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Summary

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The dissociation and reassociation reactions of hemocyanin mixtures were studied by electron microscopy. The experiments were done respectively with a mixture of Helix pomatia and Loligo pealei hemocyanin (both belonging to phylum Mollusca) and with a mixture of Helix pomatia (a Mollusc) and Limulus polyphemus (an Arthropod) hemocyanins. After reassociation many of the original molecular structures are observed together with a certain amount of smaller and irregularly aggregated material. The importance of these specific reassociation reactions between hemocyanin subunits from different classes and from different phylums is discussed.

Author

1. Introduction

Electron microscopy shows characteristic quaternary structures in hemocyanins from different biological origins. Previous work demonstrated that under certain conditions it is possible to dissociate these molecules into smaller components that will reassemble after restoration of the original conditions. We were interested in the specificity of this reaction and wondered what would happen if we repeated the experiment with a mixture of two hemocyanins. Would they reassemble, or would they form some typical hybrids or aggregate to random structures?

Electron microscopy is an excellent method for studying these reactions because it affords direct visualization. The choice of hemocyanins can be made so that it is possible to identify the two molecule types involved on electron micrographs.

Two essentially different experiments were done. Firstly, hemocyanins were taken from Helix pomatia (a Gastropod) and Loligo pealei (a Decapod), both belonging to the phylum Mollusca. Both hemocyanins dissociate into 11 s units at high pH. (Eriksson-Quensel & Svedberg, 1936; Van Holde & Cohen, 1964).

Secondly, hemocyanins belonging to different phylums were taken: Helix pomatia from Mollusca and Limulus polyphemus from Arthropoda. The first hemocyanin dissociates at high pH into 11 s components, and the second dissociates into 6 s components under the same conditions. (Eriksson-Quensel & Svedberg, 1936). Tiselius and Horsfall (1939) did similar

experiments with the more closely related Mollusca hemocyanins of Helix pomatia, Helix nemoralis and Littorina littorea. They followed, however, the dissociation and association reactions by boundary electrophoresis and sedimentation analysis.

2. Materials and Methods

The hemolymphs described in the foregoing paper were used. The experiments were done at a room temperature of 21°C, using a concentration of about 0.2% for each of the hemocyanins. In the case of Helix pomatia and Loligo pealei the dissociation was done in a solution containing 0.01 M-ammonium carbonate brought to pH 10.0 with ammonia. For Helix pomatia and Limulus polyphemus 0.01 M-ammonia of pH 10.8 was used. This was necessary because Limulus hemocyanin is not completely dissociated at pH 10.0. The mixtures were kept at their high pH values for thirty minutes. Then part of the solution was used for preparing a specimen of the dissociated hemocyanin mixture, while the pH of the remaining solution was immediately lowered with acetic acid to a value between 6 and 7. To favor the association, the mixture was then dialysed against a solution containing 0.01 M-acetic acid, 0.005 M-calcium acetate and 0.005 M-magnesium acetate brought to pH 6.0 with ammonia. Specimens of the mixture in the association state were made about twenty-four hours after lowering the pH.

Specimens were mainly prepared with the droplet method described in the foregoing paper that also contains the details of the electron microscopy.

