STUDIES ON THE FORMATION OF COLLAGEN

I. Properties and Fractionation of Neutral Salt Extracts of Normal Guinea Pig Connective Tissue*

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(Received for publication, September 18, 1957)

The discovery of a form of collagen which can be extracted from fresh connective tissue in cold, neutral, and mildly aklaline media (1–5) has suggested that a fraction of the total collagen exists in a dispersed or loosely aggregated state in the extracellular, extrafibrillar matrix of the connective tissues. The process whereby a dispersion of collagen molecules (tropocollagen (6)) might be polymerized in the tissues was suggested by the observation that simple warming of the extract to physiological temperatures results in the precipitation of fibrils with the typical detailed fine structure of native collagen (4, 5).

In addition to describing a new procedure for isolating and purifying the extracted collagen, this paper presents some results of an exploration of the properties of crude neutral salt extracts of fresh guinea pig corium as a base line for further studies on the role of the extracted components in the fibrogenesis of collagen and the physiology of connective tissues.

Materials and General Procedure

Guinea pigs of both sexes ranging in weight from 100 to 500 gm. were fed Purina rabbit chow pellets (containing antibiotics) supplemented with lettuce daily and 25 mg. of vitamin C three times weekly.

The hair was removed with an electric clipper and the animals sacrificed by intraperitoneal administration of nembutal followed by exanguination *via* intracardiac puncture. The skin of the trunk was removed, the epidermis and subcutaneous tissue dissected away and the corium ground in a hand driven meat grinder, the cutting edges of which were kept cold with

* This is publication No. 216 of the Robert W. Lovett Memorial for the Study of Crippling Disease, Harvard Medical School, at the Massachusetts General Hospital, Boston. This study was aided by grant A90 (C6-7) from the National Institute of Arthritis and Metabolic Disease of the United States Public Health Service. This work was presented in part at the 8th annual meeting of the Histochemical Society at Atlantic City in April, 1956. (Gross, J., *Histochem. and Cytochem.*, 1956, **4**, 408. [abstract]).

‡ This work was done during the tenure of an Established Investigatorship of the American Heart Association.

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chips of dry ice. After removal of aliquots for analysis, the ground corium was suspended in two volumes of extracting medium (*v/w*) and shaken for 16 hours at 3°C. Ionic strength of the extracting medium was 0.45 in all cases except in some experiments in which NaCl ($\mu = 0.14$) was also employed. If complete mixing with total tissue is assumed, the final ionic strength would be about 0.41. The tissue mash was then centrifuged in the Spinco model L preparative ultracentrifuge at 50,000 *g* for 60 minutes and the supernatant fluid filtered with suction through medium and fine sintered glass. All operations were performed at 3–5°C. In several instances the fresh subcutaneous tissue was treated in a similar manner.

Aliquots of each extract were taken for hydroxyproline analysis and the remainder was dialyzed overnight in the cold against large volumes of extracting medium with stirring. Changes in volume on dialysis were not significant.

Indications of the amounts of certain categories of substances in the extracts were obtained by the following analyses after dialysis against the medium: Hydroxyproline for collagen[1]; tyrosine for non-collagenous protein, (this being less than 1 per cent in purified collagen); hexose, hexosamine, and uronic acid for carbohydrates as polysaccharides and glycoproteins. The following colorimetric methods were used:—

1. Hydroxyproline—Neuman and Logan (7) 1 ml. aliquots were hydrolyzed with 1 ml. 12 N HCl in a sealed tube in an oil bath at 138° for 3 hours. Glycine and proline were determined on the same hydrolysates when required.
2. Glycine—Christensen, Riggs, and Ray (8).
3. Proline—Troll and Lindsley (9).
4. Tyrosine—Bernhardt (10).
5. Hexosamine–Boas (11) modification of Elson and Morgan (12).
6. Hexose—Friedman (13) (orcinol).
7. Uronic Acid—Fishman *et al.* (14) (naphthoresorcinol).[2]

For electrophoretic analysis 4 ml. of extract were dialyzed against large volumes of veronal citrate–NaCl buffer, (pH 8.4, $\mu = 0.14$ or 0.2) and examined in the Perkin-Elmer electrophoresis apparatus at 4°C. A run required 4 hours for adequate separation, particularly at $\mu = 0.2$. At lower ionic strength, too much distortion of boundaries occurred in viscous extracts. At $\mu = 0.1$, the collagen fraction precipitated. Sedimentation studies on the crude and dialyzed extracts were performed in the Spinco model E analytical ultracentrifuge (using the

[1] Hydroxyproline is an accurate measure of collagen, since there are no other known animal proteins containing any appreciable amounts of this amino acid. It has also been demonstrated in this study that the hydroxyproline containing fraction when isolated in highly purified state can be precipitated as morphologically typical collagen fibrils (by electron microscopy) and contains the glycine, proline, and hydroxyproline content characteristic of pure mammalian skin collagen. It is worth mentioning that under abnormal conditions a collagen might be formed with reduced or increased hydroxyproline content. For this reason glycine and proline should also be measured for comparison. It is necessary, however, to isolate the collagen fraction first, since other proteins also contain varying, although much smaller, amounts of these two amino acids.

Control analyses under the conditions of acid hydrolysis described gave 97 to 102 per cent recovery of hydroxyproline. Tyrosine produces about 1 per cent of the color of an equal amount of hydroxyproline (7). The relatively large amounts of tyrosine present in these extracts are nearly completely destroyed in the acid hydrolysate.

