Biochem. J. (1968) **109**, 857
Printed in Great Britain

The Effect of Chondroitin Sulphate–Protein on the Formation of Collagen Fibrils *in vitro*

By B. P. TOOLE and D. A. LOWTHER
Department of Biochemistry, Monash University, Clayton, Vic., Australia

(*Received* 8 *March* 1968)

1. It was found that the precipitation of collagen fibrils at 37° from mixtures of chondroitin sulphate–protein and tropocollagen at physiological ionic strength and pH takes place in two distinct phases. The first occurs immediately on mixing either at 4° or at 37°, and the second occurs only at 37° and after a lag phase whose magnitude depends on the proportions of components. 2. When the second stage of precipitation was inhibited by mixing the reactants at 4°, the initial precipitate was found to contain 'native-type' collagen fibrils and chondroitin sulphate–protein. 3. On the basis of kinetic experiments it was concluded that aggregates of chondroitin sulphate–protein and tropocollagen form instantaneously and that these act as sites for the second stage of precipitation of fibrils. 4. The gels that result after continued incubation at 37° are fibrous in appearance if formed in the presence of the initial precipitate of chondroitin sulphate–protein and tropocollagen. 5. On the basis of these experiments *in vitro* the authors propose a sequence of events for collagen fibrogenesis *in vivo*.

There is considerable evidence that tropocollagen molecules are precursors of collagen fibres *in vivo* (Jackson, 1957; Harkness, Marko, Muir & Neuberger, 1954) and that such fibres are formed in the extracellular phase of connective tissues (Jackson & Smith, 1957; Porter & Pappas, 1959; Revel & Hay, 1963; Goldberg & Green, 1964). At physiological pH and ionic strength *in vitro* tropocollagen molecules are capable of self-assembly into 'native-type' fibrils when solutions are warmed to 37° (Gross, 1956; Wood, 1964). Several workers have therefore concluded that other soluble components of the extracellular phase may only have an indirect effect on fibre formation (Gross, 1956; Lowther, 1963; Goldberg & Green, 1964). However, Kuhn, Schuppler & Kuhn (1965) demonstrated that polymerization occurred less readily if a carbohydrate-containing contaminant was removed by repeated precipitation of the tropocollagen; Mathews (1965) has observed the formation of a reversible complex between protein-free CS* and tropocollagen at neutral pH and Wood (1960*b*) showed that the addition of protein-free AMPS modified the kinetics of fibril precipitation, though this effect was minimal at physiological ionic strength.

It is now well established that sulphated AMPS occurs in the extracellular phase of connective tissues as complexes with protein (Muir, 1964; Schubert, 1964). These macromolecules contain several AMPS chains covalently bound to protein (Mathews & Lozaityte, 1958; Partridge, Davis & Adair, 1961; Luscombe & Phelps, 1967) and probably exist as aggregates of such units under physiological conditions (Mathews & Lozaityte, 1958; Jackson, 1964). The physical properties of these complexes differ considerably from the protein-free AMPS preparations (Laurent, 1966). The present paper describes the instantaneous precipitation of a fraction of tropocollagen by CS–protein isolated from nasal cartilage by mild methods designed to minimize alteration of the physical properties of the native molecule. The precipitates that form at physiological pH and ionic strength both at 4° and 37° contain 'native-type' collagen fibrils. The effect of this reaction on the subsequent precipitation of remaining tropocollagen is also described. It is suggested that interactions between AMPS–protein and tropocollagen take place *in vivo* at the surface of connective-tissue cells, thus providing a mechanism enabling these cells to control the orientation and packing of fibrils into collagen fibres. A preliminary report of some of this work has been published (Toole & Lowther, 1967).

* Abbreviations: CS, chondroitin sulphate; AMPS, acid mucopolysaccharide.

MATERIALS AND METHODS

Materials

Buffers. Unless otherwise stated, the buffer used contained 0·14 M-NaCl in 8 mM-Na_2HPO_4–KH_2PO_4, pH 7·3. Krebs–Ringer bicarbonate buffer was prepared as described in Umbreit, Burris & Stauffer (1959).

Protein–polysaccharides. Extracts were obtained by brief homogenization in water of nasal cartilage from young cattle (Goh & Lowther, 1966) and subsequent centrifugation to remove 'protein–polysaccharide heavy fraction' (Gerber, Franklin & Schubert, 1960). CS–protein prepared from these extracts by density-gradient centrifugation in CsCl (Franek & Dunstone, 1966) was used in the experiments described unless otherwise stated.

Other CS–proteins used for comparison were: (*a*) from bovine nasal-cartilage extracts, purified by quaternary ammonium salt precipitation and ECTEOLA-cellulose chromatography (Goh & Lowther, 1966); (*b*) from bovine intervertebral disks by the same method (Lowther & Baxter, 1966); (*c*) from water and salt extracts of bovine heart valves by CsCl-density-gradient centrifugation (F. A. Meyer & D. A. Lowther, unpublished work); (*d*) from salt extracts of bovine heart valves by quaternary ammonium salt precipitation.

Dermatan sulphate–proteins were precipitated from hot urea extracts of the fibrous residues of bovine heart valves (Lowther, Toole & Meyer, 1967), skin (Toole & Lowther, 1966) and tendon with 2·3 vol. of saturated ethanolic KCNS (B. P. Toole & D. A. Lowther, unpublished work).

Hyaluronate–protein was obtained from water extracts of bovine heart valves by CsCl-density-gradient centrifugation (F. A. Meyer & D. A. Lowther, unpublished work) and from bovine synovial fluid by ultrafiltration (Preston, Davies & Ogston, 1965).

All preparations were dialysed against the buffer and centrifuged at 120000*g* for 3 hr. before use. A summary of the gross compositions of these preparations is given in Table 3.

Protein-free AMPS. CS–keratan sulphate, dermatan sulphate and keratan sulphate were prepared from proteolytic digests of cartilage, skin and intervertebral disks respectively as described previously (Toole & Lowther, 1966; Lowther & Baxter, 1966).

Tropocollagens. The preparations used for most of the experiments described were obtained from 0·1 M-acetic acid extracts of bovine embryo skin and were purified by repeated precipitations with 5% NaCl at pH 3 and 17% NaCl at neutral pH. The collagen was reprecipitated by dialysis against 10 mM-Na_2HPO_4 (Green & Lowther, 1959). The final precipitate contained approx. 95% of its dry weight as tropocollagen. It was dissolved in 0·1 M-acetic acid, dialysed against three changes of the buffer for 48 hr. and centrifuged at 120000*g* for 3 hr. before use to ensure complete removal of fibrils. All operations were carried out at 4°.