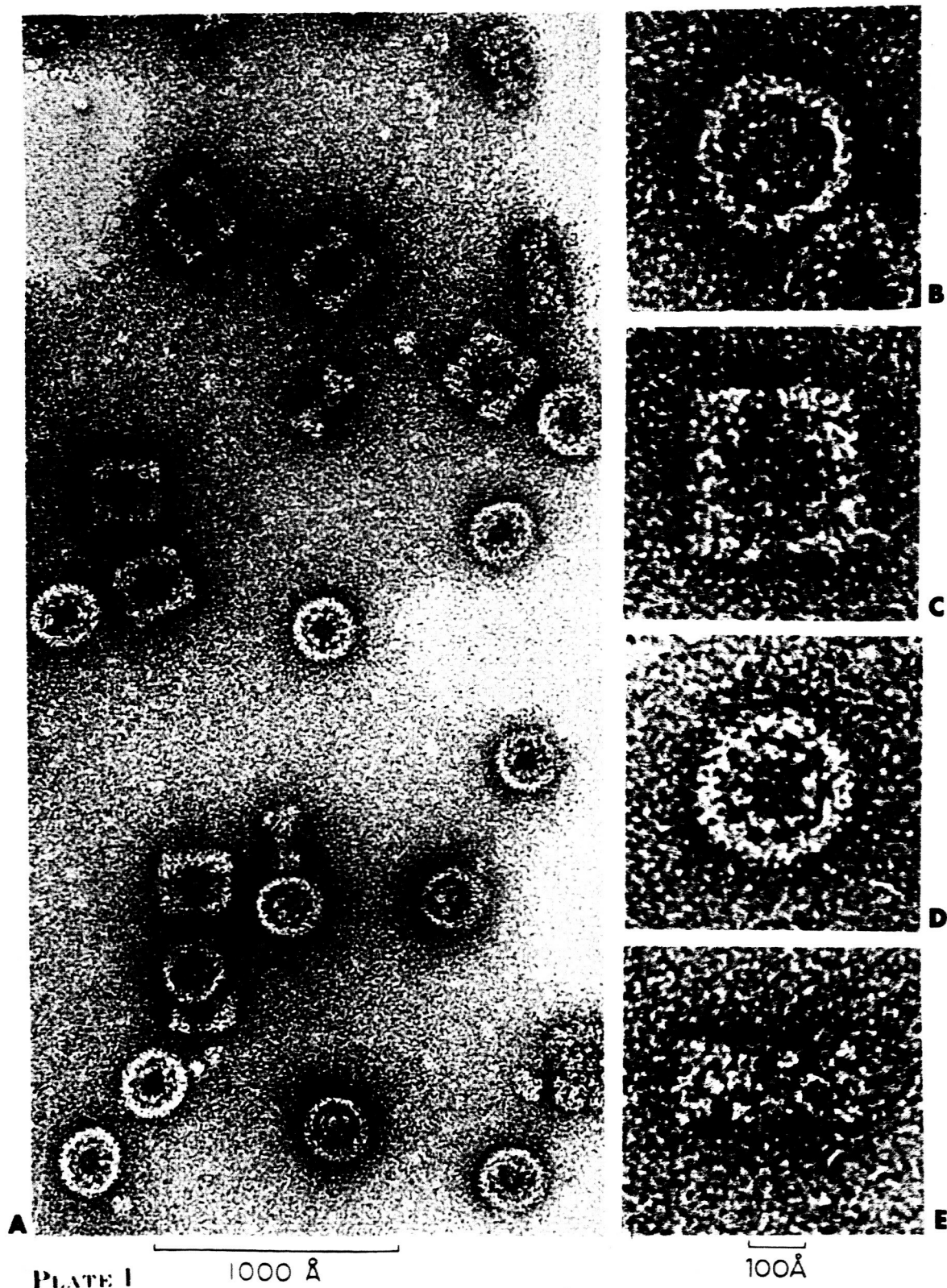
3. Results

(a) Mixture of Helix pomatia and Loligo pealei hemocyanin.

Plate I is an electron micrograph of a mixture of Helix pomatia (Mollusca, Gastropoda) and Loligo pealei (Mollusca, Decapoda) hemocyanin taken before dissociation in a medium containing 0.01 M-ammonium acetate/acetic acid, 0.005 M-magnesium acetate and 0.005 M-calcium acetate of pH 6.0. The different aspects of both types of molecules can be easily recognized. Comparison with the electron micrographs of the foregoing paper shows that the molecules marked (b) and (c) are typical projections of Helix hemocyanin, while (d) and (e) belong to Loligo hemocyanin. This plate also demonstrates clearly that the circles of both hemocyanins have the same dimensions.

Plate II shows the situation at pH 10.0, in a buffer containing 0.01 M-ammonium carbonate/ammonia. Complete dissociation is observed in subunits measuring 30 Å to 50 Å across, which still show a very distinct substructure. At this high pH these units show a tendency to irregular aggregation which can also be seen in some areas of this print.

Plate III shows the reassociation of the small units. It was taken twenty-four hours after lowering of the pH from 10.0 to 6.5 followed by dialysis against a solution containing 0.005 M-magnesium acetate and 0.005 M-calcium acetate, brought to pH 6.0 with ammonium acetate/acetic acid. Besides a rather large amount of irregular structures, some structures ((b), (c), (d), (e)) can be seen which are similar to those indicated in Plate I.



