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Electron Microscopy of Protocollagen Proline Hydroxylase from Chick Embryos*

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SUMMARY

Protocollagen proline hydroxylase was purified from chick embryos, and it was examined by electron microscopy with negative staining techniques. Enzyme prepared in Helsinki consisted almost entirely of single ring structures. Enzyme prepared in Philadelphia under essentially the same conditions consisted almost entirely of tubular forms in which four rings were stacked together. Repeated freezing and thawing of the Helsinki preparation markedly increased the number of four-ring structures, but it was not possible to establish reproducible conditions for the interconversion of these two major forms of the enzyme.

Each ring in the four-ring structure had a diameter of 100 Å, a height of 30 to 35 Å, and a central hole of about 20 Å. The single ring structure was slightly more distended. The molecular weight of each ring was about 200,000, and therefore the molecular weight of the four-ring structure was about 800,000.

The inner two rings of the four-ring structure were closer together than the outer two rings. Since the four rings appeared to be identical, the observations suggested that the rings were held together by a combination of heterologous and isologous bonds. No regular structures with more than four rings were seen, and the simplest bonding sequence consistent with these observations was (AB)—(AB)—(BA)—(BA) in which larger structures were not favored, because the formation of the inner B—B bond altered the A surfaces on the inner rings so that they were not equivalent to the A surfaces on the outer rings.

precursor of collagen called protocollagen (for reviews, see References 1 and 2). Protocollagen proline hydroxylase has been identified in connective tissue from several sources, and has been shown to require O_2 , Fe^{2+} , α -ketoglutarate, and, perhaps, ascorbate. The enzyme does not hydroxylate free proline, tripeptides such as Gly-Pro-Pro, or the polymer poly-L-proline, but it hydroxylates polymers of the structure (Gly-X-Pro) $_n$, in which X is proline, alanine, or a variety of other amino acids but not glycine (1).

Recently, protocollagen proline hydroxylase has been purified extensively from chick embryos (3, 4) and from fetal rat skin (5). The enzyme from chick embryos was obtained in a form which was homogenous by ultracentrifugation and which was over 90% pure by disc electrophoresis (4). This enzyme had a $s_{20,w}$ of 6.7 S, which suggested a molecular weight of about 150,000 (4). In the present paper we describe electron microscopic studies of the enzyme from chick embryos. The results indicate that the enzyme consists of ring structures of about 30 Å by 100 Å which are seen either as isolated rings or in tubular forms containing two or four rings.

MATERIALS AND METHODS

Enzyme Purification—Protocollagen proline hydroxylase was prepared from 12-day-old chick embryos with the procedures recently described by Kivirikko *et al.* (3, 4). About 400 embryos from white Leghorn chickens were used as a source of enzyme, and the purification procedure consisted of ammonium sulfate fractionation, fractionation with calcium phosphate gel, and chromatography on a DEAE-cellulose column (Whatman, DE-23). The column was equilibrated with 0.2 M NaCl, 0.2 M glycine, and 0.01 M Tris-HCl pH 7.0 at 4°, and the enzyme eluted with 0.30 M NaCl was concentrated by vacuum dialysis or by ultrafiltration. The enzyme was then chromatographed on a column, 2.5 × 95 cm, of 8% agarose (Bio-Gel A, 1.5 m, 100 to 200 mesh; Bio-Rad) which was equilibrated and eluted with 0.01 M Tris-HCl, pH 7.8, at 4°, containing either 0.2 M NaCl and 0.2 M glycine, or 0.05 M KCl, 0.1 M NaCl, and 0.1 M glycine. The flow rate of the column was about 80 ml per hour and 10-ml fractions were collected.

Disc electrophoresis was carried out in a 6.5% polyacrylamide gel with Tris-glycine buffer (4). The pH of the upper buffer was 9, the pH of the lower buffer was 8.2, and the gel was stained with Amido black.

Enzymatic Assay—The enzyme was assayed in an incubation system containing 125 µg per ml of the polytripeptide (Pro-Pro-

The hydroxyproline in collagen is synthesized by the hydroxylation of proline in a proline-rich and lysine-rich polypeptide

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Gly)₅ (6), 20 to 250 μ g per ml of enzyme protein, 0.5 mM α -ketoglutarate, 0.05 mM FeSO₄, 2 mM ascorbic acid, 0.2 mg per ml of catalase (Calbiochem), 0.1 mM dithiothreitol (Eastman Organic Chemicals), 2 mg per ml of bovine serum albumin, and 50 mM Tris-HCl buffer, adjusted to pH 7.8 at 25°, in a final volume of 4 ml (3-5). Samples were incubated at 37° with shaking for 1 hour, and the reaction was stopped by the addition of 4 ml of concentrated HCl. The samples were hydrolyzed overnight in sealed tubes at 120° and the hydrolysates were evaporated to dryness in a rotary evaporator. The residues were dissolved in 4.0 ml of distilled water, and the hydroxyproline content was assayed by a specific chemical procedure (7) in 3.5-ml aliquots.