Other tropocollagen preparations used for comparative purposes were: (*a*) from young rat skin as described above; (*b*) from guinea-pig granulomata (Green & Lowther, 1959) as described above; (*c*) from bovine embryo skin as described above, but excluding dialysis against Na_2HPO_4; (*d*) from 0·2 M-NaCl–0·25% EDTA (pH 7·0) extracts of bovine embryo skin, purified by NaCl precipitations only; (*e*) from the same extracts described in (*d*), purified by NaCl precipitations followed by dialysis against Na_2HPO_4 followed by precipitation with ATP (Kuhn, Fietzek & Kuhn, 1966).

Turbidity measurements

The turbidity was measured in a spectrophotometer at 400 mμ (Wood & Keech, 1960) with 0·5 ml. mixtures of tropocollagen and protein–polysaccharide. The reactants, previously dialysed against the buffer, were mixed at 4° in small test tubes and the contents transferred to 0·5 ml. cuvettes. All glassware and solutions were cooled to 4° before use and the mixtures kept at 4° until turbidity had been measured. Some variation in turbidity was observed on leaving mixtures to stand at 4° and so the initial turbidity was estimated within 2 min. of mixing.

Changes in turbidity at 37° were followed by placing the cuvettes in a constant-temperature water bath at 37° within 5 min. of mixing the components at 4° as described above. The E_{400} value was read at regular intervals as described by Gross & Kirk (1958). Occasionally the initial mixing was performed after heating each component separately at 37° for 4 min., but this caused no significant difference in the results. Precipitation curves were constructed by plotting E_{400} against the time of incubation at 37°. The lag time is defined as the period between the commencement of incubation at 37° and the onset of maximum precipitation rate. Precipitation rate was judged by the slope of the curve.

Centrifugation of mixtures of CS–protein and collagen

Mixtures of CS–protein and tropocollagen, in a final volume of 2 ml., were left for 1 hr. at 4° and then centrifuged at 120000*g* for 1½ hr. at 4°. Portions of the supernatants were assayed for CS–protein and collagen. The amount of each component in the pellets could then be calculated since the amounts present in the original mixtures were known. Changes in turbidity on incubation of these supernatants at 37° were followed as in the preceding paragraph.

Analytical methods

CS–protein. The CS–protein content of samples was calculated from uronic acid analyses (Bitter & Muir, 1962) by using the factor 3·2 (calculated from the composition of the CS–protein prepared by CsCl-density-gradient centrifugation).

Collagen. The collagen content of samples was calculated from hydroxyproline analyses (Leach, 1960; or Serafini-Cessi & Cessi, 1964) by using the factor 7·5.

Electron microscopy

Specimens were prepared by diluting suspensions of fibrils with cold buffer, drying samples on to copper-mesh grids and staining with neutral 1·5% phosphotungstic acid at 4°. The grids were examined in a Hitachi HU 11A electron microscope.

RESULTS

CS–protein was found to form a precipitate immediately after mixing with tropocollagen at physiological ionic strength and pH. This reaction was studied mainly at 4°, where no spontaneous formation of fibrils from tropocollagen alone occurs. The proportion of tropocollagen and CS–protein giving maximum precipitation, the morphology and composition of the precipitate

and some factors affecting the precipitation are described in the first section. As only a portion of the tropocollagen takes part in this instantaneous reaction, the complete precipitation of collagen at 37° is described in the second section.

Instantaneous precipitation of tropocollagen when mixed with CS–protein at 4°

Limiting ratio of reactants (Lr). When the concentration of CS–protein is increased in the presence of a constant concentration of tropocollagen at 4° the E_{400} value increases until it reaches a limiting value as shown in Fig. 1(*a*). The ratio of the two reactants at this point is reproducible and may also be determined by plotting the E_{400} obtained when increasing concentrations of tropocollagen are added to a constant concentration of CS–protein (Fig. 1*b*). This ratio of tropocollagen to CS–protein is called 'the limiting ratio of reactants' or *Lr*.

CS–protein present in crude extracts of cartilage and CS–protein isolated from these extracts either by caesium chloride-density-gradient centrifugation or by precipitation with quaternary ammonium salts and chromatography on ECTEOLA-cellulose reacted with tropocollagen to give a turbidity (Fig. 1*b*). Acid-extracted tropocollagen from bovine skin, rat skin and guinea-pig skin granulomata, and neutral-salt-extracted tropocollagen from bovine skin, prepared by a variety of methods (see the Materials and Methods section), all gave a turbidity. The *Lr* values obtained varied from preparation to preparation of CS–protein (Fig. 1*b*) or tropocollagen, but were normally in the range 3–8. However, in some cases preparations of CS–protein that had been subject to repeated precipitation or to long periods of storage in the frozen state gave low *Lr* values.

Electron microscopy of precipitates. Precipitates obtained by reaction of CS–protein and tropocollagen at 4° after being washed three times by centrifugation and redispersal in buffer at 4° were prepared for examination in the microscope. Fibrils were observed singly or in small bundles with banding of periodicity 550–650 Å (Plate 1*a*). The fibrils varied in size, being 30–50 μ in length and 300–600 Å in width. The fibrils were relatively few in number compared with the thick network obtained by heating tropocollagen at 37°. However, their number was quite significant as no fibrils of this nature were observed in the control tropocollagen solutions prepared for examination in the same way. The large amount of background material in these micrographs was similar to micrographs of the control tropocollagen solution (Plate 1*b*).

Composition of pellets resulting from centrifugation of mixtures of tropocollagen and CS–protein at 4°. When mixtures of CS–protein and tropocollagen at *Lr* were centrifuged, the pellets were found to contain 62–69% of the total collagen (Table 1). The collagen remaining in the supernatants did not

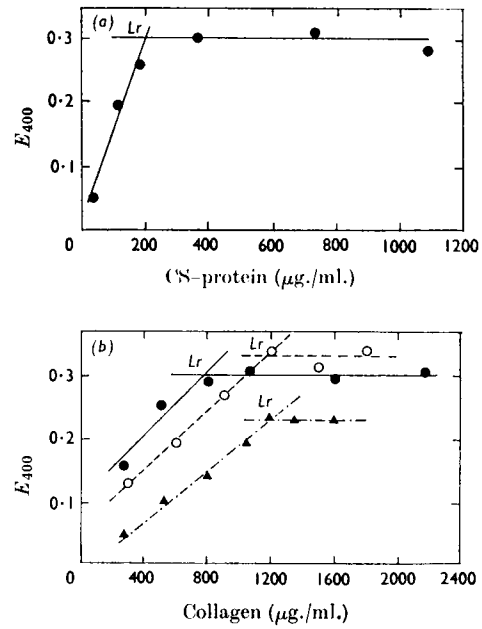


Fig. 1. Titration of CS–protein and tropocollagen. (*a*) Increasing concentrations of CS–protein (CsCl) were mixed with a constant concentration of tropocollagen (1200 μg./ml.), *Lr* 6·0. (*b*) Increasing concentrations of tropocollagen were mixed with constant concentrations of three different nasal-cartilage CS–protein preparations: ○, crude cartilage extracts (153 μg./ml.), *Lr* 7·5; ●, CS–protein (CsCl) (192 μg./ml.), *Lr* 3·8; ▲, CS–protein (QAS/ECTEOLA) (682 μg./ml.), *Lr* 1·8. See Table 3 and the Materials and Methods section for details of each CS–protein preparation.

