by the inclusion of orotic acid in a purified diet can be prevented or reversed by supplementation of the diet with adenine or by feeding dog chow. With these dietary changes, certain pathways of pyrimidine metabolism are notably altered. The possible significance of these findings, as well as the cytological changes observed, are discussed.

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§ Present address: Department of Biochemistry, University of Oxford, Oxford, England.

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*THE CHARGE PROFILE OF THE TROPOCOLLAGEN MACROMOLECULE AND THE PACKING ARRANGEMENT IN NATIVE-TYPE COLLAGEN FIBRILS**

BY ALAN J. HODGE AND FRANCIS O. SCHMITT

DEPARTMENT OF BIOLOGY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY

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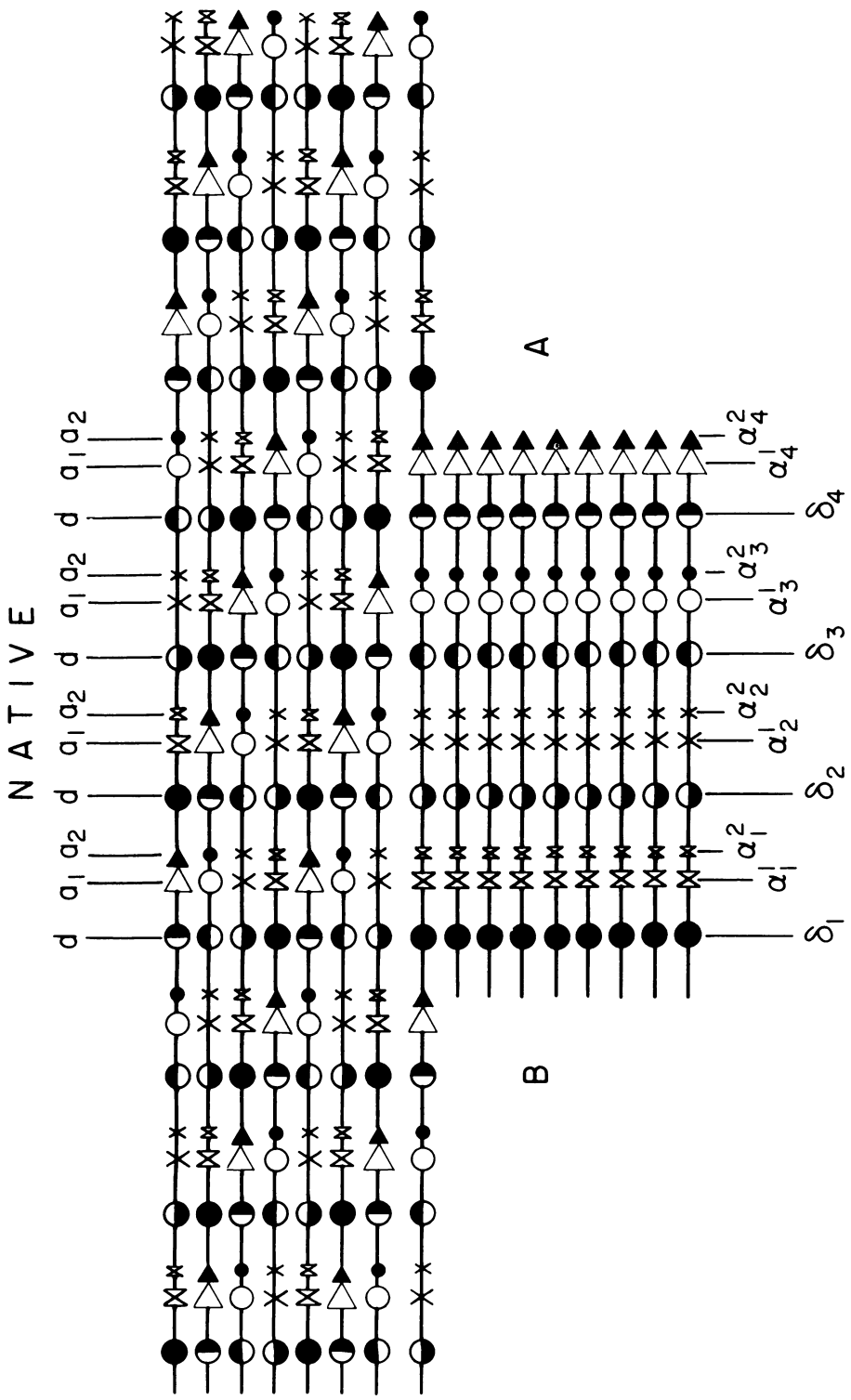
The existence of a discrete collagen monomeric unit, the tropocollagen (TC) macromolecule, in solutions of soluble collagen has been amply confirmed by a variety of techniques, the most important of which have been: (1) the demonstration by Schmitt *et al.*[1] that the macromolecules may under appropriate conditions be



TEXT FIG. 1.—Diagrammatic representation of the packing arrangement of TC macromolecules in the "dimorphic" ordered structures shown in Figs. 8 and 9. Only three of the twelve or thirteen bands usually observable in the native-type structure are shown in order to minimize complexity. Note that all staining loci are in accurate transverse alignment, i.e., in register in both packing arrangements. However, in the SLS-type packing only like features are in register, i.e., "homo-register," while in the native-type structure each band arises by alignment of the four corresponding "equivalent loci" of the TC macromolecules, i.e., the distribution of staining loci along the TC macromolecules is such that accurate "hetero-register" results when they are packed in the "quarter-stagger" arrangement.

