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The Heterogeneity of Collagen Solutions and its Effect on Fibril Formation

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The reconstitution of fibrils from the collagen in solutions extracted from developing connective tissue has attracted a great deal of attention because of the possibility that it is related to collagen fibrillogenesis *in vivo*.

Study of the kinetics of this reconstitution (Gross, 1956; Gross & Kirk, 1958; Bensusan & Hoyt, 1958; Wood, 1958; Wood & Keech, 1960; Wood, 1960*a*, *b*; Bensusan, 1960; Bensusan & Scanu, 1960) has helped to elucidate the mechanism of the process. Wood & Keech (1960) found that fibrils form in collagen solutions that have been extracted from purified calf dermis by dilute acetic acid in two distinct, effectively consecutive steps. These occupy an initial lag period and a growth phase. Kinetic and electron-microscope data were satisfactorily explained by postulating that the first step consists of the aggregation of asymmetric collagen particles in solution to form nuclei and that during the second these nuclei grow into fibrils by the accretion of further collagen from solution. The rate of the growth during the second step and the diameter of the fibrils ultimately produced depend to a large extent on the nucleation process, and are related to the number and shape of the nuclei (Wood, 1960*a*).

Nucleation and growth were regarded as closely similar aggregation reactions, the main difference between them being that nucleation is a homogeneous reaction whereas in growth both solid and liquid phases are involved. Certain observations, particularly some aspects of the effect of chondroitin sulphate on the rate of fibril formation (Wood, 1960*b*; Keech, 1961), could only be reconciled with some difficulty with this simple model; the results suggested that nucleation and growth might be fundamentally different processes. One possibility is that nuclei are the product of aggregation of only a small fraction of the total collagen in solution, the remaining collagen being unable to form nuclei but able to aggregate on nuclei to form fibrils. A similar type of heterogeneity of acid-soluble collagen had been suggested by Buzagh (1942). Boedtker & Doty (1956) found evidence from light-scattering data for the presence of a small amount of aggregated material in solutions of citrate-soluble collagen, which could only be removed by prolonged centrifuging. Fessler (1957, 1960*a*, *b*) observed that, under certain experimental conditions, collagen which was precipitated on warming solutions of neutral-salt-soluble collagen to 37° could be divided into two fractions. One of these ('fraction A') redissolved when the precipitate was kept at 0° for 48 hr.; the other ('fraction C') did not. In addition, a portion of the original collagen ('fraction B') was not precipitated. Except for these studies there appears to be little evidence from their physical properties that the collagen molecules in a solution of collagen are not identical. However, Jackson & Bentley (1960) have recently concluded that extracts of collagenous tissue contain collagen molecules of different ages. The work reported here was designed to test the hypothesis that collagen molecules in solution are heterogeneous with respect to their ability to aggregate into fibrils. A preliminary account of this work has been published (Wood, 1962).

EXPERIMENTAL

Materials

Collagen solutions. The skins of freshly killed month-old rats (Wistar strain) were freed of hair, epidermis and subcutaneous tissue, homogenized in a stainless-steel mortar after being frozen with liquid nitrogen and then extracted serially twice with 3 times their weight of each of the following solutions at 0°: 0·3M-NaCl containing sodium phosphate (0·01M) buffer, pH 7·1, 1·0M-NaCl similarly buffered and 0·2M-sodium citrate buffer, pH 3·7. The extracts were purified by repeated precipitation with NaCl (Jackson, 1957; Jackson, Leach & Jacobs, 1958) dissolved in 0·1% acetic acid and freeze-dried. Solutions of collagen (0·05–0·2 g./100 ml.) (and also collagen fractions—see below) were prepared in 3% (v/v) acetic acid, dialysed against 0·1M-NaCl, pH 4·2 (Wood & Keech, 1960), and clarified by centrifuging for 1 hr. at 28 000 rev./min. (Spinco model L ultracentrifuge, no. 40 rotor) at 0°.

Solutions of acid-soluble collagen were also prepared from purified calf dermis (Wood & Keech, 1960) and clarified by centrifuging in the same way. The purification involved exhaustive extraction with neutral NaCl solutions so that the acid-soluble collagen was substantially free of neutral-salt-soluble collagen.