[2] The uronic acid assayed by this naphthoresorcinol method is of value for comparative purposes only. Analysis of purified hyaluronic and chondroitin sulfuric acids yielded only 20 to 30 per cent of the expected amounts. However, there is little interference from hexoses; 150 μg. of hexose in orosomucoid produced no color.

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wedge cell for two simultaneous analyses) at 3–6°C. at 56,900 R.P.M. Viscosity was measured in Ostwald viscometers (flow time for buffer, 60 seconds) at 5.0 ± 0.1° prior to dialysis. Dialysis against the extracting medium had little influence on viscosity. Samples were centrifuged at 50,000 *g* for 3 hours before viscometry.

RESULTS

Some General Properties of Neutral Salt Extracts.—The average extract prepared as described above with 0.45 M NaCl is clear pink or amber colored and quite viscous. The 0.14 M NaCl solution is the same color but exhibits little viscosity on shaking. The measured relative viscosity of the stronger saline extract (obtained from normally growing 250 to 600 gm. animals) ranged between 7.5 and 20. Extracts obtained from the skins of rapidly growing animals under 150 gm. occasionally had relative viscosities as high as 56. Relative viscosities of 0.14 M extracts were never above 2.6.

TABLE I

Quantitative Indices for Collagen, Non-Collagenous Proteins and Non-Dialyzable Carbohydrate in NaCl Extracts of the Dermis of Normally Growing Guinea Pigs

Two extracts, one 0.45 M NaCl and the other 0.14 M NaCl, were made of separate aliquots of the same skin of each of eight animals.

Extraction medium	μg./ml. ± σ				
	Hydroxyproline	Tyrosine	Hexose	Hexosamine	Uronic acid
0.45 M NaCl	295 ± 50	296 ± 19	344 ± 35	67 ± 12	22 ± 5
0.14 M NaCl	65 ± 17	264 ± 19	267 ± 18	64 ± 11	23 ± 4

Table I compares the components extracted in 0.45 M and 0.14 M NaCl in terms of mean values for hydroxyproline, tyrosine, hexose, hexosamine, and uronic acid, for eight pairs of extracts, each pair obtained from separate aliquots of the same skin of normally growing animals.

The relatively wide dispersion of hydroxyproline content probably reflects differences in growth rate (15), whereas the other parameters are essentially unaffected by this factor. The amount of collagen extracted at the higher ionic strength is considerably greater as shown earlier by Gross, Highberger, and Schmitt (4). Bound hexose is somewhat lower in the 0.14 M extracts; the other parameters are about equal.

The hexoses were identified as glucose, galactose, and mannose by paper chromatography in three different solvent systems as described elsewhere (16). Hexosamine, identified by the chromatographic method of Stoffyn and Jeanloz (17) is predominantly glucosamine with only a trace of galactosamine.

Ultracentrifuge patterns revealed a relatively fast moving, rapidly diffusing peak followed by a slow sedimenting, hypersharp, non-diffusing boundary (Fig. 1 (*a*)). Occasionally a third low, broad boundary may appear between these two. The pattern for the 0.14 ionic strength

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extract was qualitatively the same, differing only in the magnitude of the slow moving peak which was much smaller (Fig. 1 (*b*)). The same type of pattern was obtained for phosphate extracts.

The electrophoretic patterns of the 0.45 ionic strength extract may be compared with that of guinea pig serum (Fig. 2 (*a*)).[3] The major difference is the addition of a large hypersharp slow moving peak in the region corresponding to the γ-globulin fraction, most prominent in the ascending limb (Fig. 2 (*b*)). There is considerable broadening of what corresponds to the α-globulin boundary in the serum pattern and also a relatively smaller "albumin" peak. Occasionally a small boundary moving more rapidly than "albumin" was observed (Fig. 2 (*c*)). The pattern is essentially the same for the 0.14 ionic strength extract except that the slow moving spike is very much smaller (Fig. 2 (*c*)).

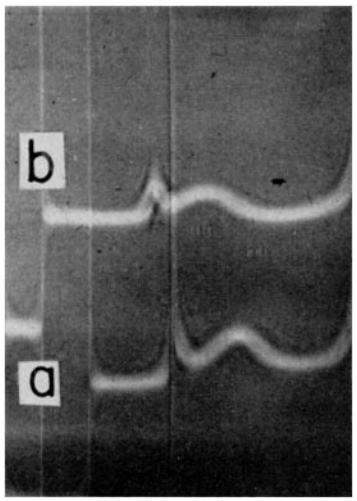

FIG. 1. Ultracentrifuge patterns of cold NaCl extracts of guinea pig corium. Simultaneous double run using wedge cell. (*a*). 0.45 M NaCl extract. (*b*). 0.14 M NaCl extract of sample of same tissue.

Identification of the hypersharp peak with the collagen fraction was demonstrated by the effect of heating the extracts to 37°, a procedure which precipitates practically all of the collagen in solution in the form of a rigid gel consisting of characteristic collagen fibrils as seen in the electron microscope (4). The ultracentrifuge pattern of the supernatant solution (after removal of the heat-precipitated collagen) revealed considerable diminution of the slow moving hypersharp boundary and is identical in appearance with that in Fig. 1 (*b*). Similarly, electrophoretic examination showed that the high spike in the "γ-globulin" region had been almost completely eliminated (Fig. 2 (*d*)) with little alteration in the rest of the pattern. This change was matched by a considerable fall in viscosity, and a comparably large drop in hydroxyproline content. In the case of the 0.14 ionic strength extract there was only a slight diminution in the peak (Fig. 2 (*c*)) correlated with a corresponding small loss in hydroxyproline content. In both extracts there was a disappearance from solution of 40 to 50 per cent of the hexosamine, hexose, and tyrosine. The fact that this loss also occurs in the lower

[3] The patterns shown in Fig. 2 were obtained with phosphate extracts (μ = 0.4 and 0.14; pH 7.6). These are identical with patterns obtained with NaCl extracts.