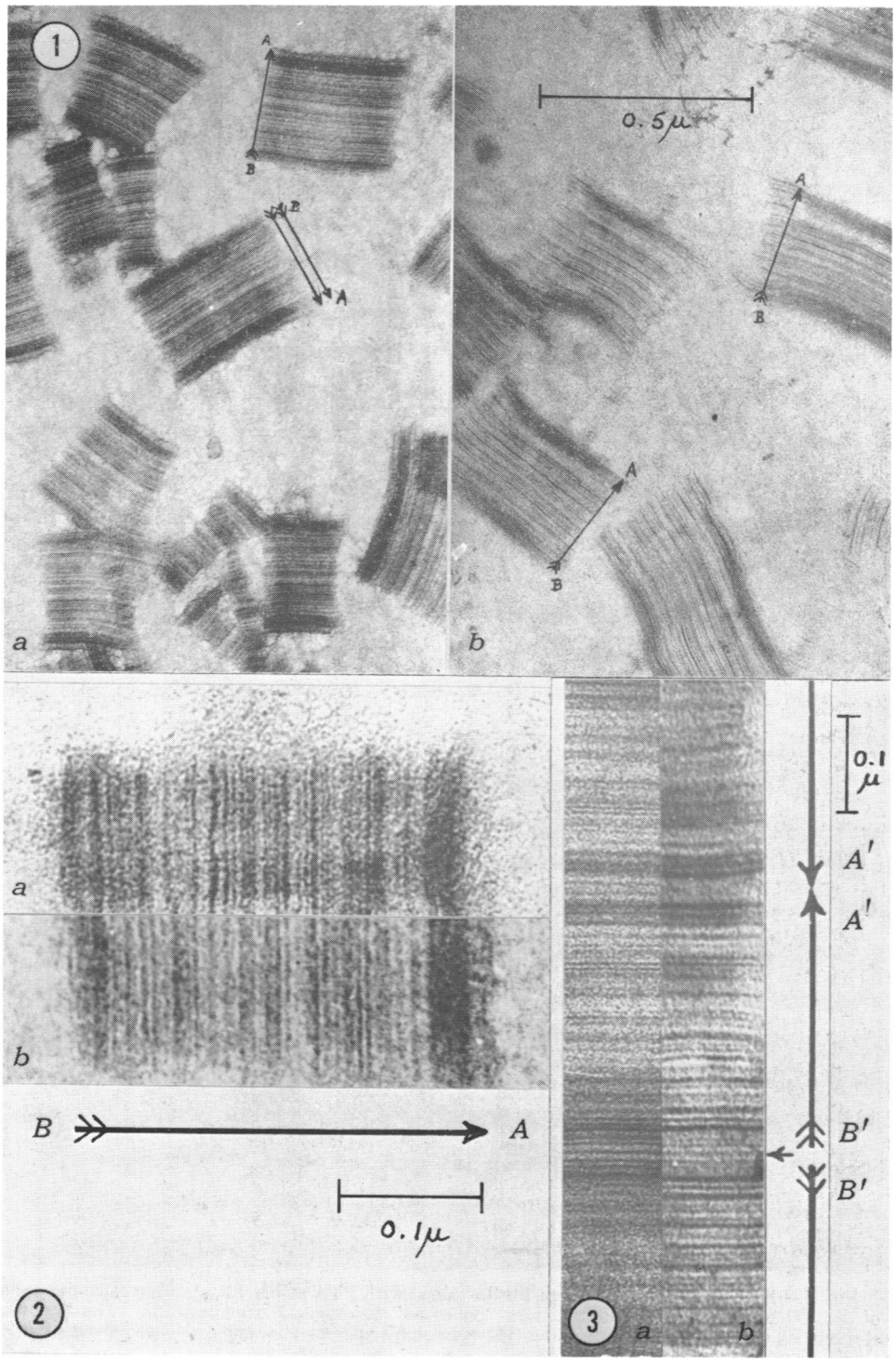
induced to pack in the segment long-spacing (SLS) form, in which the TC macromolecules are arranged in parallel array with all like features in register (see Text Fig. 1); and (2) the physico-chemical measurements of Boedtker and Doty[2] together with the direct electron microscopic evidence of Hall[3] indicating the presence in solutions of soluble collagen of particles having dimensions about 14 × 2800 A with a molecular weight of about 360,000. Since the physical properties of TC solutions indicate that the macromolecules are essentially rigid rods, it seems likely that the three-chain helical configuration indicated by large-angle x-ray diffraction studies characterizes most regions of the macromolecules. Studies on the denaturation of the native macromolecules indicate that the helical configuration is maintained by hydrogen bonding involving the hydroxyproline residues.[5-7]

