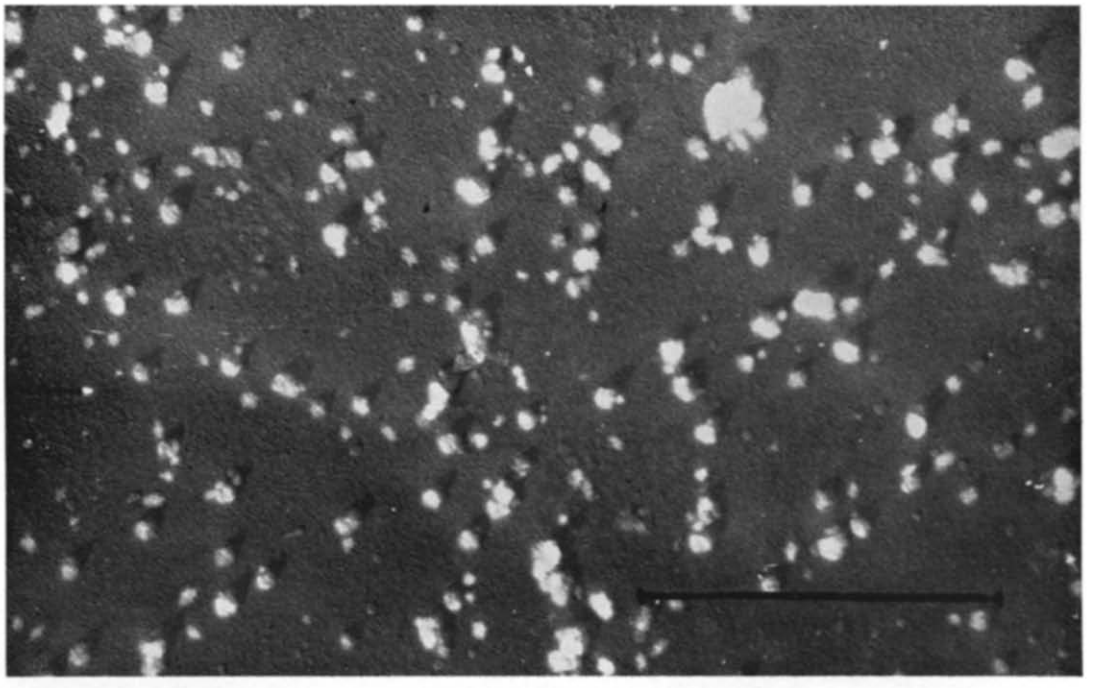
3b



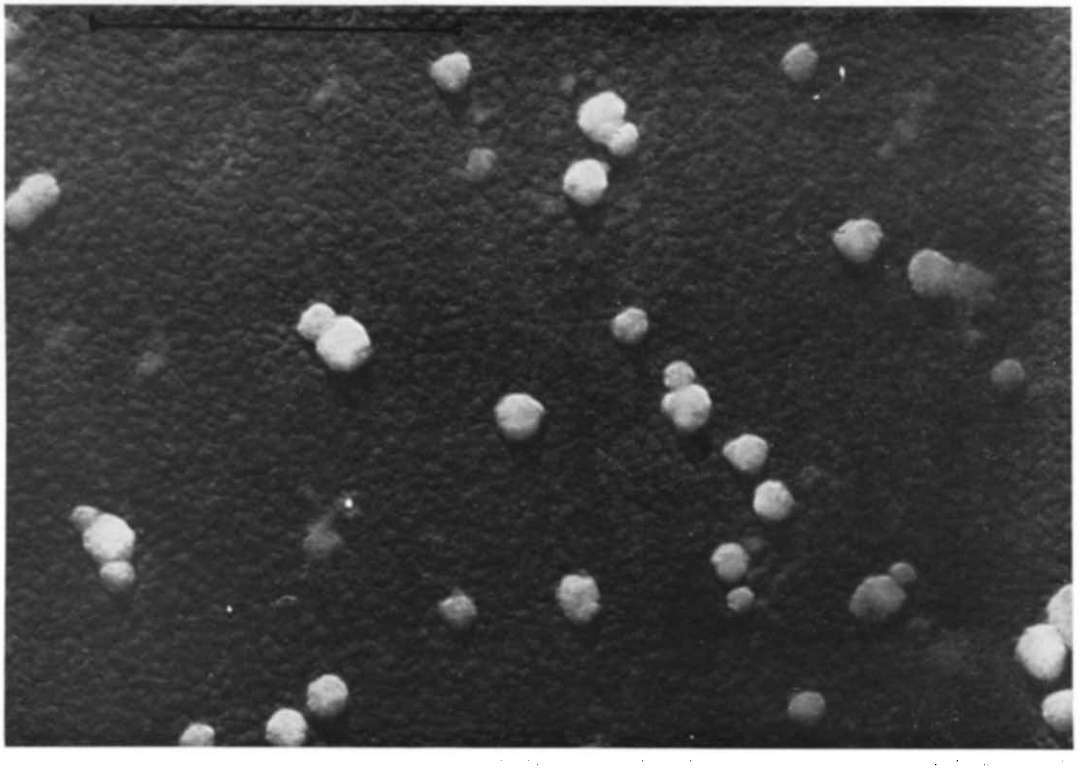
3c

3d

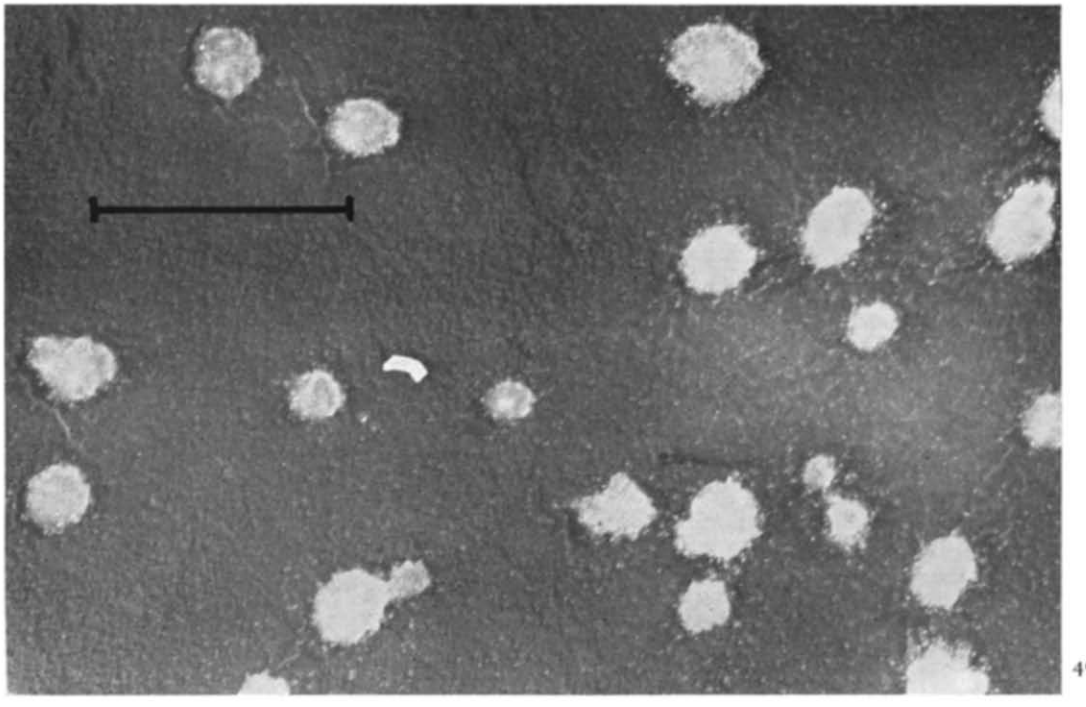
Fig. 3. Electron micrographs of artificial complexes of hyaluronic acid and blood albumin (concentration 3:1) at different pH's. Cr shadow casting. Lines indicate 1 μ length. (a) pH = 10.0; (b) pH = 6.8; (c) pH = 4.3; (d) pH = 3.0.