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ionic strength extract in which there was little precipitation of collagen indicates that the diminution of these non-collagenous substances is probably unrelated to the formation of

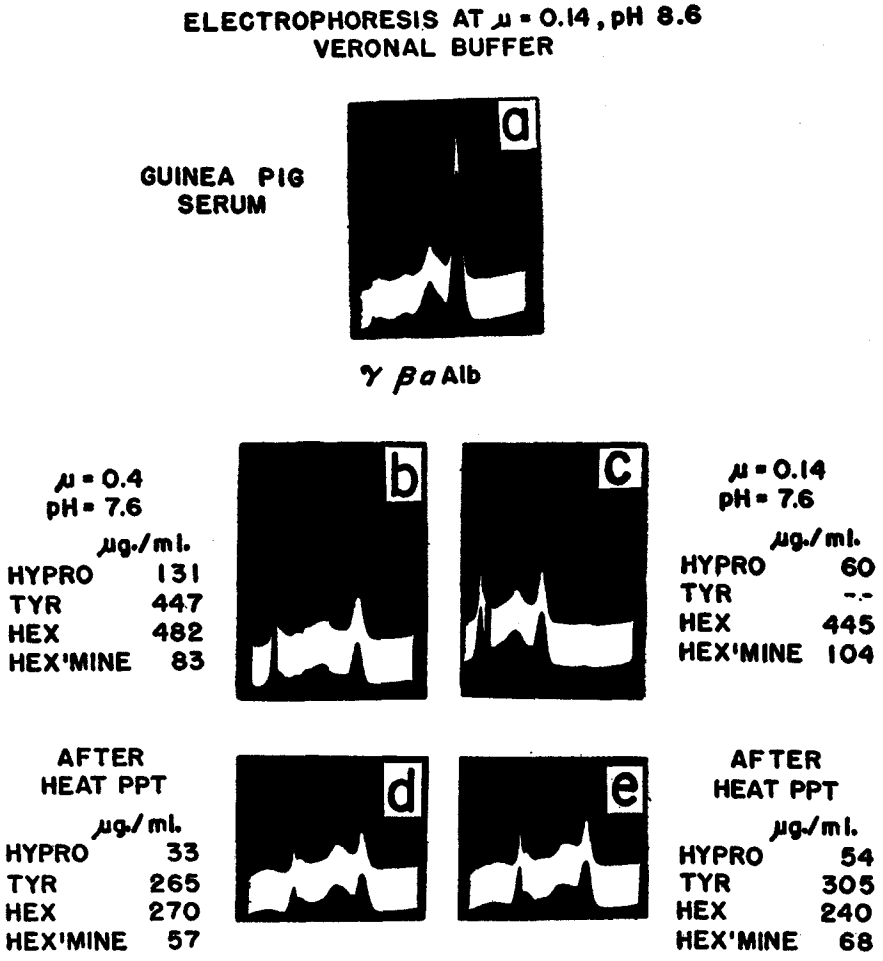

FIG. 2. Electrophoretic patterns (ascending limb) of guinea pig serum and neutral phosphate extracts of corium. (Hydroxyproline (Hypro), tyrosine (Tyr), hexose (Hex), hexosamine (Hex'mine).) All were dialyzed *versus* veronal-citrate-NaCl buffer $\Gamma/2 = 0.14$, pH = 8.6. (*a*) serum; (*b*) phosphate extract of corium ($\Gamma/2 = 0.40$, pH = 7.6); (*c*) phosphate extract of corium ($\Gamma/2 = 0.14$, pH = 7.6); (*d*) supernatant after removal of collagen from $\Gamma/2 = 0.4$ extract by heat precipitation at 37°; (*e*) supernatant after attempted removal of collagen from $\Gamma/2 = 0.14$ extract by heat precipitation at 37°.

collagen fibrils. The heat precipitation of fibrils occurs with equal ease and completeness from neutral salt solutions of highly purified collagen (described later) in concentrations of 0.1 per cent and higher.

Extracts of subcutaneous tissue contained a small collagen moiety. The electrophoretic patterns of such extracts were similar to those of the dermis.

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Effect of Enzymes on Extract Viscosity.—To further characterize the component in the extract responsible for viscosity, the effect of three enzymes on this property was measured.

Extracts of fresh guinea pig skin prepared with 0.45 M NaCl were dialyzed *versus* phosphate buffer pH 7.6, $\mu = 0.45$ at 3°C. and divided into four 10 cc. aliquots. They were brought to 27°C. in a water bath and 5 mg. of collagenase,[4] 10 mg. of hyaluronidase,[4] and 10 mg. of trypsin[4] were added to each of these tubes respectively in the form of 1 ml. of enzyme solution in phosphate buffer. The same volume of buffer alone was added to the fourth tube as a control. Immediately following mixing 3 ml. samples were transferred to Ostwald viscometers with flow times of 34 seconds and viscosity was measured at intervals up to 2 hours at 27°. In other experiments the time was extended to 4 hours with little change.