As indicated elsewhere (4), the standard assays were not carried out with saturating concentrations of substrate because of the difficulty and expense in obtaining appropriate polypeptide substrates. All values for enzyme units and specific activities referred to here were based on assays carried out under the conditions described above. One unit of enzymatic activity was defined (3, 4) as the number of micrograms of hydroxyproline synthesized in 1 hour under the standard assay conditions. The amount of hydroxyproline synthesized in 1 hour was about 2-fold greater when saturating concentrations of substrate were used (4). Because the K_m value for the (Pro-Pro-Gly)₅ substrate was somewhat higher than for polymer preparations of (Pro-Gly-Pro)_n with an average molecular weight of about 3000 (4, 8), the values for units of enzyme observed here were corrected by a factor of 1.25 in order to make the values directly comparable to those reported previously.

Techniques for Electron Microscopy—Protocollagen proline hydroxylase was dissolved in 0.01 M Tris-HCl, pH 7.8, containing either 0.2 M NaCl and 0.2 M glycine or 0.05 M KCl and 0.1 M glycine, and it was examined by negative staining (9). One drop of the solution was applied to a carbon film on an electron microscopic grid. The carbon film was prepared by evaporating carbon onto Formvar-coated grids and the Formvar was removed by placing the coated grids on a filter paper soaked in chloroform. After application of the enzyme solution to the carbon film on the grid, the edge of the grid was blotted with filter paper. The thin film thus produced was allowed to evaporate for a few seconds. Negative staining was then performed by adding 1 drop of 1% uranyl acetate (E. Merck, Darmstadt, Germany) at pH 4.8, or 1 drop of 4% sodium silicotungstate (Taab Laboratories, Reading, England) at pH 7. The excess fluid was blotted with filter paper. In some instances the enzyme solution was mixed with an equal volume of 4% sodium silicotungstate prior to application to the grid.

The grids were examined in a Hitachi HU-11C-1 electron microscope and Kodak contrast projector slide plates were exposed to the image at a magnification of 100,000. The accelerating voltage was either 75 or 100 kv.

RESULTS

Preparation of Enzyme—All of the studies reported here were carried out with protocollagen proline hydroxylase prepared from chick embryos by the most recent procedure of Kivirikko *et al.* (3, 4). Most of the work was done with enzyme prepared in Philadelphia, but, because gel filtration indicated that the molecular weight of the preparations was greater than that reported previously (4), the electron microscopy was repeated with enzyme prepared in Helsinki.

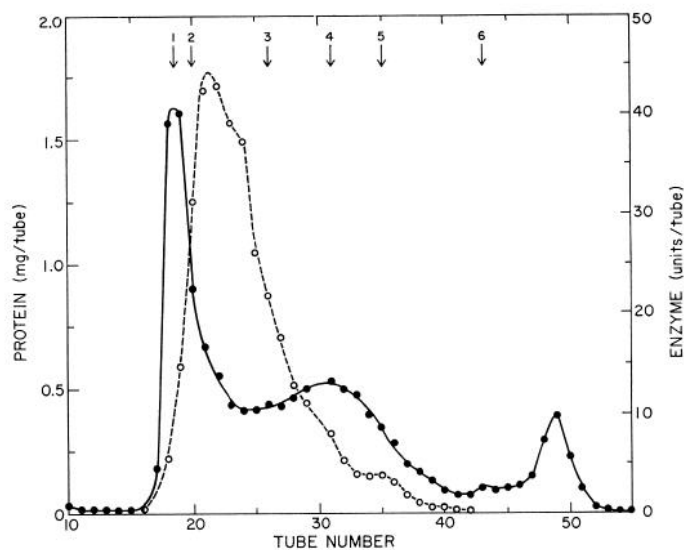


FIG. 1. Chromatography of protocollagen proline hydroxylase prepared in Philadelphia. The enzyme was purified through the DEAE-cellulose column and then it was chromatographed on an 8% agarose column as described in the text. Elution peaks of other substances in the same column: 1, blue dextran (mol wt $\sim$ 2,000,000); 2, IgM globulin (mol wt $\sim$ 1,000,000); 3, peak fraction for protocollagen proline hydroxylase prepared in Helsinki; 4, IgG globulin (mol wt $\sim$ 150,000); 5, albumin (mol wt $\sim$ 65,000); 6, cytochrome *c* (mol wt $\sim$ 13,000). Elution of protein as indicated by absorbance at 230 $m\mu$, $\bullet$ — $\bullet$; elution pattern of protocollagen proline hydroxylase, $\circ$ — $\circ$.