Mixture of negatively stained Helix pomatia and Loligo pealei hemocyanin molecules at pH 6.0. The Helix hemocyanin molecules are seen as circles (b) and six-row rectangles (c). The Loligo hemocyanin molecules appear as circles with a slightly different central region (d) and three-row rectangles (e). (a) X 360,000. (b) X 900,000.

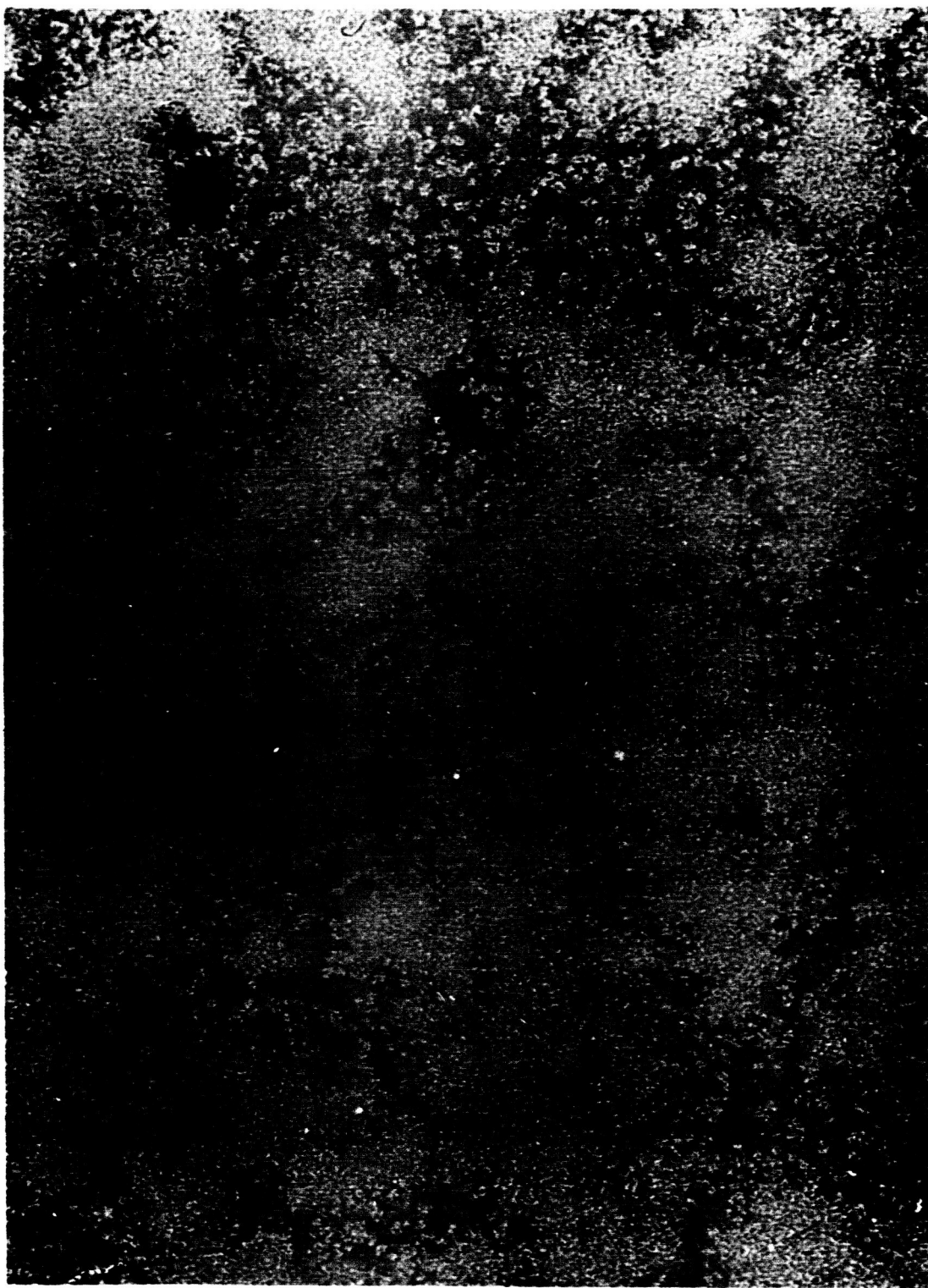


PLATE II

1000 Å

Mixture of negatively stained Helix and Loligo hemocyanins at pH 10.0. The molecules are completely dissociated into the same type of subunits measuring about 30 Å to 50 Å across. X 360,000.

