3. Yields of β_2 -lactoglobulin may be improved by crystallizing it in the orthorhombic rather than the monoclinic form.

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Connective Tissue Growth Stimulated by Carrageenin

1. THE FORMATION AND REMOVAL OF COLLAGEN

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Although connective tissue growth has been studied intensively by histological methods (for review see Cameron, 1952) and to a lesser degree by electron-optical methods, very few biochemical studies are available in the literature. Kodicek & Loewi (1955) have studied the granulation tissue in regenerating tendon, and Dunphy & Udupa (1955) have reported on a combined biochemical and histological study of connective tissue formation in healing wounds. In neither of these studies was any attempt made to fractionate the collagen into the three types known to exist in developing connective tissue. The paucity of information is probably due to the fact that the amount of new tissue formed in a given period in normal circumstances is small and cannot be separated from pre-existing tissue. Where the new tissue is separable, as for example in healing wounds, the amount of tissue formed is insufficient for detailed biochemical studies. Recently, however, Robertson & Schwartz (1953) have reported a method of producing comparatively large amounts of easily separated connective tissue. This method consisted of injecting a solution of carrageenin subcutaneously into the lower abdominal regions of guinea pigs. Carrageenin is a sulphated polygalactose which is extracted from the seaweed Irish moss (*Chondrus crispus*). Here again, however, no attempt was made to fractionate the collagen.

Quantitatively, collagen is the most important constituent of connective tissue, in which three forms of this protein are known to exist. One form, referred to as acid-soluble collagen, can be extracted either with dilute acetic acid (Zachariades, 1900;

Nageotte, 1927; Leplat, 1933) or by acid buffers (Tustanovskii, 1947). The second form was isolated by Highberger, Gross & Schmitt (1951) from skin by extraction with slightly alkaline phosphate buffers and more recently with neutral salt solution (Gross, Highberger & Schmitt, 1955; Jackson & Fessler, 1955). This fraction has been called 'alkali-soluble collagen' (Harkness, Marko, Muir & Neuberger, 1954) or 'neutral-salt-soluble collagen' (Jackson & Fessler, 1955) and will be referred to in this communication by the latter name. The bulk of the collagen in connective tissue is insoluble but can be rendered soluble by transformation into gelatin.

It has been suggested that acid-soluble collagen is the precursor of insoluble collagen because its concentration in connective tissue decreased with age and because [α - 14 C]glycine was incorporated more rapidly into acid-soluble collagen than into insoluble collagen (Orekhovitch, 1952, 1955). However, Harkness *et al.* (1954) showed that [α - 14 C]glycine is incorporated most rapidly into collagen soluble in neutral salt solution, indicating that this was the true precursor, and further that insoluble collagen need not pass through the acid-soluble stage. It has also been suggested (Bowes, Elliott & Moss, 1953, 1955) that insoluble collagen is a complex of acid-soluble collagen and a mucoprotein of high tyrosine content. That there is no sharp division between acid-soluble and insoluble collagen was indicated by the increased solubility in acid of tendon treated with hyaluronidase (Jackson, 1953).

Robertson & Schwartz (1953) reported that as much as 10 g. of relatively homogeneous tissue could be obtained by their technique, and it seemed likely that this offered a good opportunity to investigate the occurrence of the three collagen fractions enumerated above during the development of new connective tissue. The incorporation of [α - 14 C]glycine into these fractions was also studied. Preliminary communications on part of this work have already been made (Jackson, 1956*a, b*).

EXPERIMENTAL

Treatment of animals. Male and female albino guinea pigs (National Institute for Medical Research stock) were used throughout. Their weights at the time of injection varied between 600 and 700 g. The animals were fed on diet no. 18 (Bruce & Parkes, 1946), hay and greens. Ascorbic acid was supplemented by giving orally, every other day, 50 mg. dissolved in 10% (w/v) sucrose as suggested by Robertson & Schwartz (1953).