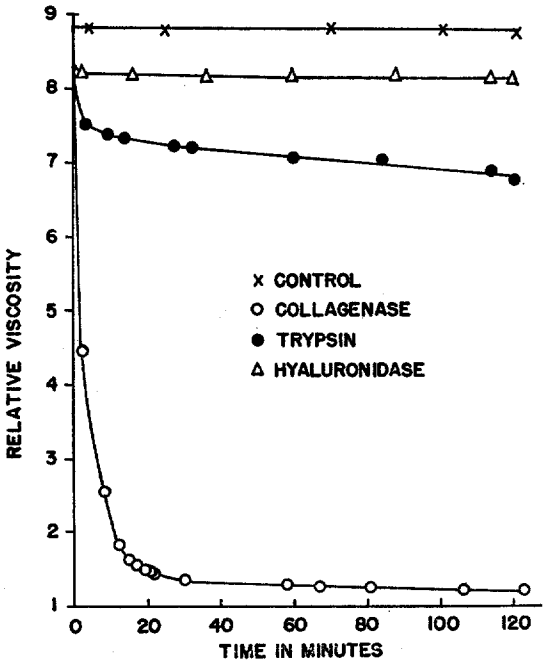

FIG. 3. Effect of enzymes on extract viscosity as a function of time of incubation. 0.45 M NaCl extract dialyzed *versus* phosphate buffer $\Gamma/2 = 0.4$, pH = 7.6, T = 27°C.

The results of a representative experiment are presented in Fig. 3. No change is noted in the control preparation (ηrel. = 8.8). An immediate and rapid fall in viscosity to ηrel. = 1.5 occurred in 20 minutes in the collagenase-treated sample, followed by a slow fall to ηrel. = 1.2 in 80 minutes without

[4] Clostridium histolyticum collagenase was obtained through the kindness of Dr. J. D. Maclennan. Wyeth hyaluronidase and Worthington 2× crystallized salt-free trypsin were used.

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further change. Viscosity of the hyaluronidase-treated sample fell within several minutes to ηrel. = 8.2 and remained unchanged thereafter; this small decrement was readily reproducible and could be obtained by adding hyaluronidase to a collagenase-treated preparation. The relative viscosity of the trypsin-treated sample fell to 7.3 in less than 10 minutes, after which it declined slowly but linearly to 6.7 in 120 minutes.

The three enzyme-treated preparations and control described above were dialyzed against veronal-citrate-NaCl buffer, pH 8.4, μ = 0.2 at 3° and examined in the electrophoresis apparatus and in the analytical ultracentrifuge. Fig. 4 illustrates tracings of the patterns so obtained. There is little differ-

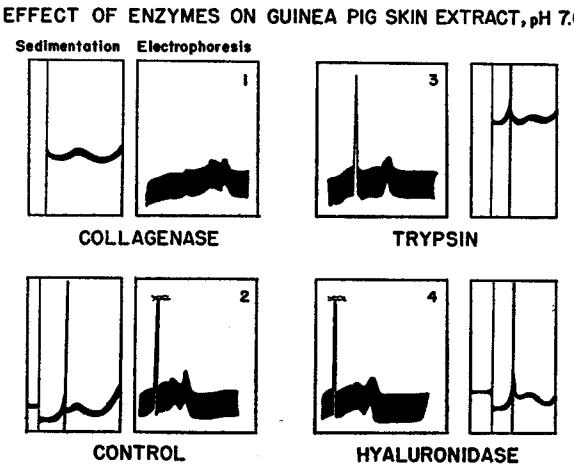

FIG. 4. Effect of enzymes on electrophoretic and sedimentation patterns. Extracts dialyzed *versus* veronal citrate NaCl buffer, $\Gamma/2$ = 0.2, pH 8.4.

ence between the control pattern and that obtained from the hyaluronidase-treated preparation. The effect of trypsin is evidenced by a partial loss of non-collagenous proteins and a diminution of the height of the collagen peak in the electrophoresis pattern. Collagenase appears to have entirely eliminated the collagen boundary in both sedimentation and electrophoretic diagrams.

Warming aliquots of all four of the above preparations to 37°C. resulted in the typical opaque gel in both the control and hyaluronidase-treated samples within the same time period. No gelation or opacity in the collagenase-treated material occurred. A heavy, fibrous, non-gelled precipitate appeared in the trypsin-treated extract, electron microscopy of which revealed normally striated fibrils and considerable numbers of non-striated fibrils and filaments. This same effect of trypsin was noted on neutral salt solutions of relatively pure collagen.

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Viscosimetric Studies of 0.45 M NaCl Extracts.—This experiment was designed to compare the intrinsic viscosity of a crude extract (based on collagen concentration) to that of a solution prepared from collagen fibrils precipitated from the same extract by warming. It has already been shown that the viscosity of the extract depends almost completely on the collagen content. The question asked here is whether the degree of asymmetry of the collagen particle in the neutral extract is radically changed by having been polymerized and redissolved.