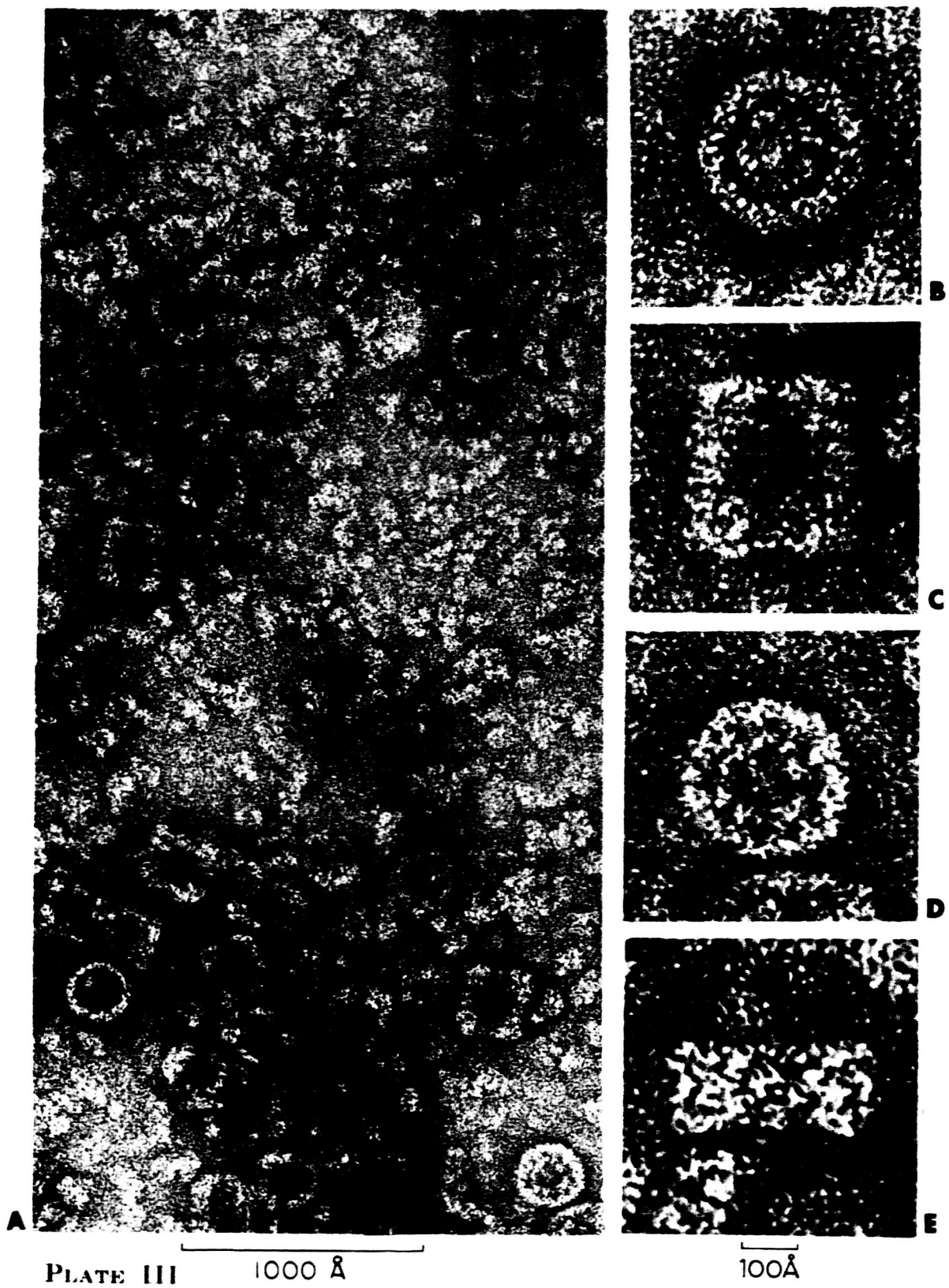


PLATE III

1000 Å

100 Å

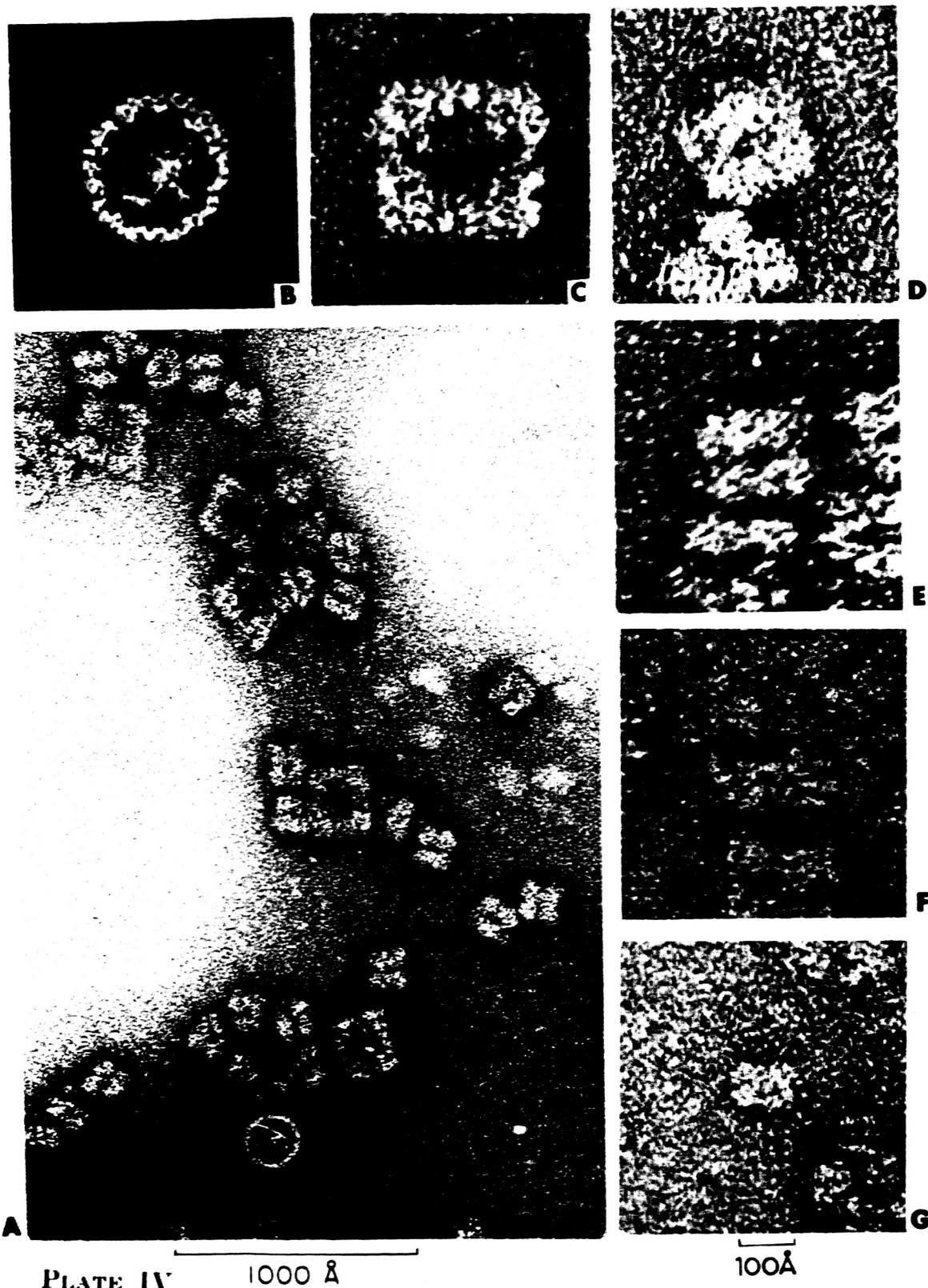
Negatively stained Helix and Loligo hemocyanin molecules reassociated from the diluted mixture of subunits. The structures shown are similar to those indicated in Plate I. (a) X 360,000. (b) X 900,000.