In the SLS type of ordered aggregate, all like features of the macromolecules are in accurate transverse register, i.e., the macromolecules are packed in "homo-register." It follows, therefore, that the band patterns observed in the electron microscope when SLS aggregates are exposed to ionic electron-dense compounds, such as phosphotungstic acid (PTA), will accurately indicate the positions (along the length of the TC macromolecule) of the polar groups responsible for binding of the heavy metal compound. Such SLS band patterns are thus in effect "molecular fingerprints" and, as we shall see, have proved to be very useful in deducing the packing arrangements of the TC macromolecules in other ordered aggregation states. If the reasonable assumption is made that the binding is directly proportional to the concentration of ionized groups on the TC under specified conditions of pH and ionic strength, it follows that the intensities of the various SLS bands accurately indicate the concentration of ionized groups at these loci. Figure 1 shows SLS-type aggregates (single segments) stained (*a*) with PTA,[8] and (*b*) with cationic uranium.[9] It has been shown by chemical analytical and electron microscope studies on native-collagen fibrils (*ca.* 700 A axial period) that the lysine-bound PTA is easily washed out, while that held by arginine is firmly bound,[10,11] and therefore primarily responsible for the band intensities in PTA-stained specimens. The band pattern of SLS stained with uranyl ion under conditions in which the ions are all cations is thought to reflect the distribution of aspartic and glutamic acid residues in the TC macromolecule. It is immediately apparent that the two band-patterns differ considerably. However, a detailed comparison (Fig. 2) shows

FIG. 1.—Segments (i.e., monomeric SLS-type aggregates) obtained by addition of ATP (2 mg/ml) to an aged solution of calf-skin collagen in 0.008 *N* acetic acid. The virtual absence of dimeric or polymeric SLS forms in preparations from such aged solutions suggests that depolymerization of any protofibrils originally present has occurred, probably as a result of the action of proteases from the tissue or from microorganisms. A similar depolymerizing action occurs in the presence of added proteases,[19] also resulting in single segment preparations. (*a*) Stained with PTA; (*b*) stained with cationic uranium. The orientation of the TC macromolecules is indicated by the arrows labeled *A* and *B*. Note the differences in the two band-patterns. ×60,000.

FIG. 2.—Two individual segments from the same preparation as shown in Fig. 1, (*a*) stained with cationic uranium, (*b*) stained with PTA, illustrating the close correspondence in the positions and the considerable differences in intensities of staining loci in the two "fingerprints." The arrow labeled *A* and *B* indicates the molecular orientation according to the convention adopted by Hodge and Schmitt.[17] ×200,000.

FIG. 3.—Polymeric SLS-type aggregates, with the molecular orientation indicated by the labeled arrows, obtained by the addition of ATP to a solution of calf-skin collagen subjected to sonic irradiation (see Hodge and Schmitt[17]). The distortions resulting from drying are minimized in such preparations, particularly near the ends of the macromolecules, and a better comparison is possible for the positions and intensities of the various staining loci. (*a*) Stained with PTA, and (*b*) with cationic uranium. ×135,000.