Table 1. *Composition of pellets obtained by centrifugation at 4° of mixtures of tropocollagen and CS–protein at limiting proportions (Lr)*

Pellets were obtained as described in the Materials and Methods section. The original tropocollagen concentration was 1200 μg./ml. The concentration of CS–protein at *Lr* as determined by titration was: batch I, 200 μg./ml.; batch II, 320 μg./ml. TC, tropocollagen.

	% of total TC in pellet	% of total CS–protein in pellet	Ratio of TC to CS–protein in pellet
Batch I (i)	62	95	3·9
(ii)	69	97	4·3
Batch II	68	71	3·6

Table 2. *Composition of pellets obtained by centrifugation at 4° of mixtures of various proportions of CS–protein and tropocollagen*

Pellets were obtained as described in the Materials and Methods section. The original tropocollagen concentration was 1200 μg./ml. The concentration of CS–protein at *Lr* as determined by titration was: batch I, 200 μg./ml.; batch II, 320 μg./ml. TC, tropocollagen.

			Composition of pellet		
	Concn. of CS–protein	Turbidity of original mixture	CS–protein/ collagen	% of total in original mixture	
Batch	(μg./ml.)	(E_{400})	ratio	CS–protein	Collagen
I	Below *Lr*:				
	44	0·14	0·10	90–100	34
	174	0·28	0·26	90–100	56
	Above *Lr*:				
	267	0·34	0·34	96	62
	348	0·32	0·45	95	62
II	Below *Lr*:				
	32	0·08	—	67	—
	160	0·20	0·23	64	33
	At *Lr*:				
	320	0·30	0·28	71	68

show turbidity when more CS–protein was added. The results obtained for two batches of CS–protein obtained by caesium chloride-density-gradient centrifugation are shown in Table 1. From batch I 95%, and from batch II 71%, of the CS–protein was sedimented. To check that these proportions of CS–protein consistently reacted, increasing amounts of CS–protein (below and near to the *Lr* value so that the collagen was in excess) were added to a constant concentration of tropocollagen and the mixtures centrifuged. The percentage of CS–protein sedimenting was constant for each batch (Table 2). It was thus concluded that batch II contained CS–protein molecules that were unable to react with tropocollagen. When a preparation giving a very low *Lr* value, 1·8 (Fig. 1*b*), was tested under these conditions only 40% of the CS–protein sedimented, indicating that this preparation contained an even larger proportion of 'non-reactive' molecules than those above. However, most preparations consistently gave 80–100% of the CS–protein in the pellet.

When CS–protein was added at concentrations greatly in excess of that at *Lr* no more collagen was precipitated, but there was an increase in the amount of CS–protein in the precipitate. It was noted that there appeared to be an alteration in the physical structure of the precipitate such that it sedimented more slowly in the centrifugal field. By sampling the clear supernatant from the top portion of the centrifuge tube it was found that 95% of the CS–protein was consistently sedimenting even when the proportion of CS–protein to tropocollagen in the original mixture was four times that at *Lr*. The increase in the ratio of CS–protein to tropocollagen in the pellets with increase in original concentration of CS–protein occurred also at low concentrations of the latter (Table 2). It therefore seems likely that large amounts of the CS–protein were occluded within the sedimenting fibril network rather than bound stoicheiometrically to the tropocollagen. The slow sedimentation of the precipitate in the presence of excess of CS–protein would have been due to the altered physical properties of collagen networks in which protein–polysaccharide is entangled (Fessler, 1960*a*; Disalvo & Schubert, 1966).

Precipitation in physiological buffer and at 37°. The interaction described above also took place under more physiological conditions. An equivalent turbidity was obtained when tropocollagen was mixed with CS–protein at *Lr* in Krebs–Ringer bicarbonate buffer.

Similar turbidity was obtained also when small volumes of the two reactants were preincubated separately at 37° for 4 min. and mixed, and the E_{400} value was measured immediately. A control sample, in which preheated buffer instead of CS–protein was added to the tropocollagen solution, gave no extinction.

Effect of ionic strength on precipitation. When increasing concentrations of sodium chloride were added to tropocollagen and the solution was mixed with CS–protein at *Lr* in 8 mM-phosphate buffer, pH 7·3, at 4°, the turbidity decreased. No precipitate formed at sodium chloride concentrations above 0·18 M.

Specificity of the reaction between tropocollagen and various polysaccharide-containing preparations. AMPS preparations that give a turbidity on mixing

Table 3. *Composition and reaction with tropocollagen of various polysaccharide preparations*

Details of preparation for each compound are given in the Materials and Methods section. Abbreviations: QAS, prepared by quaternary ammonium salt precipitation; ECTEOLA, prepared by ECTEOLA-cellulose chromatography; CsCl, prepared by density-gradient centrifugation in CsCl; W, water extracts; S, salt extracts; KS, keratan sulphate. Compositions are calculated from detailed analyses based on dry-weight determinations in all cases except the cartilage extracts, the QAS preparation of heart-valve CS–protein and the skin and tendon dermatan sulphate–proteins, where they are based on protein/polysaccharide ratios, assuming these two components account for 100% of the preparation. The ability of the various preparations to react was determined by turbidity measurements (see the text). In each case a wide range of concentration (2–5 mg./ml.) was tested with 1200 μg. of tropocollagen/ml.

Preparation	Composition (%)		Reaction with tropocollagen
	Polysaccharide	Protein	
(*a*) CS–proteins			
Cartilage extracts	CS:59 KS:10	31	+
Cartilage (CsCl)	CS:69 KS:12	11	+
Cartilage (QAS/ECTEOLA)	CS:60 KS:10	15	+
Disk (QAS/ECTEOLA)	CS:38 KS:25	22	+
Heart valve (CsCl–W)	81	11	–
Heart valve (CsCl–S)	84	10	–
Heart valve (QAS–S)	79	21	–
(*b*) Dermatan sulphate–proteins			
Heart valve	32	53	+
Skin	32	68	+
Tendon	45	55	+
(*c*) Hyaluronate–proteins			
Heart valve	80	15	–
Synovial fluid	75	21	–
(*d*) Protein-free AMPS			
CS–KS	92	0	–
KS	95	0	–
Dermatan sulphate	96	0	–

with tropocollagen at 4°, and some that do not, are listed in Table 3. It appears that only sulphated AMPS–protein preparations react in this way since CS–protein from nasal cartilage and intervertebral disks and dermatan sulphate–protein from heart valves, skin and tendons gave a turbidity whereas preparations of hyaluronate–protein and protein-free AMPS did not. The precipitates obtained with CS–protein from cartilage and dermatan sulphate–protein from heart valves were examined in the electron microscope and found to contain 'native-type' collagen fibrils. There must be other specific requirements for the precipitation since the CS–protein preparations from cartilage and heart valves were isolated by identical procedures and both contained approx. 11% of protein (Table 3), yet only the former reacted. The cause of this difference has not yet been established; however, preliminary experiments (F. A. Meyer & D. A. Lowther, unpublished work) suggest that the heart-valve CS–protein is considerably smaller than that from cartilage.