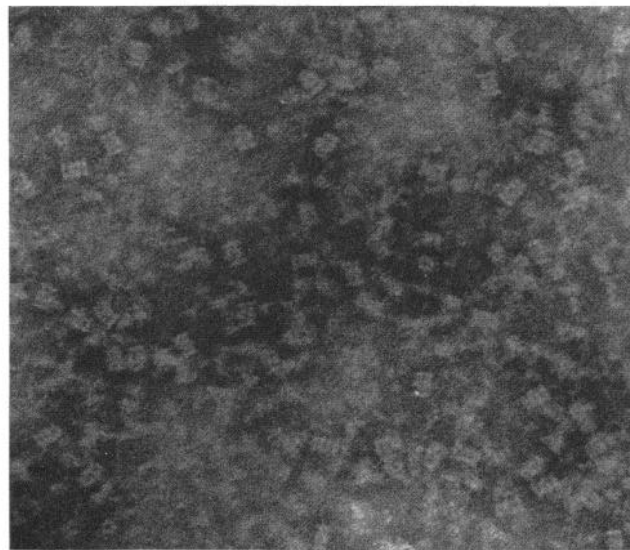


FIG. 2. Negative staining with 4% sodium silicotungstate of the enzyme recovered in tube 23 of the chromatogram shown in Fig. 1. The concentration of protein was 120 μ g per ml and the pH of the solution was 7.8. A large number of tetrad side views and a few circular top views are seen. (See text for definition of "side views" and "top views.") $\times$ 240,000.

In preparing the enzyme in Philadelphia, the results with the initial two steps of ammonium sulfate and calcium phosphate gel fractionation were about the same as those obtained in Helsinki (3, 4), but differences were found during chromatography of the enzyme on the DEAE-cellulose and on the 8% agarose columns. In Philadelphia, the recovery of enzyme