1
A
B
A
B
a
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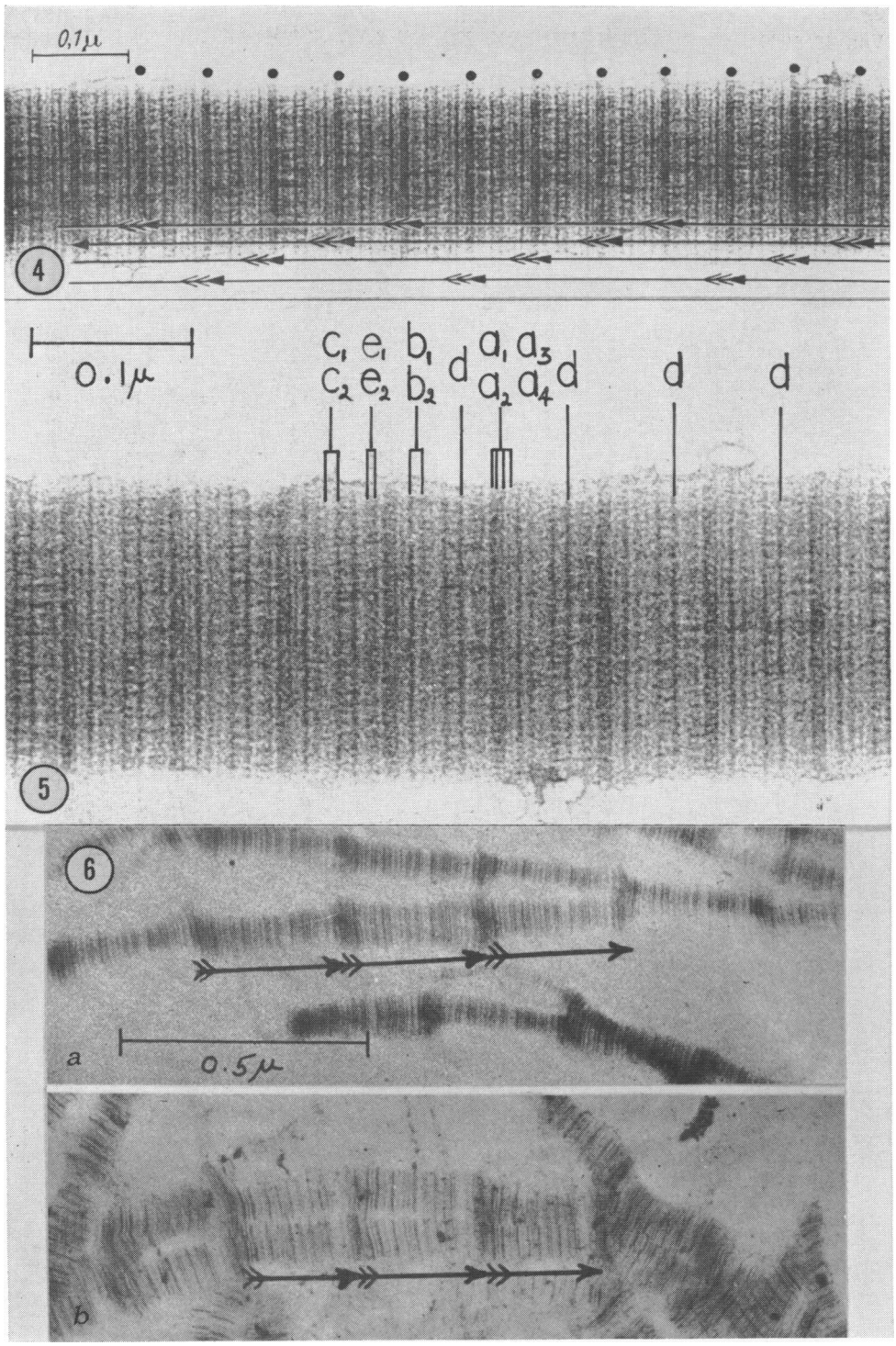

FIG. 4.—Reconstituted native-type fibril, stained with PTA at pH 4.2, showing the axial period of *ca.* 700 A, and the characteristically "polarized" intraperiod band pattern. The "quarter-stagger" packing arrangement of the TC macromolecules in this type of ordered aggregate is shown diagrammatically. ×138,000.

FIG. 5.—Generally used band nomenclature for the native-type pattern. ×230,000.

FIG. 6.—Polymeric SLS-type fibrils obtained by addition of ATP to a solution of calf-skin collagen at relatively high pH (*ca.* 5.0). Under these conditions, most of the TC is present in the form of linear polymers of the type —AB—AB—, and the addition of ATP causes them to pack in "homo-register"; (*a*) stained with PTA, (*b*) stained with cationic uranium. ×71,000.