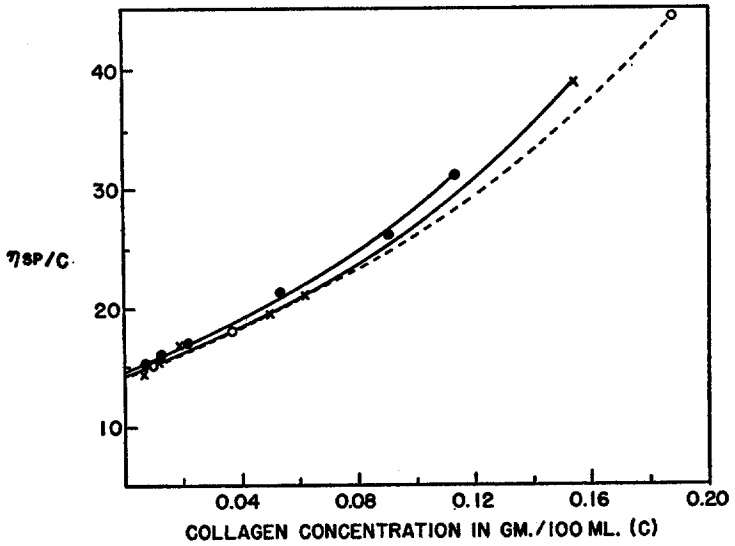

FIG. 5. Determination of intrinsic viscosity of crude 0.45 M NaCl extract as compared to neutral salt solutions prepared from heat polymerized collagen obtained from samples of the same NaCl extract. ●, Crude extract; ×, extract heat-precipitated redissolved by dialysis against 0.5 M acetic acid, then dialyzed *versus* phosphate buffer and NaCl. ○, extract heat-precipitated *and washed* collagen fibrils redissolved in 0.5 M acetic acid and dialyzed *versus* phosphate buffer and NaCl.

A 0.45 M NaCl extract was divided into three aliquots. Two of the three samples were warmed at 37° for 1 hour at which time the usual rigid opaque gels had formed with no further increase in opacity. After breaking up the gel, one sample was dialyzed against 0.5 M acetic acid in the cold for 24 hours, at the end of which time it was a faintly opalescent viscous solution and was clarified by high speed centrifugation. This solution included most of the non-collagenous materials as well as collagen. The third heat-gelled preparation was broken up, centrifuged at 10,000 *g* for 30 minutes, the supernatant fluid discarded, and the fibrous sediment rewashed in saline in the centrifuge. This partially purified sample of fibrous collagen was then dissolved in 0.5 M acetic acid as described above. Both acid solutions were then dialyzed against phosphate buffer, pH 7.6, $\mu = 0.45$ for 16 hours then redialyzed against 0.45 M NaCl (the collagen in acid solution will precipitate if dialyzed directly against NaCl). Viscosity in all three solutions was determined at a series of graded concentrations estimated

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by hydroxyproline based on 14 per cent composition for pure collagen. Ostwald viscometers of 165 seconds flow time at 5°C. were used. A plot of reduced viscosity as a function of concentration (Fig. 5) gave an intrinsic viscosity of 14.5 for all three preparations. This same value was obtained for acetic acid solutions of calfskin collagen dialyzed against phosphate (Gross, unpublished data).

Thus it appeared that the collagen particles extracted from fresh tissue in cold neutral salt solutions have the same highly elongated shape as do the collagen units extracted from fibrils by acid and then transferred to a neutral medium.

Relative Effectiveness of Different Salt Solutions as Extractants.—Gross *et al.* (4) demonstrated that neutral solutions of the sodium salts of phosphate, acetate, chloride, and oxalate were about equally effective in extracting collagen from fresh calf corium. However, further examination of this point seemed in order, using salts more widely separated in the Hofmeister series, including thiocyanate (said to be a strong solvating agent) and sulfate which, if anything, has a dehydrating effect.

TABLE II

Effectiveness of Different Extracting Media Measured by Viscosity and Hydroxyproline Content of Extract

Ionic strength of all salt solutions was 0.45. Sucrose 0.45 molar was used. Viscosity of sucrose extract is less than 1 because of dilution by tissue water.

Medium	Extract pH	η rel.	Hydroxyproline $\mu g./ml.$
Na_2SO_4	6.9	29.3	420
NaCl	6.9	22.1	375
K_2HPO_4 / KH_2PO_4	7.5	18.4	315
NaSCN	7.0	16.9	260
Sucrose	7.0	0.95	0

Equal aliquots of ground guinea pig dermis from the pooled tissues of groups of actively growing young animals were extracted with NaCl, Na_2SO_4, NaSCN, and K_2HPO_4—KH_2PO_4, (pH 7.6). Ionic strength in all cases was 0.45. Sucrose 0.45 M was also included as a non-electrolyte. Saturated Na_2HPO_4 was compared with 0.45 M NaCl in another experiment.

In three of four experiments, $SO_4^{=}$ appeared to be more effective than SCN^-, Cl^-, or $PO_4^{=}$. Saturated Na_2PO_4 (used by Harkness *et al.* (3)) extracted about one-third the amount of collagen as did NaCl ($\mu = 0.45$); electrophoretic examination of the extract revealed a much diminished yield of non-collagenous material as well as collagen. Sucrose did not extract any measurable amounts of collagen.

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Reproducibility of the amounts of collagen removed by 0.45 M NaCl was examined by extracting each of 4 aliquots of the same comminuted fresh guinea pig corium with 2 volumes of cold saline. Relative viscosity and hydroxyproline in γ/ml. are presented in Table III.