Though several workers have shown that protein-free CS can precipitate tropocollagen in various forms (Gross, 1956; Kuhn & Zimmer, 1961) the present study shows that the intact macromolecule is necessary for precipitation of 'native-type' fibrils at neutral pH and physiological ionic strength.

Formation of collagen gels at 37° in the presence of CS–protein

Effect of CS–protein on the time-course of precipitation of collagen fibrils at 37°. When solutions of tropocollagen are incubated at 37° the changes in E_{400} with time follow a sigmoid course, with an initial lag and then a precipitation phase in which the extinction rises rapidly and after which turbidity remains constant. At this point almost all of the

tropocollagen is aggregated into fibres and can be sedimented on centrifugation (Bensusan & Hoyt, 1958; Gross & Kirk, 1958; Wood & Keech, 1960).

Only 62–69% of the tropocollagen was precipitated when mixed with CS–protein at 4° and therefore it seemed likely that two kinds of tropocollagen, which may differ in the conditions required for their aggregation into collagen fibrils, were present in the preparations. The precipitation of collagen at 37° was therefore examined: (*a*) after removal of the collagen fraction that aggregates at 4° in the presence of CS–protein, (*b*) in the presence of the precipitate formed at 4° and (*c*) under conditions where the initial precipitation at 4° is prevented from occurring (i.e. by raising the ionic strength; see above under 'Effect of ionic strength on precipitation').

CS–protein and tropocollagen were mixed at limiting proportions (*Lr*) at 4°, the precipitate was removed by centrifugation and the collagen content of the supernatant was assayed. The precipitation of fibrils at 37° from the supernatant was then compared with that from the original tropocollagen solution diluted to give the same concentration of tropocollagen. The time-course obtained (Fig. 2) showed the normal sigmoid shape; however, precipitation was considerably delayed and lag times obtained in a series of three experiments were five to ten times those obtained from the controls. The precipitation rate also was lower than for controls; however, it was difficult to measure accurately owing to the fibrous nature of the precipitate (see the next section and Plate 2). These observations, together with the fact that the supernatants do not give a precipitate on addition of further CS–protein at 4°, suggest that the original tropocollagen solution contains two fractions, differing both in their ability to react with CS–protein and their ability to form fibrils at 37°.

When the original mixtures at *Lr* were incubated at 37° without prior removal of the initial precipitate, the lag times obtained (i.e. subsequent to the initial precipitation) were considerably shorter than for the supernatants (Fig. 2). Thus it would appear that the initial precipitate accelerates the polymerization of that collagen which is unable to combine instantaneously with the CS–protein at 4°, and that the latter fraction of collagen is accreted to this initial precipitate at 37°. However, this could be stated with certainty only if the pellet removed by centrifugation at 4° contained only the fibrillar precipitate and no other associated or occluded collagen fraction.

To test the effect of CS–protein without the initial precipitation taking place the precipitation of fibrils from mixtures at *Lr* was also examined in 0·2M-sodium chloride, pH 7·3, at 37°. The time-courses showed lag times and precipitation rates almost identical with those obtained in the presence of the initial precipitate in 0·14M-sodium chloride, pH 7·3 (Fig. 2). It seems, then, that an interaction occurs in 0·2M-sodium chloride similar to that in 0·14M-sodium chloride, but in the former the interaction does not give rise to a precipitate at 4°. It is possible, then, that the interaction of CS–protein and tropocollagen in 0·14M-sodium chloride gives rise to both fibrils and smaller aggregates, both of which were removed by centrifugation at 4°.

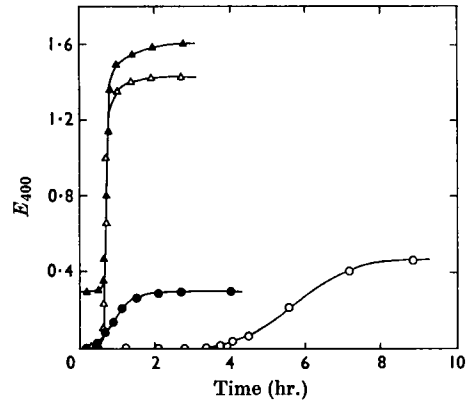
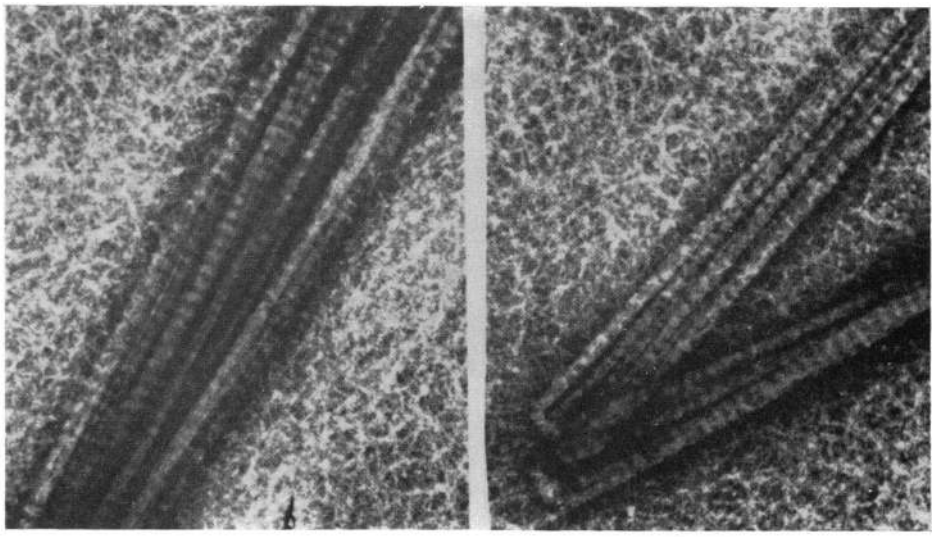


Fig. 2. Precipitation of collagen fibrils at 37° in the presence and absence of the initial precipitate formed at 4°. ▲, Tropocollagen (1200μg./ml.) plus CS–protein (200μg./ml.) at *Lr* in 0·14M-NaCl, i.e. in the presence of the initial precipitate; △, tropocollagen (1200μg./ml.) plus CS–protein (200μg./ml.) at *Lr* in 0·2M-NaCl, i.e. in the absence of the initial precipitate; ○, supernatant obtained after removal by centrifugation of the initial precipitate formed at 4° in the mixture of tropocollagen (1200μg./ml.) plus CS–protein (200μg./ml.) at *Lr* in 0·14M-NaCl (this supernatant contained 420μg. of collagen/ml. and 10μg. of CS–protein/ml.); ●, tropocollagen alone (420μg./ml.).