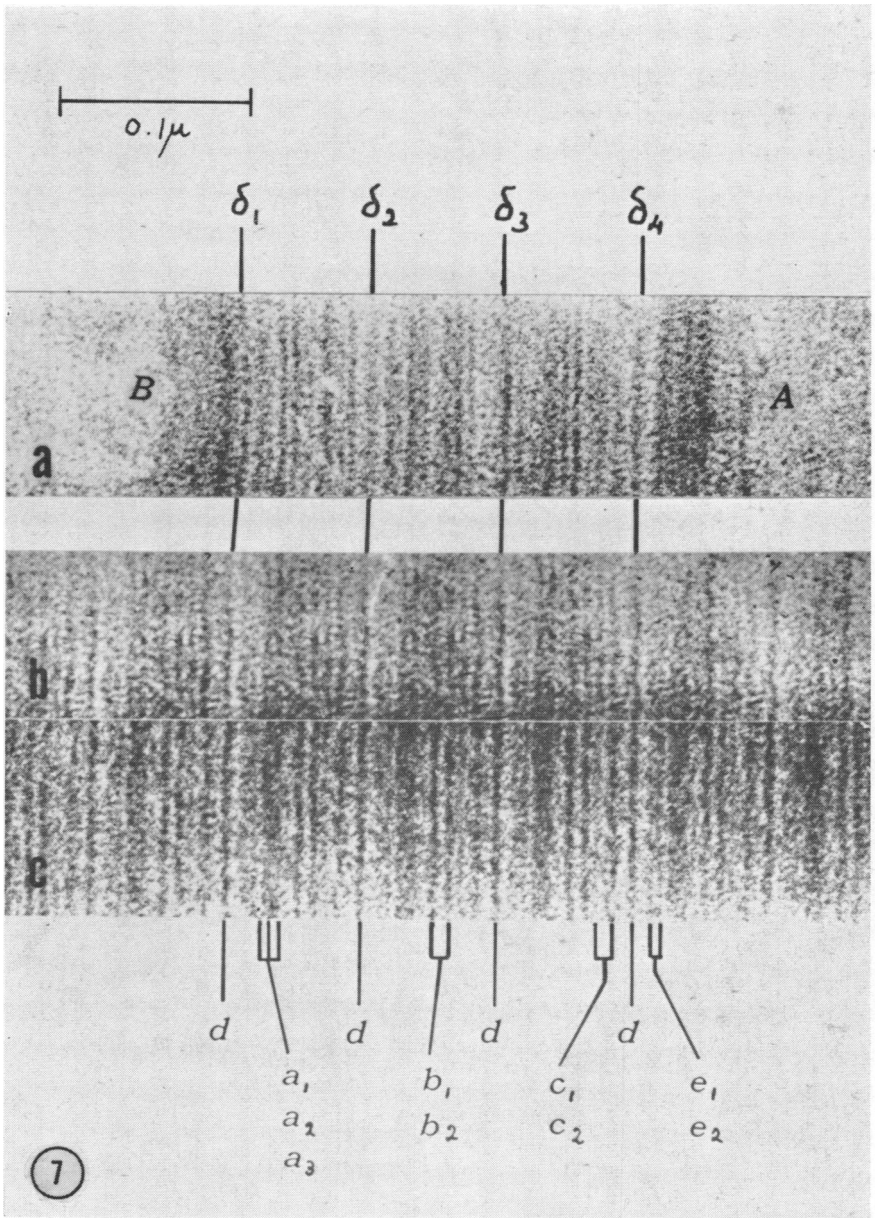

FIG. 7.—Composite electron micrograph illustrating the "optical synthesis" of the native-type band pattern from that of SLS-type aggregates. At (*b*) is shown the result of a multiple printing of the SLS image (*a*), with a longitudinal displacement between successive exposures of 1/4 of the length of the single segment shown at (*a*). The summation of band densities resulting from this procedure corresponds closely to the band pattern of native-type fibrils (*c*). Both the SLS-type aggregate (*a*) and the native-type fibril (*c*) were stained with PTA. ×250,000.

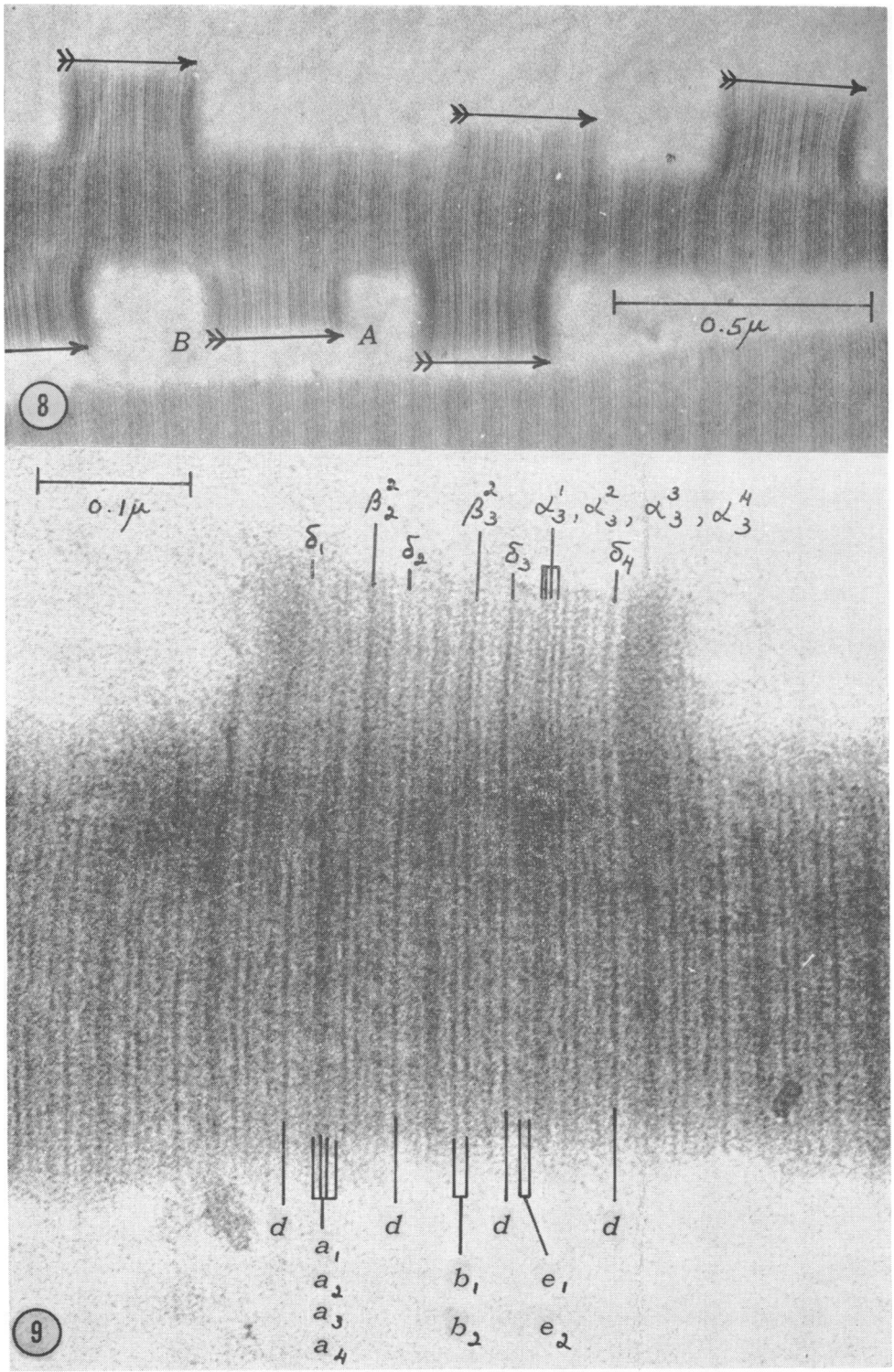

FIG. 8.—"Dimorphic" ordered aggregate of TC, produced by exposing reconstituted native type fibrils to a solution containing TC macromolecules and ATP at a pH value favoring the formation of SLS-type aggregates. Most of the segments formed under these conditions occur as outgrowths from the native-type fibrils and always exhibit a characteristic orientation and polarity with respect to the "polarized" band-pattern of the native-type fibrils. Stained with PTA. ×74,000.