(b) Mixture of Helix pomatia and Limulus polyphemus hemocyanin.

Plate IV shows a mixture of Helix pomatia (Mollusca) and Limulus polyphemus (Arthropoda) hemocyanin taken before dissociation in 0.01 M-ammonium acetate/acetic acid buffer of pH 6.0 containing 0.005 M-magnesium acetate and 0.005 M-calcium acetate. Again the molecules marked (b) and (c) are the typical projections of Helix hemocyanin; those marked (d), (e), (f), and (g) are projections of the different components of Limulus hemocyanin, as can be seen by comparison with electron micrographs of the foregoing paper. (Fernandez-Moran, van Bruggen & Ohtsuki, 1966).

Plate V gives a survey at pH 10.8 in 0.01 M-ammonia. Complete dissociation is observed. The smallest units are 30 Å to 50 Å across; in many cases, however, irregular aggregation occurs to 75 Å to 100 Å particles.

Plate VI shows the reassociation of the dissociated units twenty-seven hours after lowering the pH from 10.8 to about 6.5. This was followed by dialysis against a 0.01 M-ammonium acetate/acetic acid buffer of pH 6.0 containing 0.005 M-magnesium acetate and 0.005 M-calcium acetate. The Helix hemocyanin molecules marked (b) and (c) are easily recognized. The same is the case for the different Limulus hemocyanin components marked (d), (e), (f), and (g) that are similar to the ones shown on Plate IV:



Mixture of negatively stained *Helix pomatia* and *Limulus polyphemus* hemocyanin molecules at pH 6.0. The *Helix* hemocyanin molecules are seen as circles (b) and six-row rectangles (c). The *Limulus* hemocyanin molecules appear as a smaller type of circle (d); rectangles with two wide rows (e); a combination of a hexagon with a square (f) or as a small rectangle (g). (a) X 360,000. (b) to (e) X 900,000.