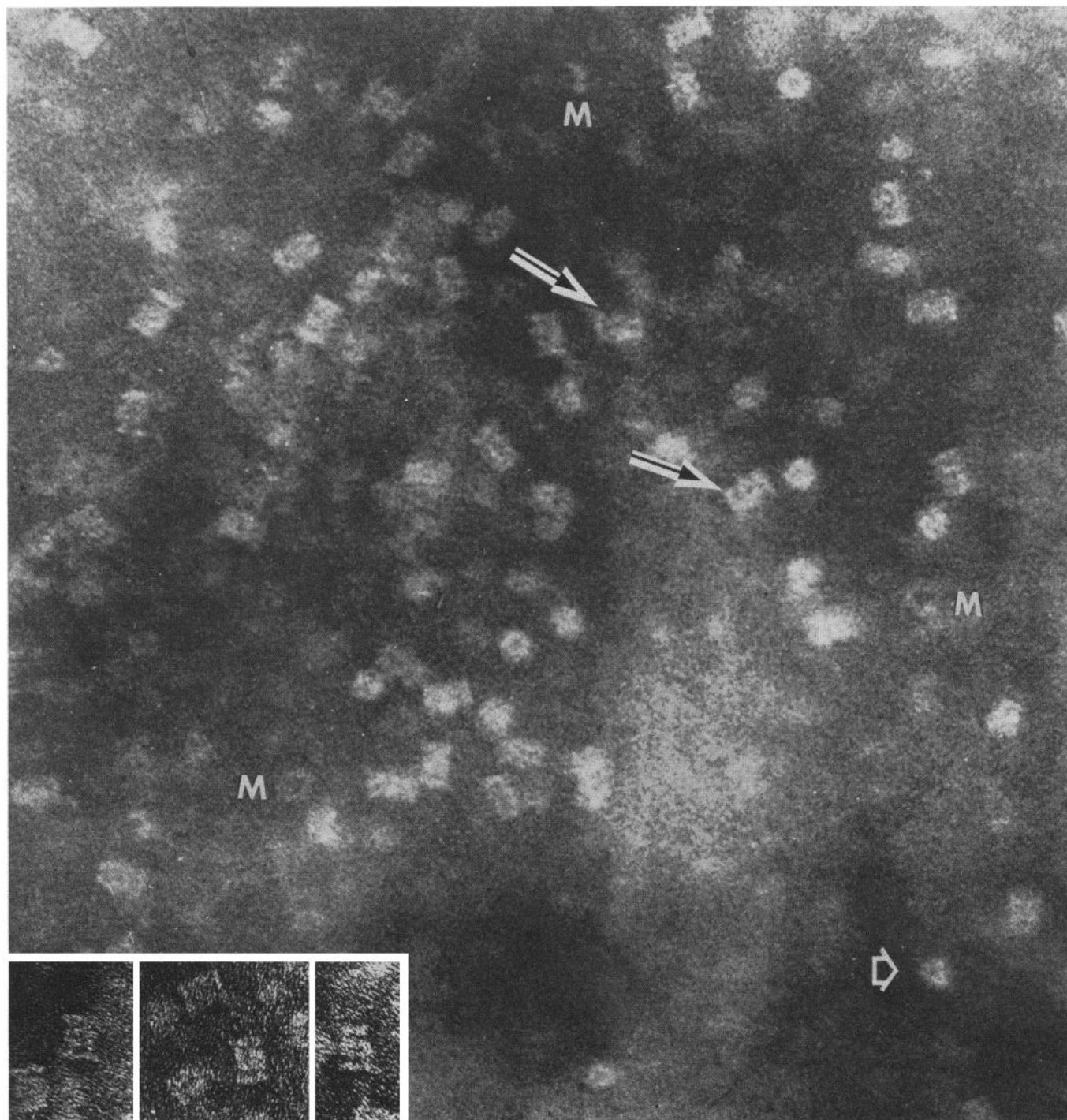


FIG. 3. Examination of the enzyme preparation shown in Fig. 2 at a higher magnification. The arrows indicate tetrads in which the central channel is seen in side view. Ring structures of low contrast which probably represent single rings (see text) are indicated by the letter *M* in the lower part of the picture. The letter *M* at the top of the picture indicates a short thread-like structure

of about 100 Å in length which could represent a single ring in side view. The open arrow indicates a top view of a structure with a triangular shape. Inset, side views of tetrads in which the contrast of the image was increased by rephotographing the prints. Note that the distance between the inner two rings is less than the distance between the outer and the inner rings. $\times 550,000$.

from the DEAE-cellulose column was better than in Helsinki, and 89 to 95% of the enzyme was recovered in the 0.3 M and 0.4 M NaCl fractions with three separate preparations. The recovery of enzyme in the agarose column was also better in Philadelphia, and 90 to 94% of the enzyme was recovered with three

separate preparations. However, the best enzyme preparations obtained after these two steps had a specific activity of only about one-third of that obtained in Helsinki (3, 4). These differences were consistent even though the procedures used were apparently identical.

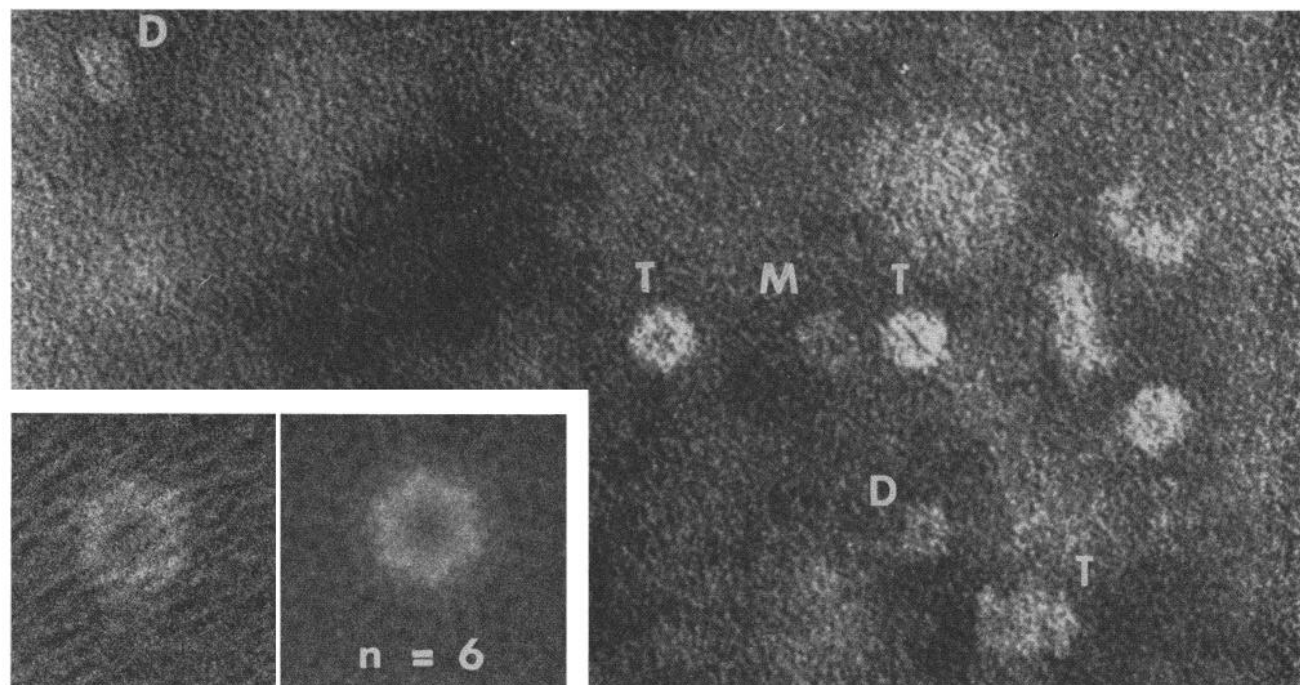


FIG. 4. Negative staining with 4% sodium silicotungstate of enzyme prepared in Philadelphia. The protein concentration was 120 μg per ml. T, top and side views of four-ring structures; D, side views of two-ring structures; M, top view of a single ring