FIG. 9.—Higher magnification view of a region in the same preparation as shown in Fig. 8, showing that in the "dimorphic forms," all bands of the SLS-type structure are continuous with the bands of the native-type fibril. In particular, it should be noted that each band in the native-structure is continuous with one of four "equivalent bands" in the SLS form (*e.g.*, *d* bands show continuity with the SLS bands δ_1, δ_2, δ_3, and δ_4; *a* bands with the α_1^2, α_2^2, α_3^2, and α_4^2 bands, and so on). Stained with PTA. ×220,000.

that the two band-patterns, corresponding to the distribution of basic and acidic residues respectively, match each other very closely in terms of the relative positions along the length of the TC macromolecule, but differ considerably in terms of the relative intensities of individual bands in the two "fingerprints." Thus we appear to have direct evidence that the polar groups, both basic and acidic, are localized in narrowly defined clusters having a characteristic ordinate with respect to the length of the TC macromolecule. The narrowest bands observed have widths in the range 15–20 A. The intensity differences between individual bands in the two "fingerprints" also indicate that while basic groups predominate in some of the polar loci, others exhibit a relative parity between basic and acidic groups, and some loci are predominantly acidic. The SLS band patterns also indicate that these polar loci are separated by regions of the macromolecule which either contain polar groups in very low concentration or lack them entirely. These regions (interband regions) presumably are constructed largely from the nonpolar glycine and pyrrolidine residues which comprise almost two thirds of the amino acids in collagen and which provide the stereochemical conditions which stabilize the three-chain helical structure indicated by x-ray diffraction.

So far as is known, collagen fibers *in vivo* exist only in the form of fibrils with an axially repeating band pattern, the axial spacing being between 640 A and *ca.* 700 A, depending on the degree of hydration. The distribution of density in this structure, which will be referred to as native type, has been extensively investigated by both electron microscopy[11,12] and small-angle x-ray diffraction.[13] Following the discovery of the long-spacing forms (SLS and FLS) having axial periods several times that of the native-type fibrils, thus indicating the existence of monomeric units much longer than the axial period of native fibrils, it was postulated[14] that these monomeric units must be packed in a "staggered array" in the native-type structure, *i.e.*, with neighboring protofibrils displaced longitudinally in relation to one another. With the demonstration of particles about 2800 A long in acid solutions of collagen,[2] it became clear that the packing rule must be that adjacent protofibrils are displaced relative to one another by $^1/_4$ of the macromolecular length (see Text Fig. 1 and Fig. 4). A somewhat similar proposal was made by Tomlin.[15]

We now have direct proof of the correctness of this hypothesis from two independent lines of evidence and as a result have been able not only to determine the precise location of the TC macromolecules in the native-type structure, but also to propose what appears to be a logical nomenclature for the bands of the SLS pattern based on this relationship.

If the "quarter-stagger" packing arrangement of TC macromolecules is correct, it should in principle be possible to "synthesize" the characteristic band pattern of native-type collagen fibrils (Figs. 4 and 5) by an appropriate overlapping of the SLS band pattern. Such a synthesis could be accomplished either by summation of the photometer curves of SLS with a displacement of $^1/_4$ of the TC length or, more directly, by multiple exposures on a photographic plate with an appropriate displacement of the recording plate between successive exposures. We have chosen the latter course, and the results of one such synthesis are shown in Figure 7. It is evident that the synthesized pattern (*b*) obtained by multiple printing of the image of the PTA-stained segment shown in (*a*) and with the same directional orientation is closely similar, in both the positions and relative intensities of the

various intraperiod bands, to the band pattern of a PTA-stained native-type fibril (*c*). The resolution obtainable by this procedure has so far been limited only by the errors in the absolute coordinates of the various staining loci introduced by shrinkage and distortion of the SLS-type aggregates during drying of the specimens for examination in the electron microscope.