A portion (5 ml.) of a 1% (w/v) solution of carrageenin in 0.9% (w/v) NaCl was injected subcutaneously into the lower abdominal region, under ether anaesthesia. After 5 days, 50 μ C of [α - 14 C]glycine in 1 ml. of 0.9% NaCl was injected intraperitoneally into each animal; 625 μ C of Na₂³⁵SO₄ in 1 ml. of 0.9% (w/v) NaCl was also injected at the same time.

The animals were killed in groups of three by ether inhalation, and a 2 mm. strip was cut out longitudinally through the skin, new connective tissue (granuloma) and abdominal muscle, and fixed for histological examination. The skin was then peeled back from the ventral surface and the mass of new tissue separated from the abdominal and chest wall, macerated with scissors, weighed and divided for collagen and mucopolysaccharide studies. The results of the latter are reported in a separate communication (Slack, 1957).

Isolation of collagen fractions

All extractions were carried out at 2° with octan-2-ol as preservative. The main steps in the fractionation procedure are shown in Fig. 1.

Neutral-salt-soluble collagen. The macerated tissue was suspended in 10 times its weight of 0.2M-NaCl (pH 7.4). The extraction was continued for 24 hr., with occasional stirring, and the mixture centrifuged for 30 min. at 1500 g. The supernatant was decanted through filter paper and re-centrifuged at 12 000 g for 30 min.

The procedure was repeated three times and the extracts were combined. Neutral-salt-soluble collagen was precipitated by addition of NaCl to a final concentration of 20% (w/v). The precipitate formed was removed by centrifuging at 12 000 g for 30 min. The clear supernatant was dialysed against frequent changes of water for several days, and insoluble material filtered off, and the solution made up to a known volume. A portion was freeze-dried and examined by filter-paper electrophoresis at pH 8.6 by the method of Durrum (1950). The remainder was evaporated to dryness, the residue being designated 'water-soluble protein' (Fig. 1, fraction 1). The solid obtained by salt precipitation was purified by the method previously described (Jackson & Fessler, 1955; Jackson, Neuberger, Leach & Jacobs, unpublished data) and the product was

designated 'purified neutral-salt-soluble collagen' (Fig. 1, fraction 2*a*). The material normally discarded during purification was retained for determination of total neutral-salt-soluble collagen (Fig. 1, fraction 2*b*).

Acid-soluble collagen. To the residue remaining after extraction with 0.2M-NaCl was added 10 times its volume of 0.2M sodium citrate buffer, pH 3.5, and the extraction was continued for 24 hr. with occasional stirring. The mixture was centrifuged for 30 min. at 1500 g and filtered. The extraction was repeated three times and the extracts were combined and made up to a known volume. One-fifth of the total volume was evaporated to dryness and the residue used for determination of total acid-soluble collagen (Fig. 1, fraction 3*b*). To the remainder NaCl was added to a final concentration of 5% (w/v). The fibrous precipitate obtained was removed by centrifuging at 1500 g for 30 min. and redissolved in 0.01N acetic acid, forming a clear viscous solution which was dialysed against water. The precipitate formed was further purified by repeating the procedure, the final precipitate being dried *in vacuo* over P₂O₅ and designated 'purified acid-soluble collagen' (Fig. 1, fraction 3*a*).

Insoluble collagen. The residue remaining after citrate extraction was extracted twice with 0.1N-NaOH (Lowry, Gilligan & Katersky, 1941), and washed on the centrifuge three times with water. The material was then autoclaved twice at a pressure of 15 lb./in.² in the presence of 25–50 ml. of water. The gelatin solutions obtained were combined and made up to a known volume. A measured fraction was taken and evaporated to dryness, the residue being used for determination of total insoluble collagen (Fig. 1, fraction 4*b*). The remainder was filtered hot through a clarifying filter (Ford's Sterimat F.C.B.). Trichloroacetic acid was added to a concentration of 5% (w/v) and the small amount of precipitate obtained after standing at 0° for several hours was removed by filtration. The clear filtrate was concentrated and poured into 10 vol. of acetone, allowed to stand at 0° overnight, and the precipitate was washed with ether and dried over P₂O₅. This material was designated 'purified insoluble collagen' (Fig. 1, fraction 4*a*).