structure. $\times 1,000,000$. Inset, left half shows a top view of a four-ring structure with an hexagonal outline. Right half shows a rotational print of the same image assuming a 6-fold symmetry. $\times 1,800,000$.

The enzyme prepared in Philadelphia consistently eluted near the void volume of the 8% agarose column (Fig. 1), and the ratio of the elution volume to the void volume (V_e/V_0) was 1.10 to 1.20. The enzyme prepared in Helsinki consistently eluted with a V_e/V_0 of 1.40. Identical V_e/V_0 values were obtained when the columns in both laboratories were standardized with blue dextran, IgM globulin, serum albumin, and cytochrome *c* (Fig. 1).

Disc electrophoresis in Tris-glycine buffer of the enzyme prepared in Helsinki gave one minor and one major band (4). The major band was well within the gel, it contained enzymatic activity, and it accounted for over 90% of the total protein (4). Under the same conditions the enzyme prepared in Philadelphia (Fraction 23 of the chromatogram shown in Fig. 1) gave four minor bands and one major band. The major band accounted for most of the total protein, but it was only 1 or 2 mm from the stacking gel. Electron microscopy of the enzyme prepared in Philadelphia showed that essentially all of the material consisted of regular enzyme structures (see below), and therefore the results indicated that the major band obtained with disc electrophoresis was the enzyme.

General Features Revealed by Electron Microscopy—The initial electron microscopy was carried out with Fraction 23 of the chromatogram shown in Fig. 1, which had a specific activity of 86 units per mg of protein. After negative staining with either uranyl acetate or sodium silicotungstate, rings about 100 Å in diameter and tetrad structures of about 140 Å in length were observed (Fig. 2). The same structures were found both when a dilute solution containing 40 μg of protein per ml was examined and after the solution was concentrated five times by vacuum dialysis. The same structures are seen at higher magnification in Fig. 3. We interpret these figures as well as those shown

below to indicate that the rings are top views and the tetrad forms are side views of the same molecule, and we conclude that most of the molecules in this preparation are tubules composed of four-ring structures stacked together.

The presence of four rings in the tetrad forms is clearly shown in Fig. 3 (inset). The same picture also shows that within the tubule the distance between an outer ring and an inner ring is greater than the distance between the two inner rings.

Most of the top views show the presence of a central hole with a diameter of 20 to 25 Å, and under favorable staining conditions a channel of this width is occasionally seen in side views of the molecule (arrows in Fig. 3).

Although most of the molecules apparently consisted of four rings, some top views were of lower contrast than others (Figs. 3 and 4) and some side views indicated only one or two rings (Fig. 4). These variations are probably explained by the enzyme existing in the form of single-ring, two-ring, and four-ring structures. No structures which could be interpreted as consisting of three rings or of more than four rings were observed.

The single-, two-, and four-ring structures seen in the Philadelphia preparations were also found in enzyme preparations made in Helsinki (Fig. 5, A and B). However, the characteristic feature of the Helsinki preparations was a high proportion of top views of ring structures which were probably single rings. The inner and outer diameters of the single rings were consistently about 20 Å greater than the diameters of the four-ring structures. The single rings may have appeared larger either because they were larger in solution or because the rings were partially distorted by the stain or by the hydrophobic surface of the carbon film. In addition, we frequently observed short thread-like structures of 30 to 35 Å by 100 to 120 Å which probably represented side views of single rings (Fig. 5B). The