The second line of evidence stems from the observation that, under carefully controlled conditions of pH and ionic strength, fibrils of native type can be induced to act as nuclei for the growth of SLS-type aggregates. In the resultant "dimorphic forms" (Figs. 8 and 9), the SLS-type outgrowths always have the same orientation relative to the "polarized" band pattern of the native-type fibrils and are located in characteristic fashion relative to the intraperiod banding of the latter. As shown in Fig. 9, each band in the native-type fibril (e.g., the *d* band) exhibits a direct transverse continuity with one of four different bands of the SLS pattern (for the *d* band, these are the δ_1, δ_2, δ_3, and δ_4 bands, according to the terminology proposed), which characteristically are of different staining intensity and separated longitudinally from one another by one-fourth of the segment length, i.e., $^1/_4$ of the length of the TC macromolecule. As shown in Figs. 5 and 9, the intraperiod bands of the native-type structure are designated as a_1, a_2, a_3 ---; b_1, b_2, b_3 ---; and so on, the pattern ending in a series of *e* bands. This type of nomenclature for native-type fibrils, which was first proposed by Schmitt and Gross[16] and has been used by other authors,[11] has the advantage that additional bands, which may be reported in the future as a result of better preservation of order, would be easily assigned an appropriate symbol by means of additional subscripts. Since each of these bands corresponds to four "equivalent bands" in the SLS pattern, it seems clear that the intensity of any particular band in the native-type pattern is the result of a summation, arising from the staggered packing arrangement, in which the binding capacities of the four corresponding "equivalent loci" on the TC macromolecule are equally represented. As shown in two-dimensional diagrammatic form in Text Figure 1, this must mean that the bands in the native-type structure arise from a "hetero-register" of equivalent staining loci, in contrast to the "homo-register" characteristic of the SLS-type aggregation state. It should be noted that the distribution of staining loci (clusters of polar residues) in the TC macromolecules is such that when packed in "parallel array," i.e., with the same directional orientation and with nearest neighbors longitudinally displaced by $^1/_4$ of the TC length, all staining loci are in accurate transverse register.

In view of the characteristic relationship between the bands of the native-type and SLS-type patterns (as demonstrated in Fig. 9 and Text Fig. 1), we propose the following terminology for the approximately 40 bands already observed in the SLS pattern. Each band will be assigned a Greek letter in keeping with its relationship to bands in the native-type pattern (α for those bands corresponding to *a* bands, β for those corresponding to *b* bands, and so on), with a superscript corresponding to the subscript of the native-type band with which it is continuous in the "dimorphic forms," and a subscript indicating which of the four "equivalent bands," is being referred to. As an example, the four SLS bands corresponding to the a_1 band of the native-type structure are a^1_1, a^1_2, a^1_3, and a^1_4, moving along the segment in the sense B to A, as defined by Hodge and Schmitt,[17] those corresponding to the a_2 band are α^2_1, α^2_2, α^2_3, and α^2_4, respectively. This type of terminology possesses

two major advantages over any arbitrary classification of the SLS bands: (1) additional bands which may be resolved in both the native-type and SLS band patterns as a result of improvements in the preservation of order of specimens for electron microscopy would be easily assigned appropriate symbols simply by providing additional subscripts and superscripts, respectively; (2) the staining intensity of any band in the native-type structure may be simply expressed as a summation of the staining intensities of the four corresponding "equivalent bands" in the SLS-type structure, thus e.g., $I_a = (k/4)\ (I_{\alpha 1}^{1} + I_{\alpha 2}^{1} + I_{\alpha 3}^{1} + I_{\alpha 4}^{1})$, where the I's indicate the staining intensities of the bands designated, and k is a constant, the value of which will depend on the degree of additivity of the "binding powers" of the four particular staining loci of the TC macromolecule. The value of k could for instance depart from unity as a result of some rotational vector involved in the packing of the TC polymers. In fact, however, the good agreement between the relative intensities of the bands in the native-type structure with those in the pattern produced from the SLS pattern by optical synthesis (Fig. 7) suggests that the intensities are closely additive, i.e., the value of k must be close to unity.

It is an interesting consequence of the band correspondence shown in the "dimorphic forms" that the SLS pattern may be divided into four regions of equal length, in each of which the distribution (but not the intensities) of the bands corresponds exactly with that in a single period of the native-type structure. However, this does not imply that the TC macromolecule comprises four identical subunits, and, indeed, all the substantial evidence available at the present time indicates that the 2800 A unit is the true monomer, both in solution and in the various ordered aggregates. Furthermore, there is evidence that the macromolecules of other fibrous proteins, e.g., some of the muscle proteins, are packed in comparable staggered arrays.[18]

Earlier determinations of the distributions of axial periods for ordered TC aggregates of FLS-, SLS-, and native-type showed rather wide spreads about the mean values, and we have accordingly reexamined the problem. It is found that much of the previous error resulting from shrinking and stretching of the substrate film under the influence of the electron beam is eliminated when the supporting plastic film is stabilized by vacuum evaporation of a thin film of carbon. Variation in magnification from one exposure to another is also a serious source of broadening of the distributions, and a further sharpening of the curves can be achieved by the simple expedient of photographing at low magnification, taking due account of the errors arising from radial distortion of magnification near the edges of the field. This procedure gives very sharp distributions for individual micrographs, with the mean values varying somewhat for each determination.[19] It is clear, therefore, that, when adequate precautions are taken to minimize distortions arising from the impact of the electron beam and to control variations in magnification arising from lens hysteresis and other effects, the sharp distributions of axial period in ordered TC aggregates are in close agreement with physical chemical determinations of dimensions and with the concept that the macromolecules are highly homogeneous with respect to their lengths. Comparably sharp distributions have been obtained for the protein paramyosin (Hodge, unpublished).