Thus solutions of three types of collagen have been used: (1) citrate-soluble collagen from rat skin; (2) neutral-salt-

soluble collagen (0·3M- or 1·0M-NaCl used as extractants) from rat skin; (3) acid-soluble collagen from calf skin.

Protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with purified pig-skin gelatin (Eastoe, 1955) as a standard.

All reagents used were AnalaR grade and octan-2-ol was used as an anti-bacterial agent.

Methods

Kinetics of fibril formation. Each precipitation was initiated by adding an equal volume of phosphate buffer, pH 7·1, at the appropriate temperature, to a sample of collagen solution previously equilibrated at 25°, and the reaction was followed turbidimetrically (Wood & Keech, 1960).

Fractionation of neutral-salt-soluble collagen. The procedure was a simplification of that used by Fessler (1960*a*). Precipitation of samples of neutral-salt-soluble collagen (0·1 g./100 ml.) at 35° was carried out as described above. The reaction was allowed to proceed until the extinction had attained its final value and the mixture rapidly cooled to 0°. It was kept at this temperature for 24 hr. and the residual precipitate (fraction C) was removed by centrifuging at 0° (Spinco model L instrument, rotor no. 40, 20 000 rev./min. for 0·5 hr.). The supernatant was brought rapidly to 35° until the extinction again reached a constant value. The precipitate (fraction A) was removed by centrifuging at 35° and the supernatant dialysed against 0·1% acetic acid and freeze-dried (fraction B). Fractions A and C were redissolved in 1% (v/v) acetic acid, dialysed against 0·1% acetic acid and freeze-dried.

Ultracentrifuging. For analytical work the Spinco model E instrument was used (An.D. rotor). Relative concentrations of components were estimated from the areas between peaks and base-lines, separately determined, and were corrected for sectorial dilution. The Johnston–Ogston effect (Johnston & Ogston, 1946) was eliminated by extrapolating, to zero concentration, results obtained in the concentration range 0·1–0·8 g./100 ml. Sedimentation coefficients were estimated and corrected to standard conditions by the usual methods (Schachman, 1959).

RESULTS

Rate of precipitation of collagen extracted by different media. Solutions of all four preparations of soluble collagen yielded precipitates at pH 7·1 of essentially the same nature. The times in which the precipitates formed differed markedly, however (Fig. 1), acid-soluble collagen precipitating most rapidly and that extracted with 0·3M-sodium chloride most slowly. (In Figs. 1–4, the extent of precipitation, p, equals E/E_∞, where E is the extinction at time t and E_∞ the extinction at 3 hr., after which no significant further change took place.) The main difference between the four curves in Fig. 1 is in the length of the lag periods, differences of rate of growth being relatively small. This suggests that the different overall rates are primarily a result of differences of the rate of the nucleation step of fibril formation.

As a result of these experiments, subsequent precipitation experiments with acid-soluble collagen were done at 25°, with citrate-soluble collagen at 30° and with neutral-salt-soluble collagen at 35°, to obtain conveniently measurable rates.

Heterogeneity of neutral-salt-soluble collagen. Differences in the form of the precipitation curve of the above type were also observed in fractions of a single preparation. In a typical experiment with a solution of collagen extracted with 1·0M-sodium chloride precipitation was initiated by adjusting the pH to 7·1, but instead of the process being allowed to go to completion it was stopped at the end of the lag period (previously determined in a separate experiment) by rapidly cooling the solution to 0°. When the solution was readjusted to 35°, 1·5 hr. later, precipitation was resumed. From a comparison of the resulting precipitation curve (curve *b*, Fig. 2) with the curve (*a*) for uninterrupted precipitation it was concluded that cooling had merely arrested precipitation. Nucleation, which is presumed to occur in the lag period, had not been reversed, for the lag period was eliminated and growth on the nuclei occurred at approximately the same rate as in uninterrupted precipitation. In a third experiment, carried out in the same way, not only was the solution cooled at the end of the lag period but in addition it was centrifuged at 0° for 0·5 hr. (Spinco model L instrument, SW 39 L rotor, 30 000 rev./min.). The top two-thirds of the contents of the centrifuge tube were transferred to a cuvette and brought back to 35°. Precipitation occurred but only after a greatly increased lag period (curve *c*). The rate of growth is again approximately the same as in uninterrupted precipitation. The protein concentration in the portion of the collagen solution used for this third precipitation was 81% of the original protein concentration.