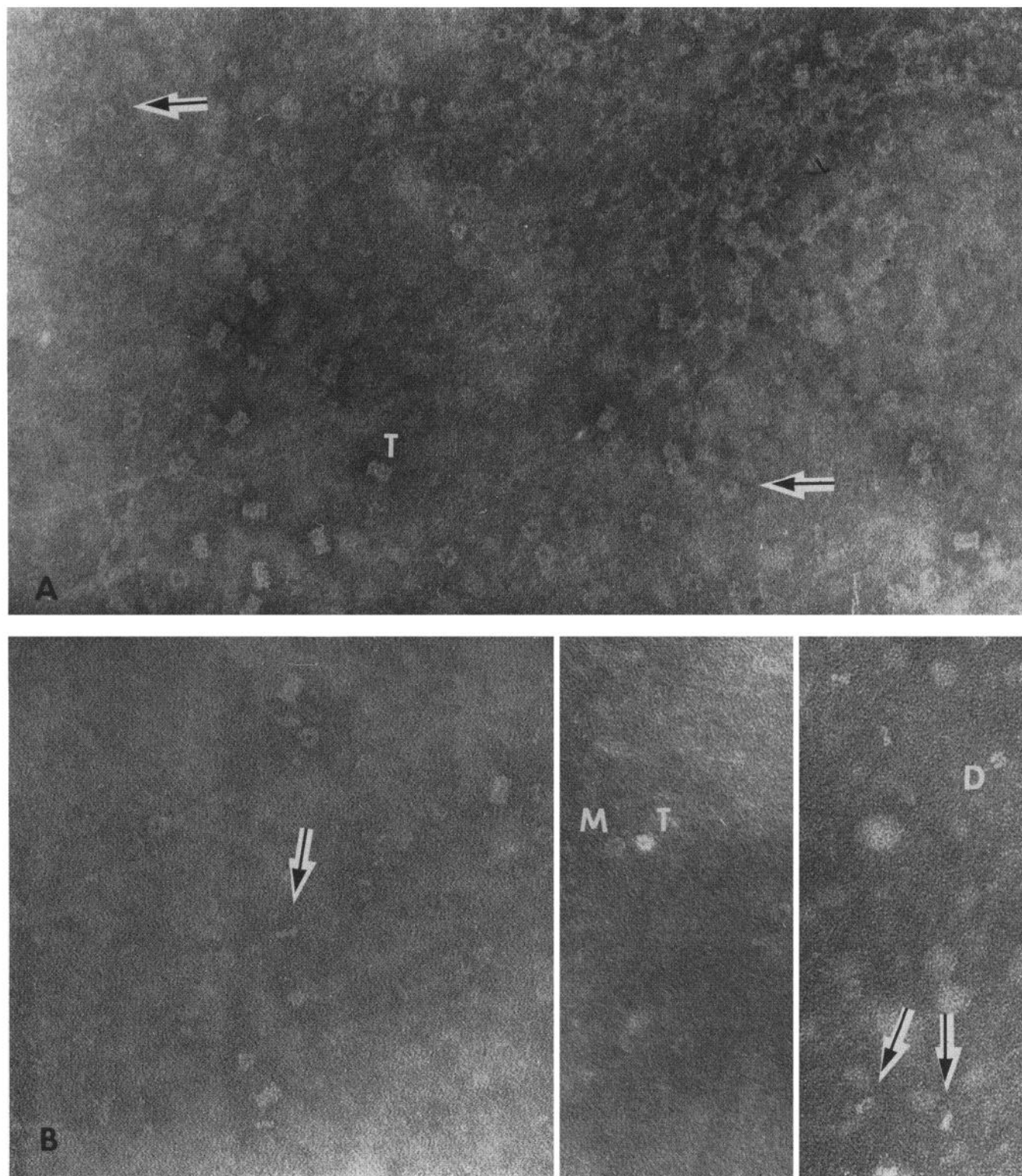


FIG. 5. *A*, negative staining with 4% sodium silicotungstate of enzyme prepared in Helsinki. The protein concentration was 40 μg per ml. A number of top views of single rings are seen (*arrows*), but the photograph was selected so as to show also a few side views of four-ring structures (*T*). The thread-like structures seen are

probably contaminants from the chromatographic columns used to isolate the enzyme, since disc electrophoresis indicated that the enzyme was about 95% pure. $\times 310,000$. *B*, Top and side views of single ring (*arrows* and *M*), double ring (*D*), and four-ring structures (*T*) in the same preparation shown in *A*. $\times 310,000$.