We wish to report one further result, which shows promise of yielding more precise information concerning the distribution of electron density along the TC

macromolecule. As has been shown by electron microscopy, the "quarter stagger" arrangement of the protofibrils results in a juxtaposition of the "equivalent staining loci," the final staining intensity of any particular band being a summation of the staining capacities of the four corresponding "equivalent loci." A comparable "summation" appears to occur in the small-angle x-ray diffraction pattern of native-type collagen fibrils, since all reflections correspond to orders of a fundamental spacing between 640 and 700 A. The observed intensities thus represent the Fourier transform of a series of accurately spaced band regions (electron-dense zones corresponding to the clustering of polar residues) each of which, however, is heterogeneous in that it is made up of a lateral juxtaposition of the four corresponding "equivalent loci"; the 700 A pattern therefore does not directly give information concerning the distribution of density along individual protofibrils, nor can this be deducted from the information contained in the diffraction pattern. As described in the following paper,[20] dialysis of an acid solution of TC versus distilled water results in a rapid increase in viscosity, the final result being the production of a "water-clear" gel, strongly indicating that end-to-end polymerization of the TC macromolecules is proceeding with only slight side-to-side aggregation. The results of Glimcher and Bonar (personal communication) using small-angle x-ray diffraction indicate that the fine filaments of the "water-gel" probably consist of protofibrils packed in the "quarter-stagger" arrangement. However, in our experiments if an ATP solution is added to the gel, or at a late stage during the dialysis, the charge distribution is altered in a way such that the SLS-type packing (i.e., homo-register of polar loci) is favored without serious depolymerization of the protofibrils. The result is a fibrous type of SLS structure (F-SLS), as shown in Figure 6, in which all features of the component TC macromolecules are in "homo-register." If fibers of sufficiently high orientation and order can be produced from these precipitates, it should be possible to utilize high resolution small-angle x-ray diffraction to obtain direct information concerning the distribution of intrinsic electron density along the length of the TC macromolecule and also by the introduction of heavy atoms linked specifically to certain residues, to determine the location of these groups.

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The substance of this paper was presented at the Sixth Congress of the International Union of the Leather Chemists Societies, Munich, Germany, on September 8, 1959 (by F. O. S.), and at a Symposium on Macromolecular Complexes held by the Society of General Physiologists, Urbana, Illinois, on September 9, 1959 (by A. J. H.).

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[8] It should be noted that the density distribution observed in stained specimens results not only from a specific binding of PTA to basic groups but also from an "intrinsic" density arising from the specific distribution of bulky polar side chains in the TC macromolecule. This intrinsic

density distribution has recently been directly demonstrated in electron micrographs of formaldehyde-fixed SLS-type aggregates by Kühn, K., U. Hofmann, and W. Grassmann (*Naturwiss.*, **46**, 512 1959).

[9] For this purpose the segments were stained by exposure to 0.002 *M* uranyl nitrate for 1 minute and washed with distilled water before drying on the grid. Under these conditions the uranium in solution is cationic (see Horne, R. A., C. D. Coryell, and L. S. Goldring, *Ann. Rev. Nuclear Sci.*, **6**, 163, 1956).

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[19] In a typical determination, the lengths of 124 segments in a single micrograph of the preparation shown in Fig. 1 were measured on photographic enlargements at a magnification of 92,000 × (determined by the use of Dow polystyrene latex spheres, with a nominal diameter of 1380 A). The arithmetic mean was 2850 A with 70% of the segments measured falling in the range 2790–2910 A, and all measurements being in the range 2670–3060 A. Sharp distributions have also been obtained for native-type fibrils.

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*THE EFFECTS OF PROTEASES ON THE TROPOCOLLAGEN MACROMOLECULE AND ON ITS AGGREGATION PROPERTIES**

BY ALAN J. HODGE, JOHN H. HIGHBERGER, GOTTFRIED G. J. DEFFNER, AND FRANCIS O. SCHMITT

DEPARTMENT OF BIOLOGY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY, AND RESEARCH DIVISION OF THE UNITED SHOE MACHINERY CORPORATION, BEVERLY, MASSACHUSETTS

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Detailed analysis of the band distribution of segment-type (SLS) ordered aggregates (in which the tropocollagen macromolecules are packed in parallel array with all like features in accurate transverse register[1]) obtained by addition of adenosine triphosphate (ATP) to sonically irradiated solutions of tropocollagen (TC) led to the suggestion[2] that "end-chains" may be involved in the formation of linear polymers of TC, i.e., protofibrils. In this picture, the TC macromolecule in solution is considered to comprise three chains coiled about one another in the characteristic helical configuration deduced from large-angle x-ray diffraction studies,[3] with relatively short (100–200 A) peptide appendages at both ends, presumably in a random-coil configuration when the monomers are free in solution. End-to-end polymerization would thus involve an orderly interaction of peptide end-chains, possibly to form an ordered helical structure. It seemed desirable, therefore, to attempt the isolation and characterization of such peptide end-chains (or fragments thereof), preferably by means of specific enzyme action.

The possibility that proteolytic enzymes could be usefully employed in such an