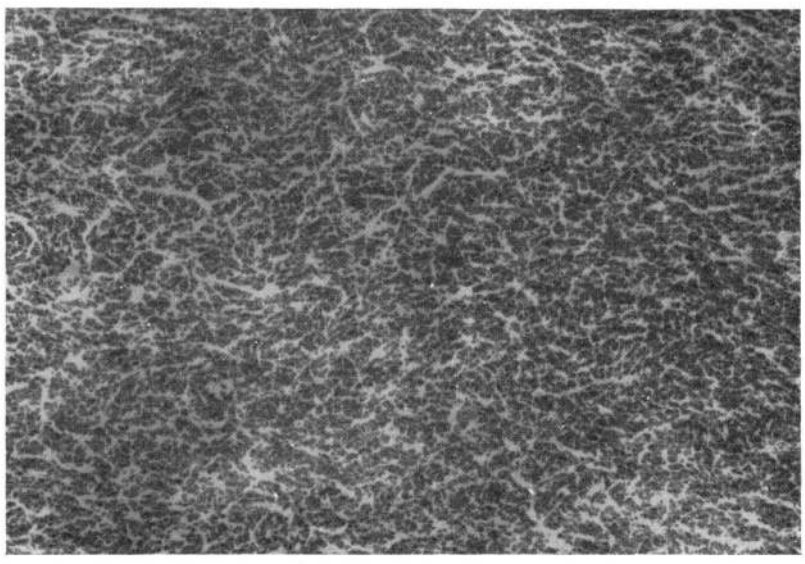
When the time-courses of precipitation at 37° of mixtures of CS–protein and tropocollagen in either 0·14M-sodium chloride or 0·2M-sodium chloride were compared with that given by tropocollagen alone at the same concentration, it was found that the lag time was increased markedly by the presence of CS–protein, though the growth rate was little affected. Fig. 3(*a*) shows a series of time-courses obtained with increasing concentrations of CS–protein and a constant concentration of tropocollagen in 0·14M-sodium chloride. As the concentration of the former increased up to the *Lr* value the lag time (subsequent to the instantaneous precipitation) increased accordingly (Table 4), but the growth rate was not affected significantly, indicating that the CS–protein may interact specifically with the tropocollagen fraction involved in the events of the lag phase.

When CS–protein was added to the tropocollagen

(*a*)

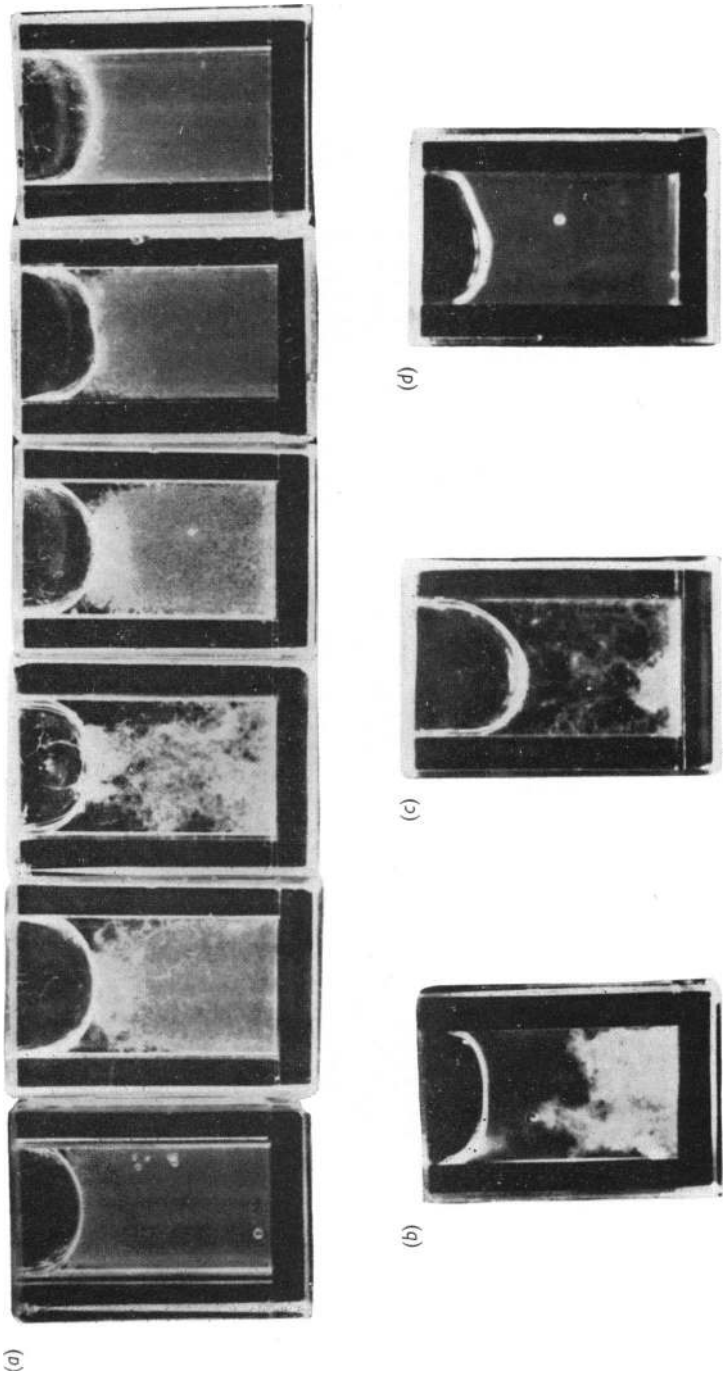


(*b*)



EXPLANATION OF PLATE 1

(*a*) Collagen fibrils formed from a mixture of tropocollagen and CS–protein at 4°. Magnification ×30000.
(*b*) Tropocollagen solution. Magnification ×38000.