With the exception of aliquot 3, the coincidence of both viscosity and hydroxyproline is surprisingly good. The lower viscosity and collagen content of aliquot 3 was accounted for by some precipitation, probably a result of accidental lowering of the pH.[5]

Effect of Temperature on Extraction.—The amounts of collagen extracted from guinea pig skin by 0.45 M NaCl at 3 and 37°C. were compared by extracting equal aliquots of pooled, ground dermis from five young (150 to 200 gm.) animals for 18 hours with toluene as a bacterial suppressant. The extract prepared at 3°C. had a relative viscosity, ηrel. = 14.0, and hydroxyproline content of 295 μg./ml. as compared with ηrel. = 1.7, hydroxyproline

TABLE III

Reproducibility of the Extraction Procedure as Measured by Viscosity and Hydroxyproline Content of Four 0.45 M NaCl Extracts of Four Separate Aliquots of the Same Tissue

Measurement	Tissue aliquot			
	1	2	3	4
η rel.	5.35	5.55	4.0	5.52
Hydroxyproline, μg./ml.	146	149	125	146

= 68 μg./ml. for that extracted at 37°. If the excised, whole dermis is incubated for 24 hours under toluene at 37°C. then minced and extracted in salt solution at 3°C. for 16 hours, very little collagen is extracted and the solution is non-viscous. Storage of freshly prepared tissue at 3°C. up to 48 hours does not affect the amount of extractable collagen, but beyond this time there was a rapid diminution. In one experiment, this diminution was noted after 24 hours.

Purification of the Collagen Fraction.—This scheme devised for fractionation of the crude NaCl extracts depends on the initial precipitation of most of the non-collagenous protein from the crude extract with 2.4 per cent trichloroacetate at pH 3.5 (in which the bulk of the collagen remains in solution) followed by precipitation of the collagen fraction with 14 per cent ethanol. The procedure is schematized in detail in Fig. 6. All the collagen-containing fractions are found in the vertical line on the left of the diagram.

[5] Reduction of the pH below 5 results in the formation of insoluble complexes between collagen and acidic high polymers such as acid mucopolysaccharides and nucleic acids. This has been observed to occur in phosphate and NaCl extracts.

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The crude extract (I_s) was dialyzed against phosphate so that the fall in pH upon dropwise addition of 25 per cent TCA (trichloroacetic acid) proceeds slowly and may be readily stopped at pH 3.5 as determined by indicator paper. Under the conditions described, this occurs at about 2.4 per cent TCA. After standing in the cold for 30 minutes, the heavy granular precipitate was sedimented in the Spinco and the collagen rich supernatant (II_s) was dialyzed immediately against phosphate buffer to remove the TCA and to return to neutral pH. Ultracentrifugal and electrophoretic examination at this stage revealed the loss of about 80 per

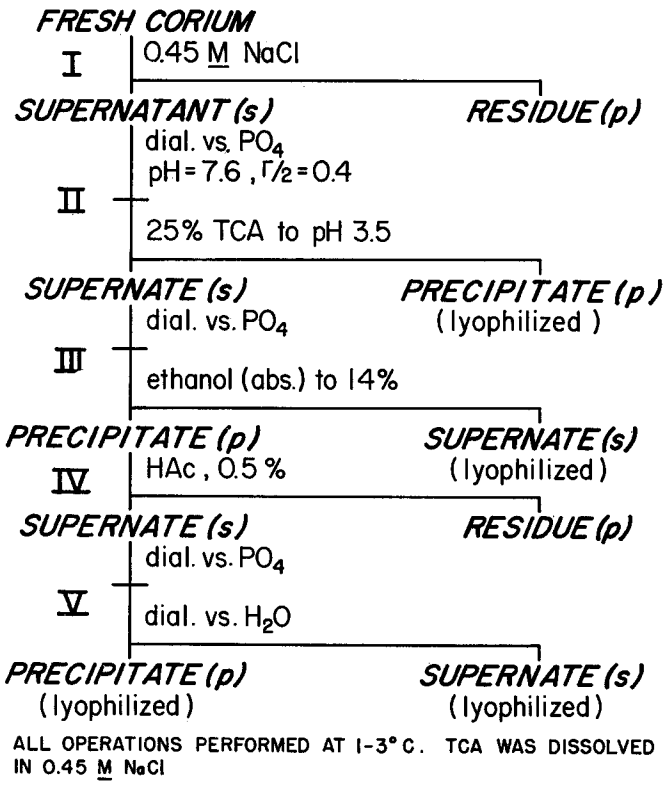

FIG. 6. Fractionation scheme applied to 0.45 M NaCl extract of guinea pig dermis

cent of the non-collagenous material. After dropwise addition of absolute alcohol to a final concentration of 14 per cent in stage III, precipitation proceeded slowly to completion in the cold in 24 hours. The collagen precipitate, fraction III*p*, was separated in the centrifuge and redissolved by dispersing in small successive volumes of 0.5 M acetic acid in a tapered Elvehjem homogenizer. It dissolved completely and the viscous solution when centrifuged 24 hours later at 60,000 *g* for 3 hours yielded a very small sediment, IV*p*, which was discarded. Extracts, solutions and reagents were kept at 1–5°C. in an ice bath throughout the operation. All precipitating reagents were added slowly, with vigorous continuous stirring with the aid of a magnetic stirrer. The reaction vessel was held in a crystallizing dish of chipped ice and water on the magnetic stirrer thus obviating the use of a cold room. The supernatant solutions in

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which the collagen fraction was expected to appear were examined by ultracentrifugation and moving boundary electrophoresis.

Precipitates and supernatant solutions were dialyzed free of salts, lyophylized, and analyzed for hydroxyproline, proline, glycine, tryosine, hexosamine, and hexose. Table IV is a typical protocol for the fractions obtained from the pooled first extracts of the dermis of three actively growing young guinea pigs.