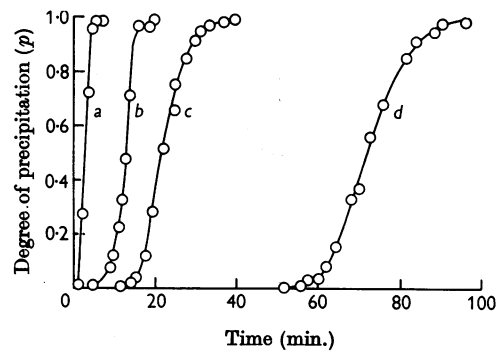

Fig. 1. Rate of precipitation from different collagen solutions at 35°, pH 7·1, I 0·23, collagen concn. 0·05%. *a*, Acid-soluble collagen from calf skin; *b*, citrate-soluble collagen from rat skin; *c*, 1·0M-NaCl-soluble collagen from rat skin; *d*, 0·3M-NaCl-soluble collagen from rat skin.

Curve *d* shows the course of precipitation of a sample of the original solution which had been cooled immediately after initiation of precipitation, centrifuged and diluted to 81% of its original concentration. It is clear that the large difference in the lag period of curves *a* and *c* is not due merely to the operations of cooling and centrifuging or to a dilution effect, but must be due to the sedimentation of a small fraction which had aggregated during the lag period, presumably the nuclei. The collagen remaining in solution was precipitated much less readily in the absence of this small fraction. This type of behaviour was also observed in solutions of collagen extracted with 0·3M-sodium chloride.

Heterogeneity of citrate-soluble and acid-soluble collagens. These preparations were alike in showing the above effect to a much less marked extent. Fig. 3 shows typical results for acid-soluble collagen.

Heterogeneity of collagen precipitated from neutral-salt extracts. To investigate the possibility that the heterogeneity of neutral-salt-soluble collagen with respect to fibril formation is related to its heterogeneity with respect to the solubility of the collagen fibrils formed from it (Fessler, 1957, 1960*a*, *b*), the following experiments were performed. The precipitation from a solution of 1·0M-sodium chloride-soluble collagen at 37°, pH 7·1 was followed (curve *a*, Fig. 4). When precipitation was complete the cuvette was cooled to 0°, and, in agreement with Fessler (1960*a*) and Gross (1958), the precipitate partly redissolved. The dissolution process appeared to be complete after 24 hr. since the turbidity no longer decreased and estimation of the protein content of the supernatant showed that 23% of the total collagen remained insoluble. When the cuvette was brought back to 35° precipitation occurred again, giving curve *b* which shows that the lag period had been almost eliminated and that the rate of growth is approximately the same as in the first precipitation (curve *a*). In a second experiment a sample of the same collagen solution was heated to 37° and the precipitate partly redissolved by cooling in exactly the same

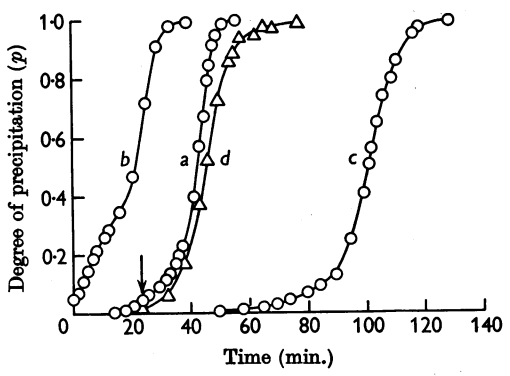

Fig. 2. Effect of removing nuclei on the rate of precipitation from a solution of 1·0M-NaCl-soluble collagen at 35°, pH 7·1, *I* 0·13, collagen concn. 0·04%. *a*, Uninterrupted precipitation; *b* and *c*, precipitation arrested by cooling to 0° at time indicated by arrow, then (*b*) brought back to 35° after 1·5 hr. or (*c*) centrifuged before returning to 35°; *d*, cooled at zero time, centrifuged, diluted and brought back to 35°.