The residue obtained after autoclaving was negligible and was discarded.

Chemical analysis

The collagen fractions were hydrolysed by heating in a sealed tube with 5.7N-HCl for 24 hr. at 100° in refluxing water. The hydrolysates were filtered and evaporated to dryness *in vacuo* over P₂O₅ and solid NaOH. The hydrolysates of the fractions 1, 2*a*, 2*b*, 3*a*, 3*b*, 4*a* (Fig. 1) were dissolved in water and made up to a known volume. Samples (1 ml.) were used to determine the hydroxyproline content by the method of Neuman & Logan (1950). Collagen was estimated by assuming a value of 13.6% hydroxyproline for all the collagen fractions (Bowes *et al.* 1955). The remainder of fractions 1 and 2*a* and the hydrolysates from the purified fractions were used to isolate 2,4-dinitrophenylglycine for radioactivity measurements.

Isolation of 2,4-dinitrophenylglycine, and radioactivity measurements. The preparation and isolation of 2,4-dinitrophenylglycine was done by the method of Perrone (1951) as described by Neuberger, Perrone & Slack (1951). The solution of 2,4-dinitrophenylglycine obtained was made up to 25 or 50 ml. and the compound was estimated colorimetrically (Kröl, 1952) with a Unicam spectrophotometer (wavelength 460 m μ). The solution was evaporated to dryness

Fig. 1. Scheme of fractionation of connective tissue proteins. All operations were carried out at 2°.

Table 1. *Variation with time, after injection of carrageenin, of the concentration of the collagen fractions expressed in mg./100 g. wet wt. of tissue*

Time (days)	Wet wt. of tissue* (g.)	mg./100 g. wet wt. of tissue				
		Total collagen	Neutral-soluble		Acid-soluble	Insoluble
			Total	Water-soluble		
3	19.4	14.9	5.5	4.7	0.2	9.2
5	37.9	74.3	18.8	9.7	11.2	44.3
6	56.7	189.3	15.5	6.0	18.2	155.6
7	70.2	333.2	18.9	9.2	28.9	285.5
9	25.5	857.7	58.9	20.3	86.9	711.9
14	23.5	986.1	103.3	41.2	115.1	767.7
21	8.7	1068.3	159.0	58.5	155.2	754.1
28	4.7	717.0	136.5	55.0	148.6	431.9

* Total from three guinea pigs.

in vacuo and the radioactivity measured on solid samples of 'infinite thinness' as in the method of Henriques, Henriques & Neuberger (1955).

RESULTS

Development of the connective tissue

The development of the connective tissue following injection of carrageenin was similar to that described by Robertson & Schwartz (1953). The wet weight (Table 1) increased rapidly up to the seventh or ninth day and then fell quite rapidly until after 6 weeks nothing remained but a thin layer of fatty tissue. Histological examination showed that there was a steady infiltration of fibroblasts and other cells from the subcutaneous tissue and from the muscle wall into the layer of carrageenin gel formed after injection. Extracellular carrageenin disappeared after about 10 days. At 3 days no fibres were visible but from 5 days onwards increasing numbers of non-orientated fibres appeared, including a proportion of thin argyrophilic fibres. From about the fourteenth day until the tissue disappeared completely the fibrous material was gradually replaced by fat. A detailed account of the histological findings will be presented elsewhere (Williams, 1957).

Determination of collagen fractions

All three collagen fractions were found, namely: neutral-salt-soluble, acid-soluble and insoluble, and in addition a previously undescribed fraction containing hydroxyproline was found in the water-soluble proteins. The total collagen concentration (Table 1) increased slowly at first and more rapidly between the sixth and ninth days. Maximum concentration was reached at 14 days, after which the concentration fell off. However, since the wet weight decreased from days 7 to 9 onwards the absolute maximum amount of collagen was present about the ninth day.

