On the Significance of the Extractable Collagens*

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ABSTRACT

This investigation has sought to determine the significance of the wide range of extractable collagen fractions which appear to exist in growing connective tissues and to determine their position in the process of fibrogenesis.

Carrageenin granulomata were induced in guinea pigs and, after injection of ^{14}C-glycine, this tissue and skin from the same animal were subjected to successive extractions with neutral salt solutions of increasing ionic strength, citrate buffer pH 3.6, and to gelatinization. The specific activity of these fractions was determined at various time intervals. At 8 hours it was found that the specific activity decreased with increasing ionic strength of the neutral salts and was still lower in the citrate extracts and gelatin. At 36 hours the situation was almost completely reversed except that the citrate extract and gelatin still had the lowest activities. The data from skin were more clear cut than that from the granuloma and the reasons for this are discussed.

It is concluded that at any given time in developing connective tissue, there is a continuous spectrum of collagen aggregates of varying degrees of strength of cross-linkage, dependent upon the time that has elapsed since their constituent molecules were synthesized. The various extraction media used remove a particular cross-section of these aggregates depending upon their disaggregating power. These extracts will thus be biologically heterogeneous. The fraction extracted with 0.14 M NaCl will contain the collagen molecules most recently synthesized and in this respect can be considered the earliest form of extracellular collagen.

INTRODUCTION

There are now many methods available to get collagen into solution and this has led to a plethora of terms to describe the various fractions thus isolated. Nageotte (23) first obtained highly viscous solutions of collagen by extracting rat tail tendon with very dilute solutions of organic acids, which he named pré-collagène. Later Orekhovitch and his coworkers (28) obtained similar solutions by extracting various tissues with acid citrate buffers and called the collagen thus extracted "procollagen." Other collagen fractions have been extracted with phosphate buffer pH 7.4, (16, 15) 0.2 M NaCl (20), 0.45 M NaCl (9) and called "alkali soluble collagen" (15) or "neutral salt extractable collagen" (9, 20). The complexity of the situation was shown by the very detailed study by Gross and his associates, (9, 12) who found that collagen could be extracted over a wide range of pH and ionic strength. They further showed that increasing amounts of collagen could be extracted with solutions of sodium chloride of increasing ionic strength from 0.2-1.5. Further confusion arises as was pointed out at a recent symposium (31), when the same name is used to describe fractions obtained by different extraction procedures. It is significant that at this same symposium no agreement could be reached on a standard nomenclature (31).

However it has become increasingly obvious

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that once extracted and purified these fractions are interconvertible (7), and have identical physico-chemical properties. Thus they have essentially the same amino acid composition (3, 21), x-ray diffraction pattern (34), and in solution behave as a single molecular species, a rod-shaped particle with a molecular weight of approximately 320,000 and having the dimensions of 3,000 A in length and 15 A in diameter (2, 20, 27). Moreover collagen fibrils having the typical 640 A spacing and interband periodicity of native collagen can be obtained by warming to 37°C. neutral solutions of collagen, obtained by any method which maintains its molecular integrity (20, 8, 12). The gel thus obtained becomes increasingly insoluble with time of incubation, first in neutral solution and later in acid solution (10). The differences in solubility must therefore be significant only in so far as they are viewed from the point of view of their physicochemical status in the tissues at the time they were extracted. In other words the various collagen fractions have only a biological significance.

It is generally accepted that the precursor of the collagen fiber is to be found among the soluble collagens which have been enumerated above, but there is some disagreement concerning the identity of the earliest precursor (19). Neutral salt solutions appear to extract the most recently synthesized collagen which, in this sense can be considered the precursor (15, 17, 9). However, even this would seem to be heterogeneous since in experiments involving ^{14}C-labeled amino acids, successive extracts with the same extraction medium contain collagen of decreasing specific activity (15, 6). It is also known that 0.45 M NaCl extracts a much larger amount of collagen than 0.2 M NaCl (9, 12) and it has been suggested that the latter is an earlier precursor (19) since it also disappears more rapidly from the skins of guinea pigs on a restricted diet (9).

It has been suggested that there is present in the extracellular ground substance a range of aggregates of its monomeric tropocollagen from which an increasing range is extracted with salt solutions of increasing ionic strength (11).

The study reported here was designed to test this hypothesis and to show that there are no clear cut collagen fractions, but a continuous spectrum of molecular aggregates of increasing age, strength of cross-linking, and hence of decreasing solubility. Determinations were made at various time intervals of the specific activity of [α-^{14}C] glycine incorporated into collagen extracted serially with different aqueous solvents from the carregeenin granuloma induced in guinea pigs (17) and from the skins of the same animals. The amount of collagen extracted by each solution was also determined.