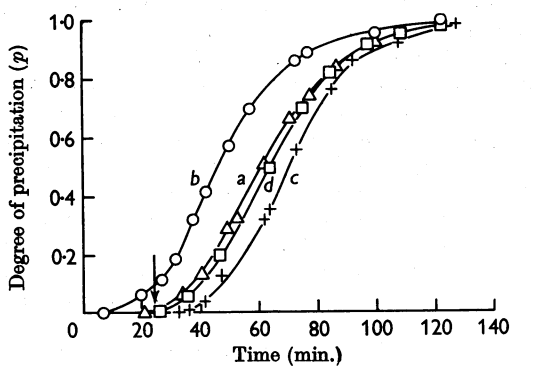

Fig. 3. Effect of removing nuclei on the rate of precipitation from a solution of acid-soluble collagen from calf skin at 25°, pH 7·1, *I* 0·23, collagen concn. 0·05%. *a*, Uninterrupted precipitation; *b*, and *c*, precipitation arrested by cooling to 0° at time indicated by arrow, then (*b*) brought back to 25° after 1·5 hr. or (*c*) centrifuged before returning to 25°; *d*, cooled at zero time, centrifuged and brought back to 25°.

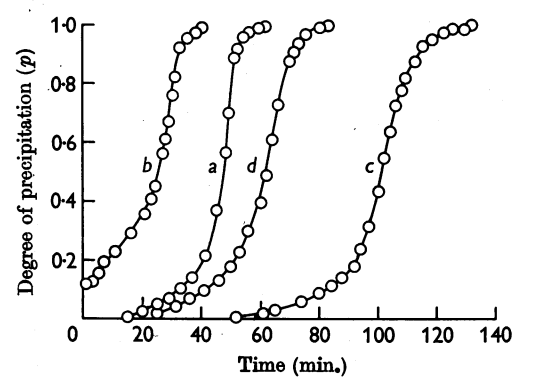

Fig. 4. Effect of removing fraction C on the rate of precipitation from a solution of 1·0M-NaCl-soluble collagen at 35°, pH 7·1, *I* 0·13, original collagen concn. 0·04%. *a*, Precipitation of untreated collagen solution; *b*, curve obtained after cooling the precipitate from *a* to 0° for 24 hr. and then returning it to 35°; *c*, curve obtained after cooling a precipitate (similar to that from *a*) to 0° for 24 hr., centrifuging off the residual precipitate (fraction C) and returning supernatant to 35°; *d*, curve obtained with original solution diluted to same concentration as the supernatant from *c*.

way. The precipitate was then centrifuged (Spinco model L instrument, no. 40 rotor, 20 000 rev./min.) at 0° for 0·5 hr. to remove the residual precipitate, and the supernatant was brought back to 35°. Subsequent precipitation occurred only after a longer lag period (curve *c*). As shown by curve *d*, which is the precipitation curve for an appropriate dilution of the original collagen solution, the difference of lag period shown by curves *a* and *c* is too great to be accounted for by a dilution effect. The series of experiments indicates that the fraction of the collagen precipitate which did not redissolve on cooling (corresponding to Fessler's fraction C) acted as nuclei during the second precipitation. When these nuclei were removed the remaining precipitable collagen (corresponding to Fessler's fraction A) only formed fibrils at a much reduced rate. The relationship between fractions C and A is thus similar to the relationship between the 'nucleus-forming' collagen and 'growth' collagen. Similar results were obtained with several preparations of neutral-salt-soluble collagen extracted with 0·3 M- or 1·0 M-sodium chloride.

Solubility of collagen precipitated from solutions of citrate-soluble and acid-soluble collagens. Precipitates formed from solutions of these preparations redissolved only slightly on cooling (about 5% re-solution in 48 hr.) and the above experiment was therefore not carried out with these materials.

Nature of fractions A and C of neutral-salt-soluble collagen. According to Fessler (1960*a*), fractions A and C do not differ significantly in their proline, hydroxyproline or hexose content; neither did he demonstrate any marked differences in molecular weight, sedimentation constant or intrinsic viscosity. To explore further any difference between them, the nature of the products of thermal denaturation of these fractions has been investigated.