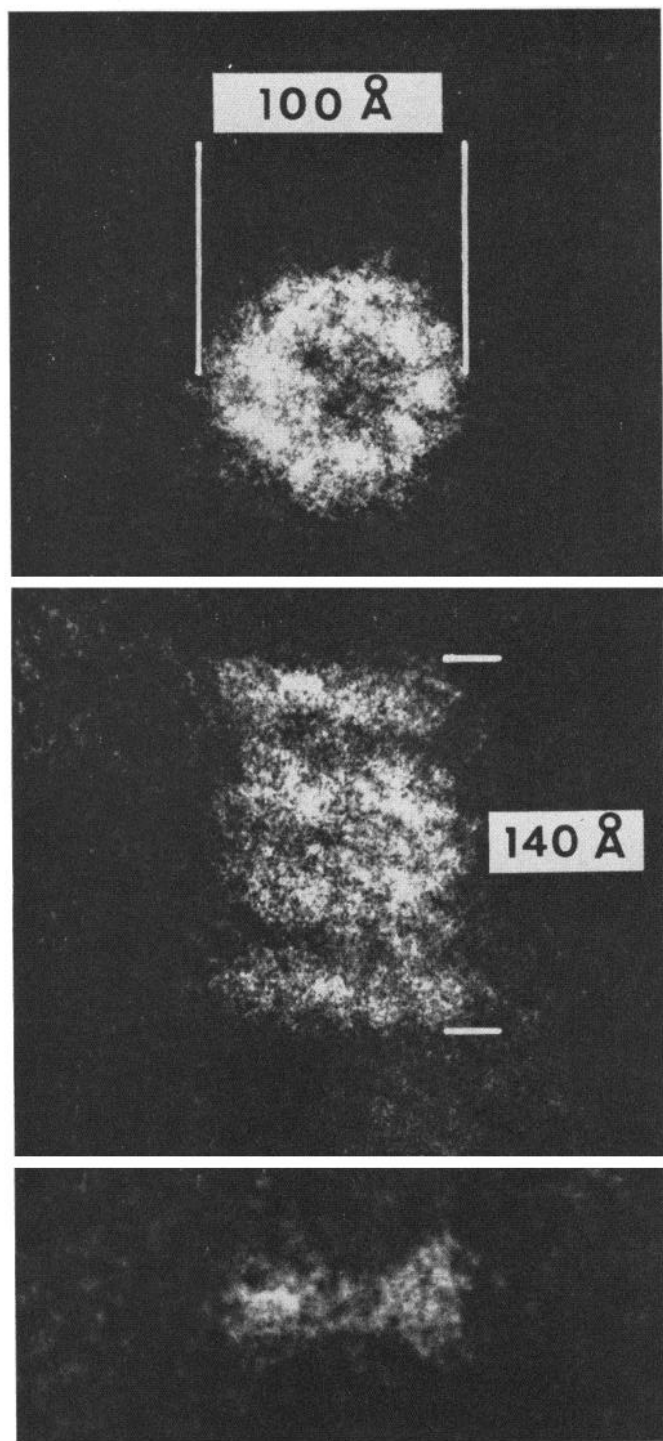


FIG. 6. Prints made by superimposing images of seven different top and side views of a four-ring structure and seven different side views of a single ring structure. No attempt was made to orient the molecules during printing of the top views. Note that the side views of the four-ring structure indicate that the inner two rings are closer together than the outer and inner rings. Magnification of the top and side views of the four-ring structure is 3,500,000. Magnification of the side view of the single ring is 3,000,000.

electron microscopic findings were essentially the same when preparations with 40 μ g or 500 μ g per ml of protein were examined.

Of special interest was the finding that the proportion of four-

ring structures in the Helsinki preparation increased when the enzyme was frozen and thawed several times for repeated electron microscopy. Also, it was found that most of the structures in tube 27 of the Philadelphia preparation (Fig. 1) which could be identified as enzyme were single rings. The V_e/V_0 value for this fraction was essentially identical with the V_e/V_0 value for the elution peak of the Helsinki enzyme in the same type of agarose column. However, both disc electrophoresis and electron microscopy indicated that Fraction 27 was too impure for it to be used for comparing the specific activities of single ring and four-ring structures from the same enzyme preparation.

The ring structure in the various preparations generally appeared round, but occasionally hexagonal structures were seen. Also, in rare instances, they had a triangular shape (Fig. 3), probably because of distortion of the ring structure. The *inset* in Fig. 4 shows a top view of a four-ring structure at high magnification, and a multiple exposure rotational print of the same image assuming a 6-fold axis of rotation. More diffuse and circular images were obtained when similar prints were made assuming a 5- or 7-fold axis of rotation.

Dimensions and Molecular Weights of Enzyme—In order to obtain average values for the dimensions of the molecules, the images of seven separate top and side views of the enzyme were superimposed during printing (Fig. 6). In printing the top views, no attempt was made to orient the molecules according to a hexagonal structure. The outer diameter of each ring is about 100 Å and the over-all length of the four-ring structure is about 140 Å. The height of each ring is therefore 30 to 35 Å, depending on the spacing distance between the rings. The diameter of the channel in the center is about 20 Å, but the size is difficult to estimate because of varying amounts of negative stain filling it. In some instances the hole is not seen, because of incomplete penetration of the stain.

Assuming a partial specific volume for the enzyme of 0.7 cm³ per g, the molecular weight for a circular disc of 100 Å $\times$ 30 Å is about 200,000. On this basis the molecular weight of the two-ring structure would be about 400,000, and the molecular weight of the four-ring structure would be about 800,000.