Materials and Methods

Male and female guinea pigs were used, weighing about 400 gm. at the start of the experiment. They were fed throughout on McDonald No. 5 scorbutogenic diet,[1] supplemented with vitamins A, D, and E, and with 50 mg. of ascorbic acid administered orally every other day. Carrageenin granulomata were induced in the animals as previously described (17), and after 5 days 35 μc. of [α-^{14}C] glycine in 1 ml. of 0.9 per cent sodium chloride was injected intraperitoneally into each animal.

The animals were killed in groups of six at 8, 10, 24, and 36 hours following glycine injection and the granulomata dissected out as previously described (17). At the same time the dorsal skin was removed after clipping and depilation and scraped as free as possible from subcutaneous fat and fascia. All manipulations were carried out at 2° with secoctanol used as a preservative. The tissues were pooled and homogenized in a stainless steel mortar and pestle after freezing in liquid nitrogen, and then extracted serially four times with twice their weight of each of several neutral salt solutions ranging in concentration from 0.14 M NaCl—2.0 M NaCl, all at pH 7.4. Each extraction was carried out for 24 hours and the extracts were pooled, centrifuged at 20,000 *g.* for 1 hour, and filtered. An aliquot of each was removed, dialyzed salt-free, and dried, for determination of total collagen extracted. The neutral salt–extracted collagens were all purified by precipitation with sodium chloride to a final concentration of 20 per cent (21), followed by resolution in 0.01 M acetic acid, and reprecipitation with sodium chloride to a final concentration of 5 per cent. This last step was repeated twice and followed by solution in 0.01 M acetic acid and dialysis against 0.01 M Na_2HPO_4. The final precipitate was disolved in 0.01 M acetic acid, dialyzed against the same solution, and dried *in vacuo*. The fractionation of the tissue was then continued as previously described (17), samples of purified collagen extracted with 0.2 M citrate pH 3.6, and of gelatin from the insoluble residue being prepared.

[α-^{14}C] glycine was isolated from all the samples and the radioactivity measured as previously described (17). The collagen content of the extracts was measured by hydroxyproline determination by the Neuman and Logan method (25).

[1] Compounded for us by Ralston Purina Company, St. Louis.

TABLE I

*Collagen Extracted Serially From the Same Sample of Skin and Granulomata by Various Extracting Media**, ‡*

M NaCl (pH 7.4)	Skin (as mg. hydroxyproline)	Granulomata (as mg. hydroxyproline)
0.14	10.2 (10.2)	23.5 (23.5)
0.28	17.5 (27.7)	7.6 (31.1)
0.45	15.1 (42.8)	3.9 (35.0)
1.0	9.2 (52.0)	2.7 (37.7)
2.0	5.1 (57.1)	2.5 (2.5)
Citrate (pH 3.6)	13.2 (70.3)	1.1 (41.3)
Gelatin	—	5.1 (46.4)

* The figures are the average of three determinations.

‡ The figures in parentheses are the accumulative totals and indicate the amount of collagen that would be extracted if each solvent were used without prior extraction.

TABLE II

Specific Activity (C.P.M./*μmole glycine*) *of Collagen Fractions from Skin and Granulomata at Various Time Intervals Following the Injection of* ^{14}C*-Glycine*

M NaCl	Skin				Granuloma			
	8 hrs.	10 hrs.	24 hrs.	36 hrs.	8 hrs.	10 hrs.	24 hrs.	36 hrs.
0.14	1970	750	—	625	2785	1072	820	747
0.28	1060	1410	993	528	1528	2655	760	1248
0.45	710	—	1145	692	1365	—	1321	831
1.0	370	420	904	615	1010	2231	2952	825
2.0	300	225	332	512	895	1451	1348	493
Citrate	62	63	172	88	—	—	976	552
Gelatin	0	0	0	0	190	—	—	550

OBSERVATIONS

Collagen Extracts.—The maximum amount of collagen was extracted from skin by 0.28 M NaCl; thereafter the amount fell steadily with salt concentration, but increased again when citrate was the extracting solution (Table I). In contrast by far the greatest amount was extracted from the granuloma with 0.14 M NaCl, the amount falling steadily with increase in salt concentration and with no increase in the citrate extract. Only a small amount of gelatin was obtained (Table I).