Solutions in 0·1 M-sodium chloride, pH 4·2, of the collagen fractions and also of unfractionated neutral-salt-soluble and citrate-soluble collagens were denatured by warming at 40–45° for 10 min. and examined in the ultracentrifuge at 38°. Unfractionated neutral-salt-soluble collagen (0·3 M- and 1·0 M-sodium chloride extracts) sedimented as one major component and two faster-moving minor ones [Fig. 5 (*a*)], none of which was observed in sedimentation patterns of undenatured collagen at 33°. The sedimentation coefficients of the two slower components ($S^0_{20,w}$ = 3·1 s and 4·1 s) identify them with the α- and β-components respectively of

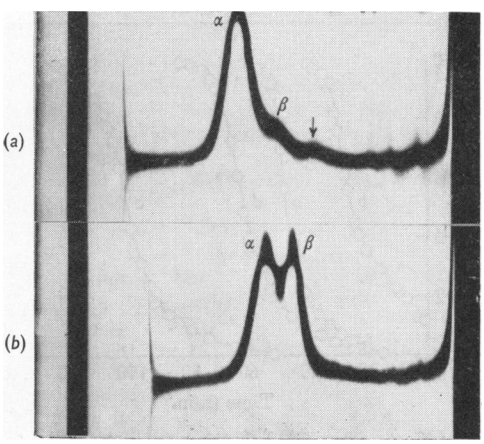

Fig. 5. Ultracentrifuge patterns obtained with heat-denatured collagen solutions. (*a*) 1·0 M-NaCl-soluble collagen (0·37 g./100 ml.) after 72 min., and (*b*) citrate-soluble collagen (0·39 g./100 ml.) after 73 min., both at 59 780 rev./min. in the Spinco model E instrument (An.D. rotor). The temperature was 38°, and the solvent was 0·1 M-NaCl containing sodium acetate (5 mM) buffer, pH 4·2 (Wood & Keech, 1960). Sedimentation was from left to right. The arrow indicates the third component—see text.

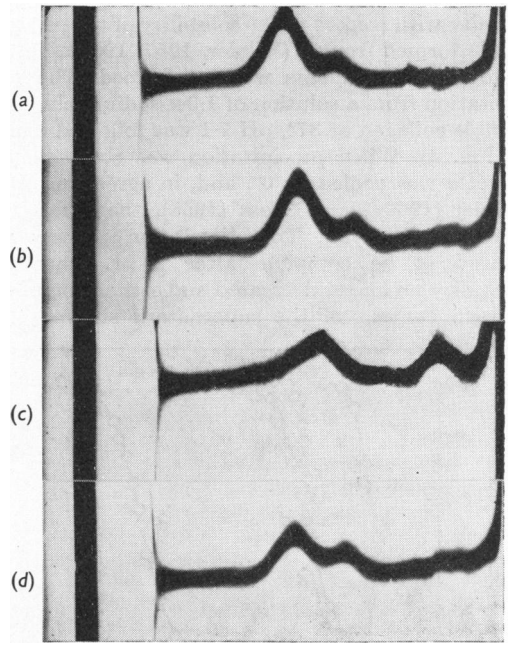

Fig. 6. Ultracentrifuge patterns obtained with heat-denatured-collagen solutions. (*a*) 1·0 M-NaCl-soluble collagen (0·13 g./100 ml.) after 78 min., (*b*) fraction A (0·14 g./100 ml.) after 82 min., (*c*) fraction B (0·10 g./100 ml.) after 85 min., and (*d*) fraction C (0·11 g./100 ml.) after 85 min., all at 59 780 rev./min. in the Spinco model E instrument (An.D. rotor). The temperature was 38°, and the solvent was 0·1 M-NaCl containing sodium acetate (5 mM) buffer, pH 4·2 (Wood & Keech, 1960). Sedimentation was from left to right.

Table 1. *Sub-unit composition of neutral-salt-soluble collagens and the fractions derived from them*

Experimental details are given in the text.

Extracting medium	Fraction	Proportion of each fraction in the unfractionated material (g./100 g.)	Composition of denatured material (g./100 g.)		
			α	β	Third component
0·3 M-NaCl	Unfractionated	—	80	15	5
	A	75	79	21	0
	B*	10	65	0	35
	C	15	68	32	0
1·0 M-NaCl	Unfractionated	—	79	19	2
	A	76	79	21	0
	B*	5	65	0	35
	C	19	69	31	0

* Calculated from single ultracentrifuge runs, concn. 0·10 g./100 ml.

denatured citrate-soluble collagen of rat skin [Fig. 5 (*b*)], first observed in this material by Orekhovich & Shpikiter (1955). The third component ($S^0_{20,w}$ approximately 6·5S) was not observed in citrate-soluble collagen but the presence of very small amounts would be obscured by the higher concentration of the β-component.