Table 2. *Relative proportions of collagen fractions expressed as percentage of total collagen*

Time (days)	Percentage of total collagen			
	Neutral-soluble			Insoluble
	Total	Water-soluble	Acid-soluble	
3	38.4	30.7	1.6	60.0
5	25.3	13.0	15.0	59.7
6	8.4	3.3	9.6	82.0
7	4.5	2.2	6.9	88.6
9	6.8	2.3	10.1	83.1
14	10.5	4.2	11.7	77.8
21	14.9	5.4	14.5	70.6
28	19.0	7.7	20.7	60.3

At 3 days neutral-salt-soluble collagen appeared (Table 1). The concentration of this fraction increased up to the fifth day and remained constant for 2-4 days before rising again coincident with the decrease in the wet weight of tissue. At 3 days the major proportion of the neutral-salt-soluble collagen was not precipitated by salt or by dialysis. This water-soluble hydroxyproline-containing material was present throughout the period of the experiment, the concentration-time curve being parallel to that for the total neutral-salt-soluble collagen (Table 1). The concentration of acid-soluble collagen, although low at day 3, increased throughout the time of the experiment. The concentration of insoluble collagen also increased steadily up to 14 days, the most rapid increase being between the fifth and ninth day. After 14 days its concentration decreased steadily.

In Table 2 the results are expressed as percentage of total collagen, showing the relative proportions of the three collagen fractions. As insoluble collagen accumulates the relative proportions of the soluble collagens decrease until the weight of tissue begins to decrease, when an increasing proportion of the total collagen is found to be in a soluble form.

Isotope experiments

The highest specific activity was found in the neutral-salt-soluble collagen. Two hours after injection its activity was already high and reached a maximum between 8 and 12 hr. (Fig. 2). The activity had fallen rapidly by 24 hr. and more slowly thereafter, substantial activity still remaining after 16 days.

The activity of acid-soluble collagen was low at 2 hr. and rose rapidly to a maximum at 12 hr., considerably below the maximum activity of neutral-salt-soluble collagen. The activity-time curve for insoluble collagen was a little lower than that for acid collagen and reached a maximum after 24 hr. Activity had fallen after 48 hr., and after levelling out continued to fall from the fourth day on.

There was some variation in the activities of comparable fractions at comparable times, in experiments carried out at different times, but the activities of the different fractions relative to each other remained the same. Similar findings have been reported in the collagen fractions from rabbits injected intraperitoneally with [α - 14 C]-glycine (Harkness *et al.* 1954).

DISCUSSION

The results presented here confirm the findings of Robertson & Schwartz (1953) that carrageenin injected into guinea pigs stimulates the formation of connective tissue, but differ in detail. These authors found that maximal amounts of tissue were obtained after 14 days and that resorption began after 3 weeks. In the present study maximal amounts of tissue were obtained after 7-9 days, resorption occurring immediately after the tissue had reached its maximum weight (Table 1).

The concentration-time curve of the total collagen content of the tissue (Table 2) is very similar to that reported by Dunphy & Udupa (1955) in healing wounds. These authors suggested that when only small amounts of collagen were produced, only the polysaccharide of the amorphous matrix and the soluble-collagen precursor were produced, the normal collagen fibres appearing rapidly thereafter. In regenerating tendon the concentration of collagen did not begin to rise until the eighth day and continued to rise steadily for over 28 days (Kodicek & Loewi, 1955).

Collagen fractions

The detailed analysis of the tissue (Fig. 1) showed the presence of all three collagen fractions known at present. Of the total collagen present on the third day 40% was neutral-salt-soluble, practically the whole of the remainder being insoluble. Since no

fibres were visible on histological examination, it is likely that no new insoluble collagen had been formed at this stage of development, the small amount found probably being derived from contamination with collagen from pre-existing loose fasciae. Thus it is probable that at this stage of development only neutral-salt-soluble collagen had been produced and that this fraction is the precursor of insoluble collagen as suggested by Harkness *et al.* (1954), since it is essentially the same fraction as the alkali-soluble collagen of these authors (Jackson & Fessler, 1955).