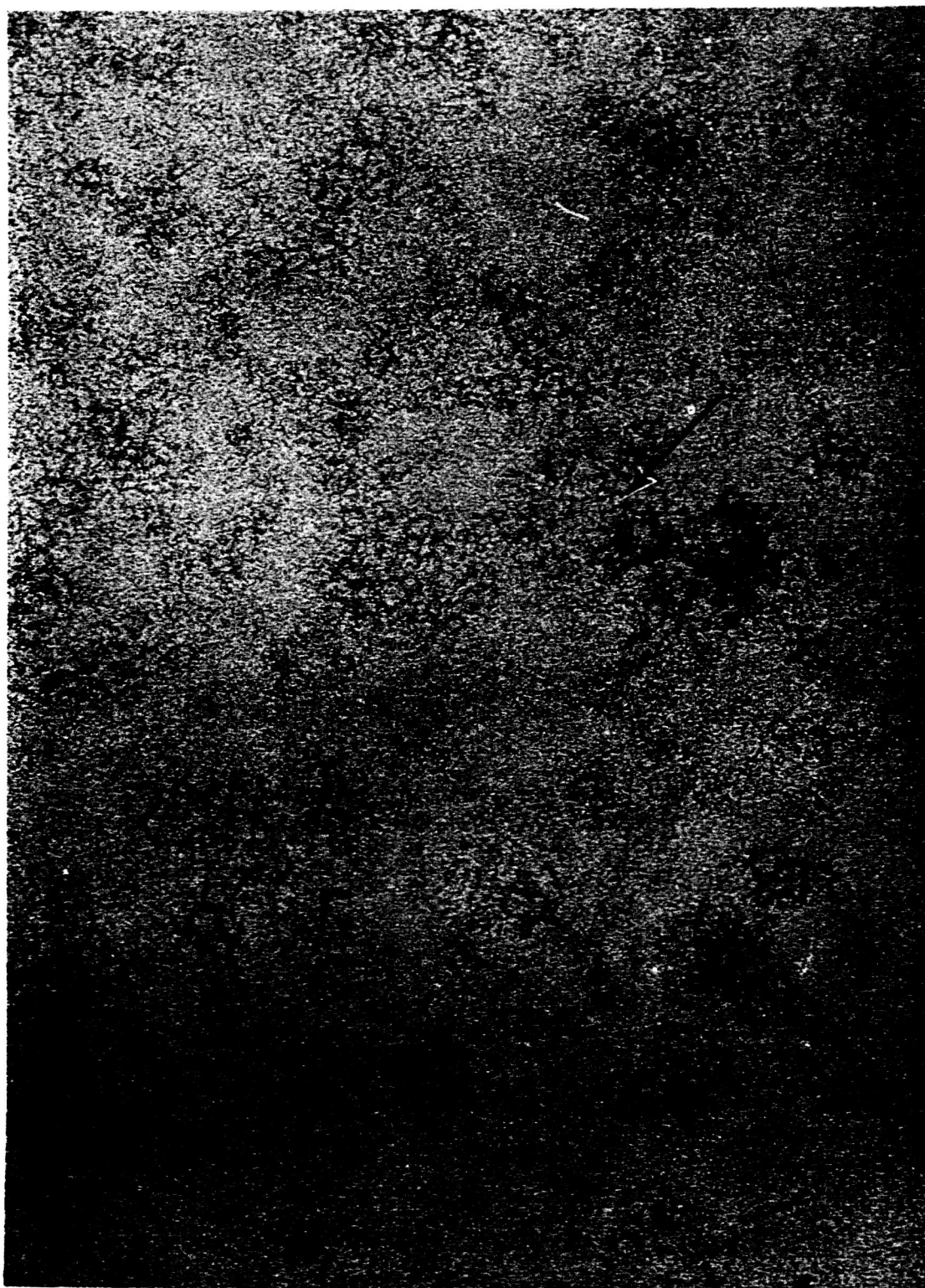
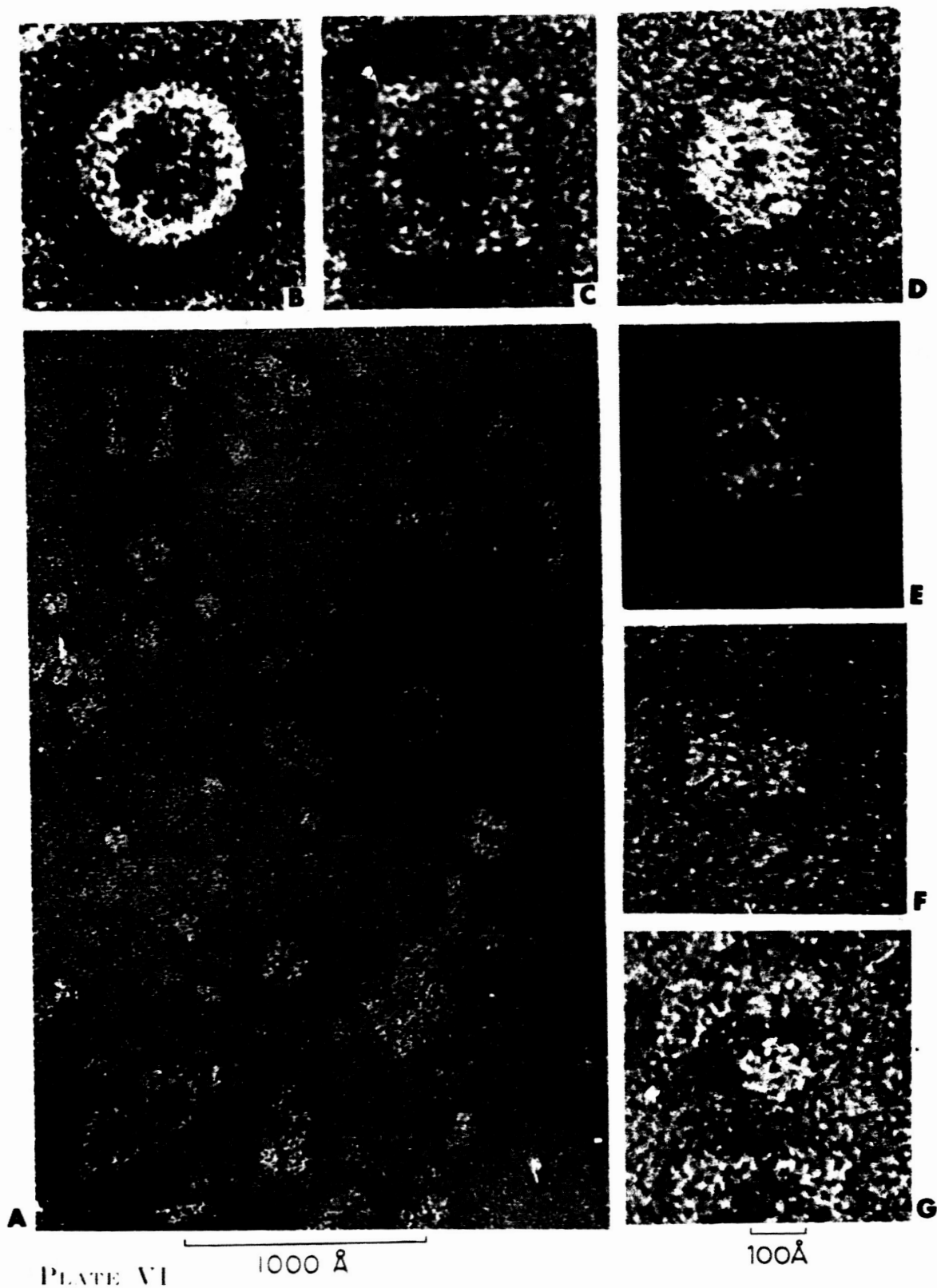