Incorporation of [α-^{14}C] *Glycine.*—In the skin the highest specific activity was found at 8 hours in the 0.14 M extract, the specific activity decreasing with increasing salt concentration (Table II). At 10 hours the highest activity was found in the 0.28 M extract, at 24 hours in the 0.45 M extract and at 36 hours in the 1 M fraction. At each time the activity of the citrate extract was lower than the neutral salt extracts rising slowly to a maximum at 24 hours and decreasing at 36 hours. No significant activity could be found in any of the gelatin fractions.

In the granuloma the results were similar to those obtained with skin, but somewhat more complicated. Again the highest specific activity at 8 hours was found in the 0.14 M NaCl extract, at 10 hours in the 0.28 M fraction, but at 24 hours the highest activity was found in the 1 M fraction and again in the 0.28 M extract at 36 hours. The lowest activities at all times were again found in the citrate fraction, but the gelatins also showed significant specific activities.

The specific activities of all the granuloma fractions were higher than their counterparts from skin, probably reflecting a higher growth rate and cellularity.

DISCUSSION

There are two major points of discussion. First, the significance of the relationship of the variation of specific activity of the collagen fractions with time, to the type of extraction used; and secondly, the effect of the extraction media on the collagen aggregates in the tissue during the extraction procedure. If these two phenomena can be related then a fairly clear picture of extracellular fibrogenesis should emerge.

It has been previously shown (4, 17), that when ^{14}C-labeled amino acids are injected into animals or are present in the media during the incubation of tissue slices (6), that they are incorporated first into collagen extracted with 0.2 M NaCl and later appear in citrate extracted collagen and in gelatin from insoluble collagen. It was therefore suggested (4, 17) that neutral salt–soluble collagen was the precursor of the other two fractions. It can now be seen that the radioactivity is not transferred directly from the 0.14 M fraction to the citrate, but from collagen extracted with neutral salt solution of low ionic strength to that extracted with neutral salts of increasing ionic strength (Table II).

In skin, from which the data is particularly clear cut, 8 hours after injection the specific activity of the collagen fractions varies inversely

with the ionic strength of the extracting media, the highest specific activity being found in the 0.14 M NaCl extract. At 36 hours the situation is reversed and the specific activity increases with increasing ionic strength. At all times the specific activity of the citrate fraction is lower than that of all the neutral salt fractions and even at 36 hours no radioactivity is found in the gelatin fraction. Thus one can say that with respect to the time since their constituent molecules were synthesized, gelatin > citrate > 2.0 M NaCl > 1.0 M NaCl > 0.45 M NaCl > 0.28 M NaCl > 0.14 M NaCl.

The function of the extraction media must basically be the dissolution of aggregates of collagen molecules. In an aqueous medium the stability of a collagen fiber is determined by the equilibrium established between swelling forces and the intermolecular cross-links (30, 13 *a*). In the fully formed fiber the major type of cross-link is believed to be hydrogen bonding between hydroxyl groups of hydroxyproline with > C = O groups on adjacent molecules (14). To obtain solution the swelling pressure must exceed the cohesive forces. Thus a good proportion of collagen fibers from a young growing animal (about 20 per cent) can be extracted readily with organic acids around pH 3, which is close to the point of maximum swelling (5). In the rest of the fiber the remaining collagen can only be rendered soluble by applying even more disruptive forces by gelatinization. Thus gelatinization extracts collagen with a greater degree of cross-linking than that extracted with organic acids.

The effect of neutral salts has been discussed in detail by Gustavson (13), who concludes that the interaction of salts and collagen will result in the weakening of intermolecular forces. Extraction with neutral salts is probably in effect a "salting-in process" and hence within certain limits the higher the ionic strength the greater will be the amount of collagen extracted. This was found by Gross *et al.* (12), and in this study. (In Table I the figures in parentheses are accumulative totals, representing the amount of collagen that would be extracted if each solvent had been used *alone without prior extraction.*)

Thus with respect to their efficiencies as extracting media, one can say that gelatinization > acid > 1 M NaCl > 0.45 M NaCl > 0.28 M NaCl > 0.14 M NaCl. We have already shown from the radioactivity data that, with respect to the age of the molecules extracted by these media, the same order applies. Thus it would appear that the longer the time that has elapsed since a collagen molecule was synthesized, the more firmly will it be bound into a collagen aggregate. This will be a continuous process and so at any given time there will be a continuous spectrum of collagen aggregates of varying degrees of cross-linking. The cross-section of these aggregates which is extracted and also the ages of their constituent molecules will depend on the extraction medium used. All extracts will be heterogeneous as regards the age of the collagen molecules they contain. The more efficient the method of extraction the greater will be the heterogeneity. The evidence of decreasing specific activity following injection of ^{14}C-labeled amino acids, in successive extracts with the same extraction media, in one case acid citrate buffers (15, 26) and in the other with 0.2 M NaCl (6), also demonstrates this heterogeneity.