Sedimentation patterns of fractions A and C were qualitatively similar to those of the unfractionated material except that the third component was missing, but fraction B consisted of the α-component and the third component, the β-component being entirely absent (Fig. 6). Analysis of the sedimentation patterns revealed, however, that fractions A and C also differed in the relative proportions of the three components (Table 1). In both preparations fraction C contains a higher proportion of the β-component than does fraction A. In addition, the results indicate that during fractionation there has been a small decrease in the total amount of the α-component and a corresponding increase in the amount of the β-component.

DISCUSSION

The precipitation experiments with neutral-salt-soluble collagen clearly show that this material is heterogeneous with respect to the ease with which its constituent molecules aggregate to form fibrils. The results support the view that the nucleation and growth steps of fibril formation are two rather distinct processes which are undergone by different fractions of the collagen. Removal of nuclei from a solution in which a precipitate is forming leaves a fraction which forms fibrils only relatively slowly. This retardation is primarily the result of slower nucleation, the rate of growth being altered very little. Moreover, the formation of nuclei is not reversed by cooling but, under the experimental condition used, the collagen which grows on the nuclei redissolves to a large extent on cooling.

In citrate-soluble collagen and acid-soluble collagen the evidence for heterogeneity is less clear, as the above effects are not so marked. Not only does the removal of nuclei have less effect on the rate of precipitation of the remaining collagen, but the precipitates from these types of soluble collagen redissolve less readily on cooling. These observations suggest that the species of collagen molecule which is able to form nuclei constitutes a greater proportion of these preparations, and the observation that the rate of fibril formation under standard experimental conditions is greater with citrate-soluble and acid-soluble collagens than with neutral-salt-soluble collagens is consistent with this idea.

Despite the marked difference in the rates at which neutral-salt-soluble and citrate-soluble collagens form fibrils and in the stability of the fibrils formed, these two types of soluble collagen are remarkably alike, both in chemical composition and physical properties (Jackson *et al.* 1958; Jackson, 1958; Jackson & Bentley, 1960; Fessler, 1960*a*, *b*). Mazurov & Orekhovich (1960), however, found that the products of denaturation of these materials differed markedly. Whereas citrate-soluble collagen yielded a mixture of approximately equal weights of two components (α- and β-) which could be resolved in the ultracentrifuge, denatured neutral-salt-soluble collagen consisted very largely of the α-component with only a very small amount of the β-component. It would be of interest, therefore, to know if 'nucleus-forming' collagen is characterized by an unusually high proportion of the β-component.

Unfortunately nuclei cannot easily be prepared in sufficient quantity for such a study. The precipitation experiments show, however, that the relation-

ship between fractions A and C of precipitates prepared from neutral-salt-soluble collagen (i.e. the fraction which redissolves on cooling and the fraction which remains insoluble at 0° respectively) is similar to the relationship between 'growth' collagen and 'nucleus-forming' collagen. They indicate, in fact, that fraction C is identical with or contains the 'nucleus-forming' collagen. It is reasonable to suppose, therefore, that if 'nucleus-forming' collagen has an unusually high content of the β-component this should be apparent in the sub-unit composition of fraction C.

Fraction C does, in fact, yield a significantly higher proportion of the β-component than fractions A or B or the unfractionated collagen. Moreover, fraction B, which is not precipitated at all under the conditions used, yields no β-component. Taken with the observations of Mazurov & Orekhovich (1960) these results strongly suggest that the heterogeneity of neutral-salt-soluble collagen with respect to fibril-forming properties is related to the heterogeneity with respect to sub-unit composition. Acid-soluble collagen from calf skin is reported to have an even higher β-component content than that from rat skin (Doty & Nishihara, 1958; Grassmann, Hannig & Engel, 1961), and our observation that this material is precipitated at an even greater rate than citrate-soluble collagen from rat skin is thus consistent with this interpretation.