PLATE V

1000 Å

Mixture of negatively stained Helix and Limulus hemocyanin at pH 10.8. The molecules are completely dissociated into subunits. (see arrow). X 360,000.



Negatively stained Helix and Limulus hemocyanin molecules reassociated from the diluted mixture of subunits. The structures shown are similar to those indicated in Plate IV. (a) X 360,000. (b) to (e) X 900,000.

4. Discussion

Tiselius and Horsfall (1939) clearly showed that the closely related Helix pomatia and Helix nemoralis hemocyanin form a broad spectrum of mixed hybrid hemocyanins after dissociation at pH 8.5 and reassociation at pH 6.8. Their experiments for Helix pomatia and Littorina littorea hemocyanin showed less clear results. They were difficult to interpret because of the polydispersity of the solutions after reassociation. Electrophoresis is therefore not a particularly useful technique in this case.

Our experiments show that it is very well possible to recognize the hemocyanins obtained from the different species chosen (see Plates I and IV). They further indicate a dissociation into units measuring 30 Å to 50 Å across (Plates II and V). It was already pointed out in the foregoing paper that these structures are compatible with a 6 s component found by sedimentation analysis of dissociated Mollusca hemocyanin. Our pictures do not show typical differences in the size of the particles. It is impossible to distinguish between subunits of different species.

The types of molecules present before dissociation are again seen after reassociation (Plates III and VI). A certain amount of smaller structures and irregular aggregated material is also present. The dissociation at a pH between 10 and 11 is probably responsible for an irreversible denaturation of a part of the protein subunits. These experimental conditions, however, were chosen to achieve as complete a dissociation as possible of the original molecules.

The fact that so many of the original structures are observed clearly demonstrates that we are dealing with a highly specific reassociation process.

The specificity of association of these globular proteins is so marked that in mixtures of dissociated hemocyanins of widely different origin the corresponding subunits will recognize their identical partners, even at relatively high dilutions, to reconstitute the complex macromolecular assemblies.

These electron microscopic observations are all the more interesting since previous work (Tiselius & Horsfall, 1939) had indicated that subunits of hemocyanin molecules from very closely related species were merely able to combine with each other to form hybrid molecular complexes.

Preliminary experiments indicate that for Helix pomatia apo-hemocyanin (copper-free) the normally occurring reassociation no longer takes place under the conditions described. Instead a wide variety of randomly associated complexes is observed, none of which even closely resembles the original Helix apo-hemocyanin molecules. The possible role of the copper suggested by these observations will be the subject of further investigations combining high resolution electron microscopy with biochemical and sedimentation analysis.

Hemocyanins may well serve as model systems for future studies bearing also on the properties of regulatory enzymes. According to the interesting concepts proposed by Monod, Wyman and Changeux (1965) the functional properties of regulatory enzymes could be accounted for on the assumption that the quaternary structures of oligomeric proteins involve an element of symmetry in many proteins made up of identical subunits.

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