The collagen extracted with 0.14 M NaCl will contain the most recently synthesized collagen molecules and also the narrowest age range. In the work reported here the conditions of preparation of the tissue would probably lead to cell destruction and since the collagen molecules must be synthesized inside the cell, intracellular collagen will also be included in the 0.14 M NaCl extract. It has been recently shown (Lowther and Green, personal communication) that a collagen fraction closely associated with the microsomal fraction and with a very rapid turnover is extracted from the microsomes by 0.14 M NaCl. Thus the collagen extracted by 0.14 M NaCl probably represents that most recently synthesized.

The relatively large amount of collagen extracted from granuloma with 0.14 M NaCl was to be expected since this tissue is younger and more cellular than the skin and would contain a lot of more recently synthesized collagen. The less clear cut evidence obtained from the granuloma is probably due to the unique feature of this tissue, that the collagen formed is rapidly resorbed by breakdown of the fibers to more easily extractable components (17, 33) which are precipitated like collagen during the isolation procedure (17). Hence there may be an admixture of recently synthesized collagen molecules and collagen molecules from the breakdown of older collagen aggregates, which will complicate the situation.

The relatively high activity of the gelatin obtained from the granuloma compared to that from

skin may be explained by the fact that at this stage of the granuloma formation, there is a considerable amount of extracellular carrageenin. This is an acid polysaccharide which forms an insoluble complex with collagen at an acid pH, (unpublished data), which would therefore not be extracted with citrate buffer. It would probably be extracted as gelatin, which would then contain radioactivity derived from the carrageenin-citrate–extractable collagen complex.

The evidence now available should enable us to construct a working hypothesis of the process of fiber formation which is an elaboration of that already put forward (12, 19, 18). Since collagen is a protein it must be assumed that the collagen molecules are synthesized by the same mechanism as obtains for other proteins and occurs intracellularly. The best evidence to date, suggests that the first collagen fibrils visible in the electron microscope are formed at the surface of the fibroblast (29) and these will probably be extracted by 0.14 M NaCl. Further increase in size does not appear to occur by aggregation of these first fibrils but by accretion of material from the extracellular space (22, 29). In a young growing connective tissue two things will be going on simultaneously: (*a*) The formation of new fibrils at the cell surface and the addition of more molecules into the fibrils formed earlier by the cell. As Porter (29) has suggested these fibrils probably act as templates to extend the polymerization of collagen. (*b*) Increasing strength of cross-linking in the older fibrils. At this point extraction with 0.14 M NaCl would remove the new fibrils and also the molecules laid down on the older fibrils at the same time. The remainder of the fibril will now only be extractable with neutral salts of higher concentration. Eventually the main process will be the increasing diameter of the fibrils by accretion of newly synthesized molecules (22, 29). The deeper in the fiber the collagen molecule is, the more firmly will it be cross-linked, as with time the molecules move into more favorable steric apposition under the influence of thermal agitation. Hence there will be an increase in proportion of the collagen fractions extracted with neutral salt of high ionic strength, acid citrate, and as gelatin. The outer layers will be more loosely aggregated and hence the more easily extractable. This would account for the "metacollagen" of Banga (1), and the "collastromin" of Tustanovski (32), since the outer layers would have a lower shrinkage temperature than the inner core, which would appear as collastromin or metacollagen under the conditions of the experiments of the above authors (1, 32). It would also account for the observed decreasing proportion of collagen extractable with aqueous solutions as connective tissue ages, since when synthesis of new collagen ceases, and collagen becomes relatively metabolically inert (24) the only process continuing is the spreading of the region of maximum cross-linking through the whole of the fiber.

The place in this scheme of the non-collagenous proteins or mucoproteins, which binds so tenaciously to collagen in the connective tissues such as skin and tendon, is uncertain. It would appear possible that, since the collagen is laid down in a matrix containing these proteins, that they become "trapped" in the collagen fiber lattice as this increases in diameter. As these proteins possess active groups capable of forming hydrogen bonds with adjacent collagen molecules, like those between adjacent collagen molecules they would become part of the collagen lattice. The conditions necessary to extract them completely therefore would be those necessary to take the collagen into solution, which is in fact the situation found (3).

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