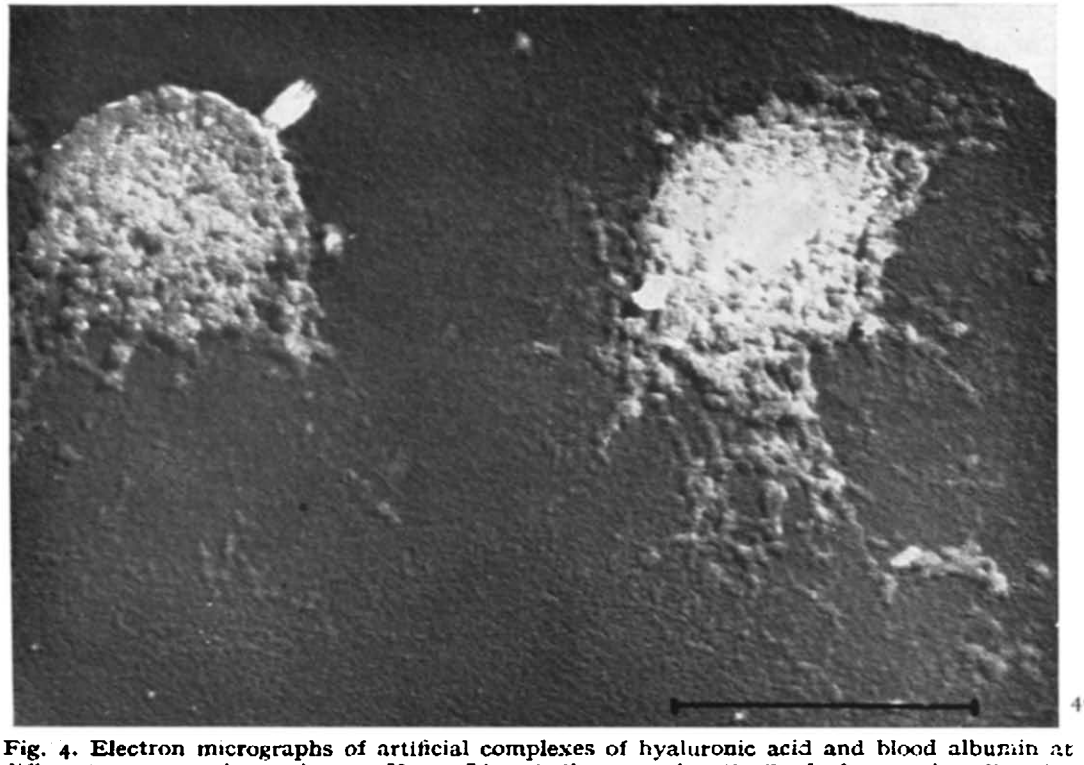
4a



4b



4c



4d

Fig. 4. Electron micrographs of artificial complexes of hyaluronic acid and blood albumin at different concentration ratios at pH 4.3. Lines indicate 1 μ length. Cr shadow casting. Protein/hyaluronic acid ratio: (a) 1:19; (b) 1:3; (c) 1:1; (d) 3:1.

that HA preparations cannot be purified completely from proteins by enzymic digestion.

These interpretations of the electron micrographs are in agreement with the histochemical observations of SYLVÉN AND MALMGREN[7] who claimed that in metachromasia of HA the actual local concentration of HA has to be above 5 % before it can be seen. The fact that they observed red staining material between the blue staining protein layers of vitreous body indicated that "the actual concentration of hyaluronic acid per micro-unit of tissue volume must be much higher than the average percentage of the hyaluronate" in the tissue. This confirms the aggregate formation of HA as seen by electron microscope.

The aggregate formation of HA, rather than a molecular complexing with proteins would require that the intermolecular attraction between HA molecules should be stronger or sterically more feasible than the polar attractions between the protein amino and the uronide carboxyl groups. The highly crystalline structure of the pure HA[9] would favor the steric hypothesis since the inter-molecular hydrogen bonding between HA molecules would be of the same magnitude or less than protein-HA bonding. X-ray diffraction data[20] indicates that the repeating unit along the HA chain is 12 Å and if this is the length of the chemical repeating unit, *i.e.*, hyalobiuronic acid, the macromolecular chain must be very stiff. This is also supported by the streaming birefringence data of many authors as reviewed critically by BLUMBERG AND OGSTON[21]. Hence, a preferred HA aggregate formation and the high anisotropy of these aggregates can be explained on the basis of steric configuration.

Anisotropic platelets of HA can serve as templates for proteins in the formation of structural units. The fibrous nature of the HA-protein complex may have its origin in the orienting effect of the HA aggregates. The experiments performed at different pH's in the present study indicate that the change in the shape of the complex aggregate under the influence of the environment might be due to the contraction and closer packing of the polyuronide material. A similar phenomenon can be observed in other polyuronides and also *in vivo* in the hyaline layers of certain tissues.