The finding that a proportion of the neutral-salt-soluble collagen was water-soluble was unexpected, since negligible amounts of hydroxyproline-containing material were found in the same fraction from the skins of young rabbits (Harkness *et al.* 1954; Jackson, unpublished results). The significance of this finding is not clear, but it is possible either that this material represents an earlier stage of collagen synthesis or that it is a lower-molecular-weight fraction of neutral-salt-soluble collagen which is known to aggregate in solution to varying degrees (Jackson & Fessler, 1955). The presence of haemoglobin makes it difficult to interpret the electrophoretic pattern and it is being further investigated.

The concentration of insoluble collagen increases with time up to 14 days. Since the wet weight of tissue decreases after the seventh to ninth day, i.e. the tissue is breaking down and is being reabsorbed, synthesis and breakdown must be taking place simultaneously at least from the seventh day. It is possible that both take place simultaneously from the beginning but that synthesis predominates at first and breakdown later. It has been found that carrageenin injected intradermally causes a rapid breakdown of the dermal-collagen fibres without preceding granuloma formation (Williams, 1957). The increase in concentration of both the soluble fractions which occurs concurrently with the decrease in wet weight suggests that the breakdown of insoluble collagen occurs first by a disaggregation into the units which make up mature collagen fibres producing acid and neutral-salt-soluble collagen, rather than by destruction of the constituent polypeptide chains, although this will probably follow disaggregation. However, in normal connective tissue development, the mature collagen fibres are stable and metabolically relatively inert (Neuberger *et al.* 1951). Hence the insoluble collagen produced by the method described here may be inherently unstable. However, in view of the results reported by Williams (1957) it seems more likely that carrageenin first stimulates the formation of normal insoluble collagen and then stimulates some mechanism which leads to its removal.

A comparison of the concentration-time curves of total bound sulphate (Slack, 1957) and total collagen shows that as the concentration of collagen increases, the concentration of bound sulphate decreases. This is compatible with histological evidence that during the development of new connective tissue there is first an accumulation of an amorphous matrix which, on the basis of its staining properties with toluidine blue, is believed to contain sulphated polysaccharides (Wolbach & Howe, 1926). As fibres appear, this matrix gradually disappears (Stearns, 1940*a*). Similar findings were reported by Dunphy & Udupa (1955), who compared the concentration-time curves for hexosamine and hydroxyproline in healing wounds.

Isotope experiments

The results are in complete agreement with the suggestion put forward by Harkness *et al.* (1954) that neutral-salt-soluble collagen and not acid collagen as suggested by Orekhovitch (1952) is the true precursor of insoluble collagen. The more recent work of Orekhovitch (1955), reiterating his belief that acid collagen is the true precursor, is invalidated because the method of extraction used would produce a mixture of the two soluble collagens; likewise the activity-time curves obtained would be the resultant of the curves for the two separate fractions.

Harkness *et al.* (1954) also suggested that acid-soluble collagen and insoluble collagen are not distinct groups of proteins but are both obtained from fibres deposited outside the cell. They also express the opinion that it is unnecessary for all the insoluble collagen to pass through the acid-soluble stage. The activity-time curves obtained in the present study support these ideas, since those of acid-soluble collagen and insoluble collagen are very similar and suggest that [α - 14 C]glycine is incorporated simultaneously into both fractions.

There are some differences in detail between the present study and that of Harkness *et al.* (1954). The rate of incorporation of [α - 14 C]glycine into the neutral-salt-soluble collagen is more rapid, maximum incorporation occurring at about 10 hr. as compared with 24 hr. for the same fraction from rabbit skin. The activity-time curves for acid-soluble collagen and insoluble collagen are also different in the two studies, maximum incorporation into acid-soluble collagen occurring in about 12 hr. as compared to 3 days for rabbit skin, and into insoluble collagen in 24 hr. as compared to 8 hr. These differences may be due to species difference or more probably to the higher rate of growth of the connective tissue stimulated by carrageenin.