The precise nature of the α- and β-components of soluble collagen is still far from clear. Grassmann *et al.* (1961) and Hannig & Engel (1961) have summarized the physicochemical results obtained by other workers as well as themselves concerning the molecular weights of the α- and β-components and the native collagens from which they are derived. On the basis mainly of their own work Grassmann *et al.* (1961) suggest that the components are derived from collagen molecules having the triple-helix structure (Rich & Crick, 1961), two polypeptide chains of which are cross-linked as shown in Fig. 7 (Type II). On denaturation, when the triple-helix breaks down, each molecule yields one single-chain (α-component) and one double-chain (β-component). They also propose that the molecules of neutral-salt-soluble collagen are predominantly Type I (Fig. 7), in which there is no cross-linking and which yields only the α-component on denaturation. If this model holds for rat-skin collagen as well as calf-skin collagen then clearly all our solutions are mixtures of Type I and Type II molecules. The citrate-soluble collagen contains 75% of Type II and the neutral-salt-soluble collagen 25–30% of Type II. Fraction C (mainly 'nucleus-forming' collagen) contains 50% of Type II and fraction A ('growth' collagen) 30% of Type II. Taken together with the results of the precipitation experiments this suggests that Type II molecules aggregate more readily to form nuclei than Type I. Some uncertainty about this conclusion arises from the indications of the ultracentrifuge results that there is a slight conversion of Type I into Type II (a decrease in the total α-component content and in increase in the total β-component content) during fractionation, and an alternative interpretation of the results would be that 'nucleus-forming' collagen shows a greater tendency to undergo conversion from Type I into Type II than does 'growth' collagen. Consideration of this possibility becomes more important in view of recent findings by Mazurov & Orekhovich (1960). They concluded, from a study of the incorporation of [^{14}C]glycine into the α- and β-components of rat-skin soluble collagen, that during the biogenesis of the collagen fibre there occurs a stabilization of the collagen molecule involving the conversion of the α-component into the β-component (in terms of the model this implies conversion of Type I into Type II but intermolecular cross-linking would presumably have the same effect), thus giving concrete expression to ideas put forward by Gross (1958) and Jackson (1958). It is likely that alternative methods of fractionation, which do not involve fibril formation, will have to be developed to resolve this question.

The nature of the third component observed in denatured neutral-salt-soluble collagen is not known. Its sedimentation coefficient is higher than that of the γ-component, small amounts of which were observed by Grassmann *et al.* (1961) and Altgelt, Hodge & Schmitt (1961) in citrate-soluble collagen and which they consider to be derived from collagen molecules in which all three polypeptide chains are joined together. The inability of fraction B of neutral-salt-soluble collagen (which is

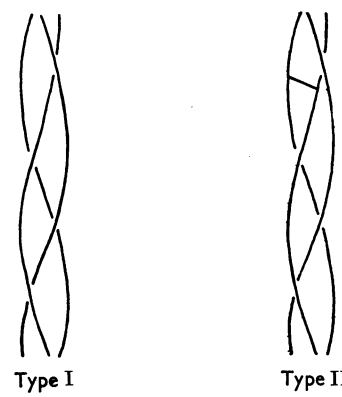

Fig. 7. Models for collagen molecules (Grassmann *et al.* 1961).

rich in the third component) to form fibrils under standard conditions and the absence of the third component from our citrate-soluble collagen also suggest that it is not built up in this way. A component with a similar sedimentation coefficient was observed by Veis, Anesey & Cohen (1960), by Veis & Anesey (1961) and by Altgelt *et al.* (1961) in some preparations of denatured calf-skin collagen.

SUMMARY

1. The formation of fibrils in solutions of neutral-salt-soluble collagen could be arrested by cooling the system at the end of the lag period. When the collagen which had aggregated to form nuclei during this period was removed by centrifuging, the supernatant formed fibrils at a much reduced rate, the effect being mainly due to a prolonged lag period (nucleation).

2. In solutions of acid-soluble-collagen, which form fibrils at a greater rate than solutions of neutral-salt-soluble collagen, the same phenomenon was observed but to a much smaller degree.

3. Removal of Fessler's fraction C from neutral-salt-soluble collagen (Fessler, 1960*a*) has a similar effect on fibril formation to the removal of nuclei.