NOTE ADDED IN PROOF

Since submitting this paper for publication PIGMAN *et al.*[22] found that the human synovial mucin is a complex of albumin and HA. Hence the model system used in this investigation has its *in vivo* counterpart and the nature of complexing elucidated here may have real physiological significance.

(Received March 23rd, 1959)

ACKNOWLEDGEMENTS

This research was supported by a Lalor Foundation award to the senior author for the summer of 1958 at the M.B.L. in Woods Hole, Mass., and by a grant (C-3984 BBC) of the National Cancer Institute, Public Health Service.

REFERENCES

1 K. MEYER, *Discussions Faraday Soc.*, 13 (1953) 271.
2 A. G. OGSTON AND J. E. STANIER, *Biochem. J.*, 46 (1950) 364.
3 A. G. OGSTON AND J. E. STANIER, *Biochem. J.*, 49 (1951) 585.

[5] A. G. OGSTON AND J. E. STANIER, *Discussions Faraday Soc.*, 13 (1953) 275.
[6] J. H. HIGHBERGER, J. GROSS AND F. O. SCHMITT, *Proc. Natl. Acad. Sci. U.S.*, 37 (1951) 286.
[7] S. S. COHEN, *J. Biol. Chem.*, 144 (1942) 353.
[8] B. SYLVÉN AND H. MALMGREN, *Lab. Investig.*, 1 (1952) 413.
[9] B. SYLVÉN AND E. J. AMBROSE, *Biochim. Biophys. Acta*, 18 (1955) 587.
[10] F. A. BETTELHEIM, *Nature*, 182 (1958) 1301.
[11] J. GROSS, *Ann. N.Y. Acad. Sci.*, 52 (1950) 964.
[12] C. E. JENSEN AND F. CARLSEN, *Acta Chem. Scand.*, 8 (1954) 1357.
[13] J. W. ROWEN, R. BRUNISH AND F. W. BISHOP, *Biochim. Biophys. Acta*, 19 (1956) 481.
[14] D. E. PHILPOTT AND A. G. SZENT-GYÖRGYI, *Biochim. Biophys. Acta*, 15 (1954) 165.
[15] R. W. JEANLOZ AND E. FORCHIELLI, *J. Biol. Chem.*, 186 (1950) 495.
[16] L. ANDERSON, *Acta Chem. Scand.*, 7 (1953) 689.
[17] Z. DISCHE, *J. Biol. Chem.*, 167 (1947) 189.
[18] J. P. JOHNSTON, A. G. OGSTON AND J. E. STANIER, *Analyst*, 76 (1951) 88.
[19] C. C. CURTAIN, *Biochem. J.*, 61 (1955) 688.
[20] A. K. PARPART, Princeton Univ., private communication.
[21] F. A. BETTELHEIM, unpublished data.
[22] B. S. BLUMBERG AND A. G. OGSTON, *Ciba Foundation Symposium on the Chem. and Biol. of Mucopolysaccharides*, 1958, pp. 22–37.
[23] W. PIGMAN, E. GRAMLING, D. PLATT AND H. L. HOLLEY, *Biochem. J.*, 11 (1959) 201.

BRADYKININ FROM TOTAL BOVINE PLASMA

ULLA HAMBERG

Department of Pharmacology, Faculty of Medicine, Ribeirão Prêto (Brazil)

(Received September 13th, 1958)

SUMMARY

1. The biological homogeneity of bradykinin prepared from whole bovine plasma and purified on an aluminium oxide column was studied. The ratio of uterus activity/guinea pig activity of a total plasma bradykinin and of a standard bradykinin prepared from a precipitated plasma globulins was determined. Results showed that in the case of total plasma bradykinin this ratio was 2–3:1, whereas in the case of standard bradykinin it was 1:1.

2. The effect upon the fall in the cat's arterial blood pressure was approximately equal to that of the standard.

3. The purified bradykinin appeared as a single substance on paper chromatography and paper electrophoresis when localized by the effect upon the rat's uterus or guinea-pig ileum.

4. The plasma bradykinin may be associated with a phospholipid when eluted from aluminium oxide at 50% methanol concentration.

INTRODUCTION

The biological homogeneity of bradykinin prepared on a large scale from whole bovine plasma[1] and purified on an aluminium oxide column has been studied. As indicated

References p. 146.