Harkness *et al.* (1954) believed that the comparatively high activities which they obtained for insoluble collagen at 8 and 24 hr. were due to

contamination with non-collagen protein of high activity. In the present study this difficulty did not arise, the activity of insoluble collagen being low at 2 hr., when the activity of the neutral-salt-soluble collagen was already very high (Fig. 2).

The persistence of radioactivity in the soluble collagens for as long as 16 days (Fig. 2) is probably due to the breakdown of insoluble collagen. If, as is suggested above, acid-soluble collagen and neutral-salt-soluble collagen are produced during breakdown, then these fractions would contain a component derived from insoluble collagen into which [α - 14 C]glycine had been previously incorporated, and a component produced by synthesis involving inert glycine. The fact that the activity-time curves of all three collagens are very similar from the seventh to the twenty-eighth day after carrageenin injection would support this suggestion.

The morphological origin of insoluble collagen is well known, but the origin of the two soluble fractions is less clear. Harkness *et al.* (1954) suggest that acid-soluble collagen is derived from collagen recently laid down: the outer layers of larger fibres and the thin fibrils which are prevalent in developing connective tissue (Gross, 1950) and which are argyrophilic and known as reticular. Preliminary experiments (Jackson & Williams, unpublished results) involving histological examination of connective tissue stimulated by carrageenin after the various stages of extraction (Fig. 1) have been carried out. After extraction with 0.2 M-NaCl, the thin argyrophil fibres no longer took up silver, but stained red with the van Gieson stain as do the thick mature fibres. Thus the argyrophil properties of these thin fibrils may be due to a coating of neutral-salt-soluble collagen not yet incorporated into the fibre structure, although

Fig. 2. Specific activity-time curves of collagen fractions from connective tissue. Δ , Neutral-salt-soluble collagen; $\circ$, acid-soluble collagen; $\times$, insoluble collagen.

other proteins which are removed by neutral-salt solutions, could be the responsible factors. After citrate extraction the argyrophil fibres were almost completely absent, suggesting that these are in fact acid-soluble collagen and hence different from basement-membrane reticulin (Kramer & Little, 1953; Windrum, Kent & Eastoe, 1955). The absence of microscopically visible fibres 3 days after carrageenin injection, when neutral-salt-soluble collagen is present (Table 1), suggests that this fraction also occurs in the non-fibrillar part of connective tissue.

It cannot be decided from the present study whether any of the neutral-salt-soluble collagen isolated was originally intracellular. Studies by Porter & Vanamee (1949), using the combined techniques of tissue culture and electron microscopy, have shown that early fibrils are formed at the cell surface, and may be clearly connected with intracellular fibroglial fibres. These early fibrils reach a mature form extracellularly by accretion of material from the ground substance.

These findings have been confirmed (Jackson, 1954*a, b*) in tissue culture of embryonic bone. It was not possible to decide whether the fibroglial fibres were actually intracellular (Jackson & Smith, 1955). However, collagen fibres fail to develop in tissue culture in the presence of anti-collagen serum (Robbins, Watson, Pappas & Porter, 1955), forming instead amorphous masses having the tinctorial properties of collagen; these are probably antigen-antibody complexes. This would suggest that a non-fibrous precursor is secreted by the cell, forming fibrous collagen extracellularly. Cytoplasmic granules in connective tissue cells have been put forward as possible precursors of both amorphous matrix and collagen fibres (Jackson & Smith, 1955). Stearns (1940*a, b*) concluded on the basis of her observations of fibre formation in a transparent chamber in the rabbit ear that these granules secreted a soluble precursor into the extracellular space, where this material was transformed into a fibrous form. It has recently been shown that neutral-salt-soluble collagen can precipitate spontaneously into typical collagen fibres (Jackson & Fessler, 1955). It would seem probable that intracellular collagen is non-fibrous and is secreted into the extracellular spaces, from which it can be extracted as a neutral-salt-soluble collagen.