EXPLANATION OF PLATE 2

Gross appearance of collagen gels obtained after incubation for 16hr. at 37°. (*a*) Tropocollagen (1200μg./ml.) plus a series of CS–protein concentrations, these being from left to right: 38μg./ml., 115μg./ml., 192μg./ml. (*Lr*), 384μg./ml., 768μg./ml., 1152μg./ml. (*b*) Tropocollagen (2900μg./ml.) plus dermatan sulphate–protein (700μg./ml.). (*c*) CS–protein plus collagen at *Lr* after removal of initial precipitate at 4°. (*d*) Tropocollagen alone (1200μg./ml.). Mixtures of collagen plus hyaluronate–protein or protein-free AMPS or mixtures of collagen and CS–protein in 0·2M-NaCl, pH7·3, are identical in appearance with this gel.

in excess of the *Lr* value, the lag time at 37° decreased in comparison with that at *Lr*, suggesting that the first stage of accretion of tropocollagen to the initial aggregate of CS–protein and tropocollagen was accelerated by excess of CS–protein. However, the precipitation rate was also slightly increased and excess of CS–protein may therefore accelerate accretion in both phases.

When a series of time-course studies similar to the above was performed in 0·2M-sodium chloride, the lag times at concentrations of CS–protein near and above the *Lr* value were almost identical with those in 0·14M-sodium chloride, despite the fact that for tropocollagen alone a longer lag time was obtained in 0·2M-sodium chloride than in 0·14M-sodium chloride (Table 4 and Fig. 3*b*). Thus the decrease in interaction between tropocollagen molecules that appears to result from raising the ionic strength (see Bensusan & Hoyt, 1958; Gross & Kirk, 1958; Wood & Keech, 1960) has not influenced the interaction of CS–protein and tropocollagen. At concentrations of CS–protein well below the *Lr* value the lag times in 0·2M-sodium chloride were longer than in 0·14M-sodium chloride, presumably owing to an additive effect of the interaction of CS–protein and tropocollagen and the decrease in interaction between 'free' tropocollagen molecules.

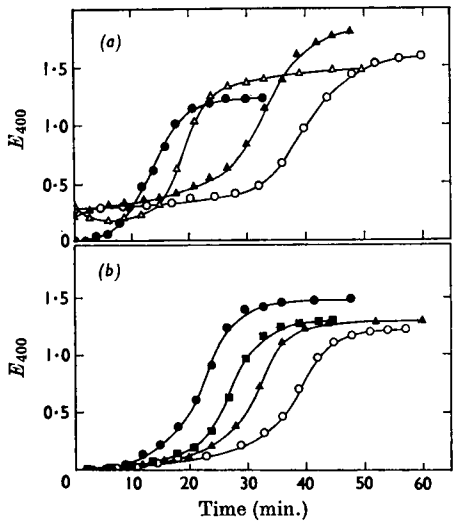


Fig. 3. Precipitation of collagen fibrils at 37° from mixtures of increasing concentrations of CS–protein and a constant concentration of tropocollagen. (*a*) In 0·14M-NaCl, i.e. in the presence of the initial precipitate at 4°; (*b*) in 0·20M-NaCl, i.e. in the absence of the initial precipitate at 4°. The collagen concentration was 1200 μg./ml. The concentration of CS–protein was: ●, zero; ▲, 115 μg./ml.; ○, 192 μg./ml. (*Lr*); ■, 768 μg./ml.; △, 1152 μg./ml.

Effect of CS–protein on the appearance of collagen gels formed at 37°. Plate 2 shows photographs of a series of gels obtained with different ratios of CS–protein and collagen. With increase in concentration of the former as far as *Lr* the gels became more fibrous in appearance. In the presence of excess of CS–protein the suspension appeared more homogeneous, but structured precipitates were still evident. These fibrous gels could be lifted out of the cuvettes as clots, leaving behind a large proportion of clear liquor. A mixture of tropocollagen and dermatan sulphate–protein incubated at 37° yielded gels with a similar fibrous appearance. Control experiments with solutions of collagen alone or collagen mixed with 'non-reactive' preparations (i.e. which gave no instantaneous precipitate at 4°), e.g. hyaluronate–protein or protein-free CS and dermatan sulphate (Table 3), were homogeneous, opaque and 'jelly-like' (Plate 2) (see also Gross & Kirk, 1958).

In the preceding section it was shown that the changes in turbidity at 37° of mixtures of tropocollagen and CS–protein were not significantly affected by preventing the initial instantaneous precipitation at 4° with 0·2M-sodium chloride. However, the gels formed by incubation of these mixtures in 0·2M-sodium chloride at 37° resembled those formed by collagen alone. They were homogeneous, opaque and firm. It therefore appears that the fibrous appearance of the gels depends on the initial precipitation.

Table 4. *Lag times obtained on incubating mixtures of tropocollagen and CS–protein at* 37° *in* 0·14M- *and* 0·20M-*sodium chloride*

Lag times were obtained from time-course graphs as described in the Materials and Methods section. The initial precipitate formed at 4° on mixing the components in 0·14M-NaCl does not form in 0·20M-NaCl. The concentration of tropocollagen was 1200 μg./ml. *Lr* as determined by titration occurred at approx. 200 μg. of CS–protein/ml.

Concn. of CS–protein (μg./ml.)	Lag time (min.) 0	38	77	115	192	288	384	768	1152
In 0·14M-NaCl	8	13	16	28	36	36	27	18	16
In 0·20M-NaCl	20	—	—	30	35	—	26	21	—

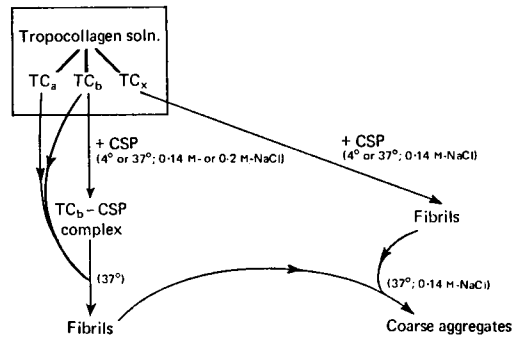
It was also stated in the preceding section that the collagen remaining in the supernatants obtained by centrifugation at 4° of mixtures of CS–protein and tropocollagen at *Lr* did not polymerize readily, and it was concluded that this collagen fraction was dependent on an initial aggregation of CS–protein and tropocollagen for rapid polymerization. When these supernatants were incubated at 37° it was also observed that a small amount of flocculent precipitate formed during the lag phase and appeared to constitute the locus for further precipitation in the precipitation phase (Fig. 2). This precipitate was similar in appearance to the initial precipitate obtained on mixing CS–protein and tropocollagen, and its final form at the completion of incubation was fibrous (Plate 2*c*). It appears, then, that the small amount of precipitate forming in the lag phase may have comprised the residual CS–protein in the supernatant (see Tables 1 and 2) and a small amount of collagen able to react with the CS–protein on heating at 37° for some time.

All of the gels formed were seen to contain a meshwork of collagen fibrils with 550–650 Å banding when examined in the electron microscope. However, no systematic study of the orientation or size of fibrils has been performed as yet.