DISCUSSION

Most large enzymes have been shown to be oligomers or polymers of small subunits. High resolution electron microscopy has shown that some of these large enzymes consist of open, thread-like structures, while others have structures which can be described as "closed crystals" or rings. As discussed by several authors (10, 11), the latter type of structure has several theoretical advantages in that it probably increases the solubility and stability of the enzyme. Also, it may help to amplify the effect of modifiers of enzymatic activity. At least seven proteins have previously been shown to contain ring structures. Glutamate dehydrogenase (EC 1.4.1.3) contains a tetrahedral structure which may be formed from bars parallel to the edges of the tetrahedron (12). Pyruvate carboxylase (EC 6.4.1.1) is a single tetrameric ring (13), and dihydrolipoyl transacetylase (EC 2.3.1.12) consists of two tetrameric rings stacked on top of each other (14). Hemoglobin from several invertebrates (15, 16) and glutamine synthetase (EC 6.3.1.2) (9) contain hexameric rings. Both the invertebrate hemoglobins and glutamine synthetase are hollow cylinders consisting of 12 subunits organized into two rings. Polynucleotide phosphorylase (EC 2.7.7.8) may also have a ring structure made up of trimeric

units, but the details of its structure have been difficult to show clearly (17).

The results presented here indicate that procollagen proline hydroxylase contains a ring structure. In all of the enzyme preparations examined, rings were seen either as isolated structures or as two or four rings stacked together into a hollow cylinder. The relative amount of ring structures increased as the enzyme was purified and as the specific activity of the preparations increased. The purest preparations of enzyme showed only minor contaminants on disc electrophoresis and, when examined by electron microscopy, they consisted almost entirely of ring structures. We can therefore conclude that the ring structures were the enzyme and not contaminants in the enzyme preparations.

Although the enzyme was prepared in Helsinki and Philadelphia under essentially identical conditions, the enzyme prepared in Philadelphia had a higher molecular weight by gel filtration and consisted mainly of four-ring structures. The enzyme prepared in Helsinki consisted mainly of single rings and only a few two-ring and four-ring structures were seen. The small number of single rings seen in the enzyme prepared in Philadelphia could not account for the relatively high specific activity of these preparations, and therefore it is clear that both the four-ring and single-ring structures were enzymatically active.

The results suggested that all of the ring structures observed were formed from a single type of ring. The Helsinki preparation which gave a single major band on disc electrophoresis and which initially contained mostly single rings was found to form four-ring structures after it was frozen and thawed several times. The single ring structures were slightly more distended than the four-ring structures, but this may be explained by partial distortion of the single rings by the stain or by the hydrophobic surface of the carbon film on which they were supported.

The typical ring of the four-ring structures had a diameter of 100 Å, a height of 30 to 35 Å, and a central hole of about 20 Å. Most of the rings had circular outlines, but in some of the pictures an hexagonal outline was seen. Because the ring was smaller than the hexagonal rings in the invertebrate hemoglobins (15, 16) or glutamine synthetase (9), it was not possible to see the detailed subunit structure. However, rotational studies with pictures of individual rings supported the impression that the ring contained six subunits. Since the estimated molecular weight of the ring was about 200,000, the molecular weight of each of six identical subunits forming the ring would be about 30,000.

What we interpreted to be side views of the four-ring structures indicated that the distance between the two inner rings was less than the distance between the outer and the inner rings. If we are correct in assuming that all of the rings are identical (above), this observation indicates that the two surfaces of a single ring are not identical. If the two different binding surfaces of the rings are represented by A and B, eight different binding sequences of the type (AB)—(AB)—(AB)—(AB) are theoretically possible for the four-ring structure. Of these, three can be ruled out because they require the middle bond to be identical with one or both of the outer bonds. Three additional possibilities are unlikely, since they require the two outer bonds to be different, but electron microscopy did not indicate any asymmetry in

the side views of the four-ring structures. Of the two remaining possibilities, the bonding sequence (AB)—(BA)—(AB)—(BA) would have a greater tendency to form higher polymers than the sequence (AB)—(AB)—(BA)—(BA). In the sequence (AB)—(BA)—(AB)—(BA), the A surfaces on the inner and outer rings are symmetrically positioned around B—B bonds, and therefore there is no formal restriction on the formation of higher polymers by means of additional A—A bonds. In the sequence (AB)—(AB)—(BA)—(BA), the formation of the inner B—B bond could mean that the A surfaces on the inner rings are not equivalent to the A surfaces on the outer rings, and this condition could prevent the formation of larger polymers by means of additional A—B bonds. Since no larger polymers were seen in any of the preparations examined, the simplest bonding sequence which is consistent with our observations is one in which the two outer bonds are heterologous and the middle bond isologous (10, 18).

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