4. Fessler's fractions A and C of neutral-salt-soluble collagen yielded different proportions of the α- and β-components on thermal denaturation. Fraction B yielded the α-component plus a third component which had a higher sedimentation coefficient. There was some indication of the conversion of the α-component into the β-component during fractionation.

5. It is concluded that neutral-salt-soluble collagen and also, possibly, acid-soluble collagen are heterogeneous with respect to the ability of their constituent collagen particles to aggregate to form fibrils, and it is suggested that the fibril-forming ability is related to the sub-unit composition of the collagen molecules.

6. The results are discussed in relation to a mechanism of fibril formation put forward previously and to the model proposed by Grassmann *et al.* (1961) for collagen molecules.

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REFERENCES

Altgelt, K., Hodge, A. J. & Schmitt, F. O. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 1914.
Bensusan, H. B. (1960). *J. Amer. chem. Soc.* **82**, 4995.
Bensusan, H. B. & Hoyt, B. L. (1958). *J. Amer. chem. Soc.* **80**, 719.
Bensusan, H. B. & Scanu, A. (1960). *J. Amer. chem. Soc.* **82**, 4990.
Boedtker, H. & Doty, P. (1956). *J. Amer. chem. Soc.* **78**, 4267.
Buzagh, A. von (1942). *Kolloidzschr.* **101**, 149.
Doty, P. & Nishihara, T. (1958). In *Recent Advances in Gelatin and Glue Research*, p. 92. Ed. by Stainsby, G. London: Pergamon Press Ltd.
Eastoe, J. E. (1955). *Biochem. J.* **61**, 589.
Fessler, J. H. (1957). *Fed. Proc.* **16**, 37.
Fessler, J. H. (1960*a*). *Biochem. J.* **76**, 452.
Fessler, J. H. (1960*b*). *Biochem. J.* **76**, 463.
Grassmann, W., Hannig, K. & Engel, J. (1961). *Hoppe-Seyl. Z.* **324**, 284.
Gross, J. (1956). *J. biophys. biochem. Cytol.* **2**, 261.
Gross, J. (1958). *J. exp. Med.* **108**, 215.
Gross, J. & Kirk, D. (1958). *J. biol. chem.* **233**, 355.
Hannig, K. & Engel, J. (1961). *Leder*, **12**, 213.
Jackson, D. S. (1957). *Biochem. J.* **65**, 277.
Jackson, D. S. (1958). *New Engl. J. Med.* **259**, 814.
Jackson, D. S. & Bentley, J. P. (1960). *J. biophys. biochem. Cytol.* **7**, 37.
Jackson, D. S., Leach, A. A. & Jacobs, S. (1958). *Biochim. biophys. Acta*, **27**, 418.
Johnson, J. P. & Ogston, A. G. (1946). *Trans. Faraday Soc.* **42**, 789.
Keech, M. K. (1961). *J. biophys. biochem. Cytol.* **9**, 193.
Lowry, O. H., Rosebrough, N. J., Farr, A. I. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Mazurov, V. I. & Orekhovich, V. N. (1960). *Biokhimiya*, **25**, 814.
Orekhovich, V. N. & Shpikiter, V. O. (1955). *Dokl. Akad. Nauk S.S.S.R.* **101**, 529.
Rich, A. & Crick, F. H. C. (1961). *J. molec. Biol.* **3**, 483.
Schachman, H. K. (1959). *Ultracentrifugation in Biochemistry.* New York: Academic Press Inc.
Veis, A. & Anesey, J. (1961). *Arch. Biochem. Biophys.* **94**, 20.
Veis, A., Anesey, J. & Cohen, J. (1960). *J. Amer. Leath. Chem. Ass.* **10**, 548.
Wood, G. C. (1958). *Abstr. Commun. 4th int. Congr. Biochem., Vienna*, 2–92, p. 26.
Wood, G. C. (1960*a*). *Biochem. J.* **75**, 598.
Wood, G. C. (1960*b*). *Biochem. J.* **75**, 605.
Wood, G. C. (1962). *Biochem. J.* **82**, 2P.
Wood, G. C. & Keech, M. K. (1960). *Biochem. J.* **75**, 588.