DISCUSSION

Formation of collagen gels in vitro. The tropocollagen solutions used in this study were found to contain at least two fractions differing in their ability (*a*) to react with CS–protein (Table 1) and (*b*) to form fibrils at 37° (Fig. 2). Other workers have produced independent evidence for the existence of chemically different types of tropocollagen molecules (Fessler, 1960*b*; Piez, Lewis, Martin & Gross, 1961; Wood, 1962; Drake, Davison, Bump & Schmitt, 1966) as well as aggregates of tropocollagen (see Wood, 1964; Davison & Drake, 1966) in normal tropocollagen solutions.

Bensusan & Hoyt (1958) and Wood (1960*a*) performed kinetic analyses on the time-course of precipitation of collagen fibrils from solutions of purified tropocollagen and concluded that two distinct processes, which Wood (1960*a*) called 'nucleation' and 'growth', were involved. During the lag phase the 'nucleus-forming' fraction of tropocollagen (Wood, 1962) gave rise to small aggregates of 'nuclei', which were the sites for further accretion of the 'growth' tropocollagen during the precipitation phase and were necessary for the occurrence of the latter phase. The number and shape of the 'nuclei', which appeared to be influenced by protein-free CS (Wood, 1960*b*), determined the rate of subsequent growth and the diameter of the fibrils in the final gel. In the present study the time-course of precipitation of fibrils from



Scheme 1. Interactions between CS–protein and components of a tropocollagen solution. Abbreviations: TC_a and TC_b, 'growth' and 'nucleus-forming' tropocollagens respectively (Wood, 1964); TC_x, an aggregate of tropocollagen molecules; CSP, CS–protein. Further explanation of this Scheme appears in the text.

mixtures of tropocollagen and various amounts of CS–protein was compared with that obtained for tropocollagen alone at the same concentration. It was found that the initial aggregation of tropocollagen and CS–protein affected the lag time, but not the subsequent precipitation rate (Fig. 3). Thus the tropocollagen fraction that reacts instantaneously with CS–protein may contain the 'nucleus-forming' fraction described by Wood (1962, 1964), and an aggregate of CS–protein and tropocollagen appears to act as the nucleus for the precipitation of fibrils. The lag phase would represent the slow accretion of a tropocollagen coat to these CS–protein–tropocollagen nuclei and is followed by a rapid accretion of further tropocollagen in the precipitation phase. Excess of CS–protein was seen to accelerate this process (Fig. 3 and Table 4), probably owing to an effect on the charge density, shape or size of the nuclei. These factors would then determine the ease of attachment of further tropocollagen and the diameter of the final fibrils.

CS–protein was found to have the same effect on the lag times in 0·2 M-sodium chloride as in 0·14 M-sodium chloride, despite the fact that no initial precipitation occurred in the former (Fig. 3 and Table 4). It appears, then, that aggregates of CS–protein and collagen that acted as nuclei formed immediately in 0·2 M-sodium chloride, but did not form a precipitate. In view of the small number of fibrils formed instantaneously in 0·14 M-sodium chloride and of their relatively large diameter (300–600 Å) (Plate 1*a*) it seems unlikely that they were the nuclei, but that smaller aggregates also were formed in 0·14 M-sodium chloride and were the loci for the second stage of precipitation. On

centrifugation before incubation at 37° these aggregates must have sedimented to explain the relative inability of the collagen in the supernatant to give rise to fibrils (Fig. 2). This may have been due to their size or to occlusion or by electrostatic linkage to the fibrils.

Scheme 1 presents a possible explanation of the phenomena observed above. The two fractions of tropocollagen, TC_a and TC_b, are assumed to be 'growth' and 'nucleus-forming' tropocollagen respectively. TC_b reacts with CS–protein at 4° or 37° and the complex formed is the proposed 'nucleus' around which further TC_a and TC_b are accreted. It is also proposed that an aggregate of tropocollagen, TC_x, which may be, for example, the δ-component described by Veis, Anesey & Mussell (1967), may react with CS–protein to give rise to fibrils instantaneously. Mathews (1965) has shown previously that at neutral pH a gel is formed when CS–protein is mixed with a soluble polymerized form of collagen prepared by Veis (Veis, Anesey & Cohen, 1961).

Several workers, e.g. Weiss (1956), Meyer (1960) and Partridge (1948), have suggested that the AMPS could be involved in the ordering of collagen fibres. The present study indicates that certain AMPS–protein preparations promote the adhesion of fibrils into coarse bundles (Plate 2). The adhesion depends on the type of AMPS–protein, the relative proportions of AMPS–protein and tropocollagen, and the ionic strength of the buffer employed (Plate 2).

Fibrogenesis in vivo. Since there is considerable evidence that both AMPS–protein and tropocollagen are produced by connective-tissue cells (see Jackson, 1964; Green & Goldberg, 1965) and that the initial polymerization of tropocollagen into fibrils occurs in grooves or folds on the surface of these cells (Porter, 1964; Goldberg & Green, 1964), it is likely that similar events to those observed *in vitro* may occur also *in vivo*. Consequently the following sequence of events *in vivo* is proposed.

(*a*) The connective-tissue cell, which is mobile and sensitive to stresses in the tissue (Stearns, 1940; Porter, 1964), aligns itself in the appropriate direction.

(*b*) The cell secretes tropocollagen (Porter, 1964; Goldberg & Green, 1964) and AMPS–protein (Godman & Lane, 1964) into grooves or folds on the cell surface in an order and concentration suited to the specific requirements of the particular tissue.

(*c*) A series of interactions takes place at or near the cell surface giving rise to a gel comprising a network of fibrils, nuclei of tropocollagen and AMPS–protein, and further occluded AMPS–protein and tropocollagen. This network would act as a restrictive framework for the accretion of tropocollagen around the nuclei; the diameter and orientation of fibrils within the framework would be controlled by the shape and number of nuclei, which in turn are dependent on the type and concentration of AMPS–protein in and surrounding these aggregates.

(*d*) Those AMPS–protein complexes that react with tropocollagen (Table 3) will become associated with the insoluble fibres of the tissue, e.g. dermatan sulphate–protein in skin (Toole & Lowther, 1966) and heart valves (Lowther *et al.* 1967). Those AMPS–protein complexes that do not react with tropocollagen (Table 3) will be destined to remain in the interfibre matrix, e.g. hyaluronate–protein and CS–protein in heart valves (Lowther *et al.* 1967) and hyaluronate–protein in skin (Toole & Lowther, 1966). In cartilage, where a very large amount of CS–protein is secreted, part becomes associated with the fibres, part with the matrix (Schubert, 1966; Toole, Goh, Lowther & Baxter, 1965). The CS–protein in the matrix can then participate in interactions with polymerized forms of collagen, as suggested by Mathews (1965) and Disalvo & Schubert (1966), thus contributing to the mechanical properties (Milch, 1965) and permeability properties (Laurent, 1966) of the tissue.