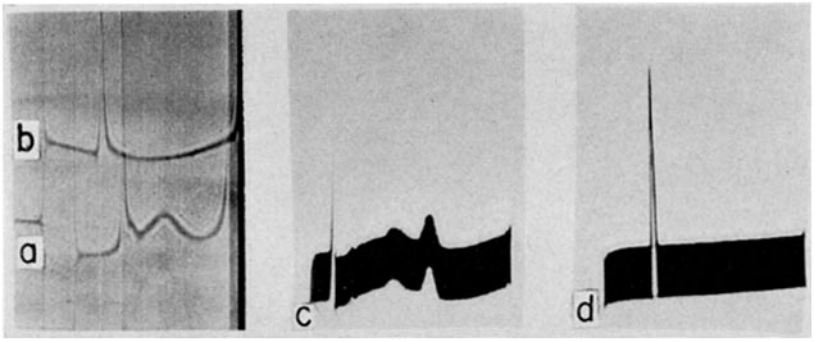

FIG. 7. Ultracentrifuge and electrophoretic patterns of crude NaCl extract and purified collagen fraction V*p* isolated therefrom. (*a*) Sedimentation pattern of crude 0.45 M NaCl extract. (*b*) Sedimentation pattern of purified collagen fraction V*p* in phosphate buffer $\Gamma/2 = 0.45$, pH 7.6. (*c*) Electrophoresis pattern of same crude NaCl extract in veronal-citrate-NaCl buffer $\Gamma/2 = 0.2$, pH 8.4. (*d*) Electrophoresis pattern of purified collagen fraction V*p* under same conditions as (*c*).

TABLE IV

Analyses of dialyzed, lyophilized fractions separated from a cold 0.45 M NaCl extract by TCA-ethanol fractionation

Fraction	Amount of solid	Solid, *gm./100 gm.*						Collagen recovered
		Hydroxyproline	Proline	Glycine	Tyrosine	Hexose	Hexosamine	
	mg./100 ml.							*per cent*
II*p*	310.0	1.72	4.66	6.35	3.46	2.64	0.61	23.9
III*s*	34.4	0.8	4.3	4.1	0.17	6.2	4.97	1.2
V*s*	4.4	3.64	—	8.68	—	—	—	0.7
V*p*	121.1	13.6	14.3	25.9	0.66	0.5	<0.1	74.0

Fig. 7 illustrates the electrophoretic and ultracentrifuge patterns of the first crude NaCl extract and the final solution of purified collagen.

About 74 per cent of the collagen in the extract was recovered in the purified fraction V*p*. Roughly 20 per cent is precipitated by TCA in fraction II*p*. Probably some of it is complexed at the low pH with acidic high polymers such as mucopolysaccharides and nucleic acids and the remainder carried down mechanically in the precipitate. The collagen in fraction II*p* can be

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recovered by reextracting the wet precipitate with phosphate buffer. Collagen accounts for about 33 per cent of the total non-dialyzable solids extracted from the tissue (in the first extract).

Evidence for the "intactness" of the collagen molecules in the final solution was obtained by demonstrating the ability of these solutions to form cross-striated collagen fibrils in a fibrous gel on warming to 37°C. and by the optical rotation. More than 90 per cent of the collagen was precipitated in fibril form. Optical rotation was performed at 24.5 and 4°C. (The specific rotation for gelatin is strongly temperature-dependent, whereas that for collagen in solution is not.)

The values obtained for Fraction V*p* redissolved in 0.5 M acetic acid, concentration = 0.39 per cent collagen determined by hydroxyproline anlaysis were:—

$$\alpha_D^{24.5°C.} - 372°., \qquad \alpha_D^{4°C.} - 367°$$

The purity of Fraction V*p* is indicated by the analyses shown in Table IV.

DISCUSSION

Skin collagen has a broad isoelectric range from about pH 5 to 10 (18–20). Solubilization of bovine hide collagen at 20°C. begins at pH 13 after many days of incubation, amounting to about 5 per cent after 14 days (21). That which occurs between pH 2.5 and 5 represents a dissolution of the fibrils to their component molecules and aggregates. Because acid-solubilized collagen may be readily precipitated *in vitro* by various means as fibrils with the characteristic electron microscope morphology of those in the tissue, it would appear that the dissolved molecules have not been significantly altered.

In the isoelectric range there is little swelling or solution of fibrils from mammalian skin (18–20). For this reason, solubilization of collagen from fresh connective tissues by neutral or near neutral salt solutions suggests the presence of a collagen fraction either in a random molecular dispersion, small aggregate state, or in some non-random organization different from the typical cross-striated fibril. Gustavson (20) has discussed the swelling and solubilizing effect of concentrated salt solutions, 1 M and above, in the isoelectric range on hide collagen, observing a Hofmeister-type series in which thiocyanate was the most effective solvating agent and sulfate the least, the latter causing less swelling than distilled water. In the experiments reported in this paper, in which relatively low salt concentrations were used, sulfate was found to be a more effective extracting agent than thiocyanate. Sucrose, which is said to be a strong swelling agent for hide collagen even at relatively low concentrations (*ca.* 0.3 M) (20), extracted no collagen from guinea pig dermis. It would appear that solvation of fibrils is not responsible for the extraction of collagen by cold neutral salt solutions as employed here.