On the evidence available a tentative scheme for the formation of collagen in developing connective tissue may be put forward. Neutral-salt-soluble collagen (or an intracellular precursor) is secreted into the extracellular space by the fibroblast. This type of collagen is then transformed into fibrillar collagen in various ways:

(1) By forming new fibrils which are argyrophilic, which behave as acid-soluble collagen.

(2) These fibrils accumulate more neutral-salt-soluble collagen to increase their length and diameter and become insoluble collagen. Banfield (1955) has shown in electron-microscope studies in normal developing connective tissue and in granulation tissue, that the width and length of collagen fibres increase with age.

(3) By direct incorporation into pre-existing insoluble collagen fibres, further increasing their length and diameter.

The first two ways would be the most important in the early stages of development, whereas the last would predominate in the later stages.

The reason for the final insolubility of mature collagen is still obscure. Bowes *et al.* (1953, 1955) have suggested that it is due to the interaction of acid-soluble collagen with a non-collagenous protein. Recently these authors have suggested that this protein may even form an integral part of the collagen fibres (Bowes, Elliott & Moss, 1956). Since neutral-salt-soluble collagen is probably the precursor and can apparently add on directly to insoluble collagen, the latter suggestion seems more likely. The increasing insolubility of collagen with age might then be due to increased aggregation of collagen molecules, as was suggested by Harkness *et al.* (1954).

Despite the complexity introduced by the breakdown of the connective tissue formed under the stimulus of carrageenin, the phenomenon described would appear to offer a basis for studies of the factors which influence the formation of the various components of connective tissue and, what may be more important from the pathological point of view, the factors influencing its breakdown, connective tissue breakdown being a feature of many connective tissue diseases.

SUMMARY

1. Subcutaneous injection of carrageenin into guinea pigs stimulates the formation of new connective tissue which is resorbed after 4-6 weeks.

2. The change in concentration with time of neutral-salt-soluble collagen, acid-soluble collagen and insoluble collagen, and the incorporation of [α - 14 C]glycine into these collagens, have been studied.

3. Maximum wet weight of new tissue is produced between the seventh and ninth day and disappears after 4-6 weeks, although the total concentration of collagen in the tissue does not reach a maximum until the fourteenth day.

4. Most of the insoluble collagen is formed between the fifth and ninth day. Neutral-salt-soluble collagen appears before acid-soluble collagen, the concentration increasing up to the fifth day, remaining constant and then increasing rapidly as the tissue is resorbed. The concentration of acid-

soluble collagen increases throughout the experimental period.

5. The highest specific activity is found in neutral-salt-soluble collagen, maximum activity occurring at 10 hr., with a half-life of 18–20 hr. The activity–time curves of acid-soluble collagen and insoluble collagen are similar, maximum activity occurring at 12 and 24 hr. respectively and being very much lower than the activity of neutral-salt-soluble collagen.

6. It is concluded that neutral-salt-soluble collagen is the precursor of collagen fibres and can either be transformed into acid-soluble collagen or add on to pre-existing acid-soluble collagen and insoluble collagen.

7. A water-soluble protein containing hydroxyproline has also been found.

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The Stabilization of Purified Human Albumin to Heat

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Scatchard, Gibson, Woodruff, Batchelder & Brown (1944) recorded changes in the turbidity and viscosity caused by heating solutions of purified human albumin at temperatures up to 57°. They found that at pH 6.8 a 25% solution of albumin in 0.3M sodium chloride could be heated at 57° for 10 hr. without demonstrable change in turbidity.

The clinical need for albumin solutions low in salt concentration which could be sterilized by heat led to the studies of Ballou, Boyer, Luck & Luen (1944) on the effect of organic ions on the thermal stability of albumin. They found, using nephelometric methods, that caprylate, phenylacetate and acetyltryptophan improved the thermal stability of