Some of this work has been presented by B.P.T. in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Monash University. The authors are most grateful to Mr J. Humphrey for technical assistance, to Mr B. Veitch for the electron microscopy and to Mrs A. Goh, Miss E. Baxter, Mr F. A. Meyer and Dr B. N. Preston for gifts of protein–polysaccharide. This project was supported in part by the Australian Meat Research Organisation.

REFERENCES

Bensusan, H. B. & Hoyt, B. L. (1958). *J. Amer. chem. Soc.* **80**, 719.
Bitter, T. & Muir, H. (1962). *Analyt. Biochem.* **4**, 330.
Davison, P. F. & Drake, M. P. (1966). *Biochemistry*, **5**, 313.
Disalvo, J. & Schubert, M. (1966). *Biopolymers*, **4**, 247.
Drake, M. P., Davison, P. F., Bump, S. & Schmitt, F. O. (1966). *Biochemistry*, **5**, 301.
Fessler, J. H. (1960*a*). *Biochem. J.* **76**, 124.
Fessler, J. H. (1960*b*). *Biochem. J.* **76**, 452.
Franek, M. D. & Dunstone, J. R. (1966). *Biochim. biophys. Acta*, **127**, 213.
Gerber, B. R., Franklin, E. C. & Schubert, M. (1960). *J. biol. Chem.* **235**, 2870.
Godman, G. C. & Lane, N. (1964). *J. Cell Biol.* **21**, 353.
Goh, A. T. W. & Lowther, D. A. (1966). *Nature, Lond.*, **210**, 1270.
Goldberg, B. & Green, H. (1964). *J. Cell Biol.* **22**, 227.
Green, H. & Goldberg, B. (1965). In *Structure and Function of Connective and Skeletal Tissue*, p. 288. Ed. by Jackson, S. F., Harkness, R. D., Partridge, S. M. & Tristram, G. R. London: Butterworths Scientific Publications.

Green, N. M. & Lowther, D. A. (1959). *Biochem. J.* **71**, 55.
Gross, J. (1956). *J. biophys. biochem. Cytol.* **2** (Suppl.), p. 261.
Gross, J. & Kirk, D. (1958). *J. biol. Chem.* **233**, 355.
Harkness, R. D., Marko, A. M., Muir, H. M. & Neuberger, A. (1954). *Biochem. J.* **56**, 558.
Jackson, D. S. (1957). *Biochem. J.* **65**, 277.
Jackson, S. F. (1964). In *The Cell*, vol. 6, p. 387. Ed. by Brachet, J. & Mirsky, A. E. New York: Academic Press Inc.
Jackson, S. F. & Smith, R. H. (1957). *J. biophys. biochem. Cytol.* **3**, 897.
Kuhn, J. & Zimmer, E. (1961). *Z. Naturf.* **16**b, 648.
Kuhn, K., Fietzek, P. & Kuhn, J. (1966). *Biochem. Z.* **344**, 418.
Kuhn, K., Schuppler, G. & Kuhn, J. (1965). In *Structure and Function of Connective and Skeletal Tissue*, p. 64. Ed. by Jackson, S. F., Harkness, R. D., Partridge, S. M. & Tristram, G. R. London: Butterworths Scientific Publications.
Laurent, T. C. (1966). *Fed. Proc.* **25**, 1128.
Leach, A. A. (1960). *Biochem. J.* **74**, 70.
Lowther, D. A. (1963). *Int. Rev. conn. Tissue Res.* **1**, 63.
Lowther, D. A. & Baxter, E. (1966). *Nature, Lond.*, **211**, 595.
Lowther, D. A., Toole, B. P. & Meyer, F. A. (1967). *Arch. Biochem. Biophys.* **118**, 1.
Luscombe, M. & Phelps, C. F. (1967). *Biochem. J.* **103**, 103.
Mathews, M. B. (1965). *Biochem. J.* **96**, 710.
Mathews, M. B. & Lozaityte, I. (1958). *Arch. Biochem. Biophys.* **74**, 158.
Meyer, K. (1960). In *Molecular Biology*, p. 69. Ed. by Nachmanson, D. New York: Academic Press Inc.
Milch, R. A. (1965). *Monogr. Surg. Sci.* **2**, 261.
Muir, H. (1964). *Int. Rev. conn. Tissue Res.* **2**, 101.
Partridge, S. M. (1948). *Biochem. J.* **43**, 387.
Partridge, S. M., Davis, H. F. & Adair, G. S. (1961). *Biochem. J.* **79**, 15.
Piez, K. A., Lewis, M. S., Martin, G. R. & Gross, J. (1961). *Biochim. biophys. Acta*, **53**, 596.
Porter, K. R. (1964). *Biophys. J.* **4** (Suppl.), p. 167.
Porter, K. R. & Pappas, G. D. (1959). *J. biophys. biochem. Cytol.* **5**, 153.
Preston, B. N., Davies, M. & Ogston, A. G. (1965). *Biochem. J.* **96**, 449.
Revel, J. P. & Hay, E. D. (1963). *Z. Zellforsch.* **61**, 110.
Schubert, M. (1964). *Biophys. J.* **4** (Suppl.), p. 119.
Schubert, M. (1966). *Fed. Proc.* **25**, 1047.
Serafini-Cessi, F. & Cessi, C. (1964). *Analyt. Biochem.* **8**, 527.
Stearns, M. L. (1940). *Amer. J. Anat.* **67**, 55.
Toole, B. P., Goh, A. T. W., Lowther, D. A. & Baxter, E. (1965). *Proc. Aust. biochem. Soc., Adelaide*; in *Aust. J. Sci.* **28**, 90.
Toole, B. P. & Lowther, D. A. (1966). *Biochim. biophys. Acta*, **121**, 315.
Toole, B. P. & Lowther, D. A. (1967). *Biochem. biophys. Res. Commun.* **29**, 515.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1959). *Manometric Techniques*, p. 149. Minneapolis: Burgess Publishing Co.
Veis, A., Anesey, J. & Cohen, J. (1961). *Arch. Biochem. Biophys.* **94**, 20.
Veis, A., Anesey, J. & Mussell, S. (1967). *Nature, Lond.*, **215**, 931.
Weiss, P. (1956). *Proc. nat. Acad. Sci., Wash.*, **42**, 819.
Wood, G. C. (1960*a*). *Biochem. J.* **75**, 598.
Wood, G. C. (1960*b*). *Biochem. J.* **75**, 605.
Wood, G. C. (1962). *Biochem. J.* **84**, 429.
Wood, G. C. (1964). *Int. Rev. conn. Tissue Res.* **2**, 1.
Wood, G. C. & Keech, M. K. (1960). *Biochem. J.* **75**, 588.