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Since cold extraction is required it would appear that insolubilization of this particular collagen fraction is prevented at low temperature. The nearly complete disappearance of extractable collagen during incubation under toluene of the excised dermis at 37°C. for 24 hours prior to extraction indicates that either this fraction is insolubilized irreversibly or is removed by metabolic degradation. It is a plausible hypothesis that polymerization to fibrils occurs *in vivo* in a manner similar to that in tissue extracts warmed *in vitro* (4).

Fessler (22) has recently demonstrated that the collagen extracted from fresh rabbit skin with neutral phosphate buffer, $\mu = 0.14$, when purified consists of three fractions, one of which (*A*) shows completely reversible fibril formation as a function of temperature, *i.e.* soluble when cold and precipitated when warm, a second fraction (*C*) which is irreversibly precipitated as fibrils by warming, and a third fraction (*B*) which cannot be precipitated on warming. This new development suggests that at least a portion of the salt-extractable collagen (fraction *A*) may be present in fibrillar form.

The collagen particles extracted in cold neutral salt solutions appear to be highly asymmetric structures as indicated by the high intrinsic viscosity *ca.* 14.5. This is the same value as that derived for the acid-soluble collagen of calfskin at pH 7.6 (Gross, data unpublished) and somewhat higher than that obtained by Boedtker and Doty (23) for ichthyocol at pH 3.7 ($[\eta] = 11.5$). It seems unlikely that the act of extraction with cold neutral saline could bring about polymerization of small globular sub-units (24–26) to form the highly asymmetric particles present in the extract. It is very probable that the collagen particle extracted in neutral salt solutions has the same dimensions as tropocollagen postulated by Gross, Highberger, and Schmitt (6), measured in solution by Boedtker and Doty (23, 27), and more recently visualized directly by Hall in the electron microscope (28).

The primary purpose of the enzyme digestion experiments was the specific identification of the component or components responsible for the high viscosity of the extracts and the slow moving, hypersharp boundaries observed in the sedimentation and electrophoretic patterns. The collagen fraction is obviously responsible for both. However, one unexpected observation was the abrupt but limited fall in viscosity resulting from the action of trypsin, since fibrous collagen is said to be resistant to this enzyme (see Gustavson (20) for a detailed review). If it were not for the change observed in the heat gelation phenomenon, one might explain this result by assuming that trypsin has attacked a small amount of gelatin, which had contributed to the over-all viscosity. Recently Gallop *et al.* (29) in a report on some properties of purified ichthyocol in neutral solutions (tris, buffer pH 7.0) observed little effect of trypsin at 20°C. on viscosity. However, they were unable to check

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the effect on heat gelation since their solutions did not exhibit this phenomenon.

Fibril formation resulting from warming neutral salt solutions which had been purified as described does not appear to require an interaction with non-collagenous substances. Although a small amount of carbohydrate (hexose) remains in the purified collagen, there is no reason as yet to assume that it takes part in the polymerization to fibrils. Neutral solutions of purified collagen prepared *via* an entirely different fractionation scheme by Jackson and Fessler (5) behaved in a similar manner.

Whether or not some alteration of the collagen molecule takes place rapidly in the crude extract by a thrombin-like enzyme in a way analogous to that in the fibrin-fibrinogen system is still conjectural.

It should be emphasized that the content and properties of the neutral salt extracts are probably only a partial reflection of the intact ground substance, since it is very likely that only a portion of the components are extracted. How the non-collagenous protein components are related to the serum cannot be ascertained by the electrophoretic patterns presented here. Humphrey, Neuberger, and Perkins (30) have recently demonstrated the presence of plasma proteins in rabbit skin in amounts equal to 25 to 30 per cent of the circulating plasma. They also found evidence of considerable amounts of non-plasma proteins.

Finally, it is worth noting that the distribution of components in neutral extracts of skin of different species may not be the same. The ultracentrifuge pattern of phosphate and saline extracts of calf corium obtained by Gross (2) differed markedly from that of guinea pig extracts reported here, although the collagen boundary was present in both.

The invaluable technical assistance of Miss Betty Dumsha and Miss Norma Glazer, is gratefully acknowledged. Ultracentrifuge and electrophoretic analyses were performed in the laboratory of Dr. Karl Schmid.

SUMMARY

Some properties of cold neutral salt extracts of fresh guinea pig dermis have been described in terms of viscosity, electrophoresis and sedimentation patterns, partial composition, the collagen content, conditions for extraction of collagen, and the effect of certain enzymes.

Viscosity of the extracts depended on the collagen in solution as demonstrated by removal of this protein by precipitation or enzymatic degradation. The intrinsic viscosity of the crude 0.45 M extract, as well as that of the isolated collagen was 14.5, identical with that for collagen dissolved by dilute acid, indicating the same high asymmetry ratio for both.

Electrophoresis of the skin extracts revealed a slow moving, high, sharp,

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poorly diffusing boundary in addition to a pattern superficially resembling that of serum. The ultracentrifuge pattern revealed a slowly sedimenting, hypersharp boundary following a large rapidly diffusing peak. The slow moving boundaries in both patterns were abolished by collagenase or heat precipitation of the collagen fraction. Hyaluronidase had no effect on either pattern.

Neutral sulfate, chloride, and phosphate extracted more collagen than did thiocyanate. Very little collagen was extracted at 37°C. as compared with that removed at 3°C.

A two stage fractionation procedure employing dilute trichloroacetic acid and ethanol is described for the isolation and purification of soluble collagen from crude extracts.

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