Some Properties of Neutral-Salt-Soluble Collagen. 1

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Neutral-salt-soluble collagen is a protein, or group of proteins, involved in collagen anabolism (Harkness, Marko, Muir & Neuberger, 1954; Gross, 1958a; Jackson, 1957) and showing the unusual behaviour of aggregating to typical collagen fibrils when its solutions are warmed to body temperature (Jackson & Fessler, 1955; Gross, Highberger & Schmitt, 1955). One available method of isolation was investigated in the present study and the product characterized. The temperature-dependent ability to form fibres was in part reversible and this led to the fractionation of the material into three parts: fraction A, which formed fibres reversibly; fraction B, which did not form fibres; fraction C, which formed fibres irreversibly. The three fractions were characterized. Coupled with the investigation of the isolation procedure was an estimation of the maximum possible phosphorus content of neutral-salt-soluble collagen, and the apparent rate of incorporation of ^{32}P . This part of the work was prompted by the report of phosphohydroxylysine in calf embryo (Gordon, 1948), together with the occurrence of hydroxylysine in collagen (Van Slyke, Hiller & McFadyen, 1941; Desnuelle & Antonin, 1947), and the presence of neutral-salt-soluble collagen principally in rapidly growing animals (Gross, 1958a). Some of these findings have been briefly reported (Fessler, 1957), and further studies on the temperature-dependent behaviour of neutral-salt-soluble collagens are given in the next paper (Fessler, 1960).

Unless specifically stated to the contrary, the term neutral-salt-soluble collagen implies material extracted from the skins of young rabbits and purified by the methods described.

EXPERIMENTAL

Materials

Animals. (1) Steadily growing rabbits, 5–7 weeks old; (2) young rats from the laboratory stock of The Department of Biochemistry, Oxford, weighing about 80 g. and injected intraperitoneally with $50\ \mu\text{C}$ of $[\text{}^{32}\text{P}]\text{phosphate}/100\ \text{g.}$ body wt.

Reagents. These were of analytical grade, except disodium ethylenediaminetetra-acetic acid, which was of best available grade. Glass-distilled water saturated with toluene was used.

Buffers. Phosphate buffer: $0.067\ \text{M-KH}_2\text{PO}_4$ and $0.067\ \text{M-Na}_2\text{HPO}_4$ or K_2HPO_4 were mixed to give buffers of pH 7.0, 7.4 and other values as required; citrate buffer was $0.067\ \text{M-citric acid}-0.033\ \text{M-NaCl}-0.033\ \text{M-NaOH}$, pH 3.7; acetate buffer was $0.067\ \text{M-acetic acid}$ and $0.067\ \text{M-sodium acetate}$ mixed to pH 3.6 (glass electrode); ethylenediaminetetra-acetic acid (EDTA) buffer was disodium salt, $0.62\ \text{g./l.}$, and $0.2\ \text{M-NaCl}$, adjusted with $2\ \text{N-NaOH}$ to pH 6.8 (glass electrode); this EDTA buffer was used in all experiments involving ^{32}P .

Methods

Isolation and purification of neutral-salt-soluble collagen from skin. The scheme of Jackson & Fessler (1955) was used with the following modifications. At all stages, except where mentioned, the Spinco preparative ultracentrifuge was used for clarifying solutions (usually rotor no. 20, 20 000 rev./min. for 1 hr.) and often also for collecting precipitates, particularly when EDTA buffer was used. Materials were precipitated four times by salting-out. In later preparations no sodium salts were used, only potassium compounds, so as to avoid the formation of a crystalline sodium phosphate at high salt concentrations. This substitution of cation had no effect on any of the observed properties of collagen. It was found best to add only conc. KCl solution, and not solid salt, towards the end of each salting-out process. Solutions of collagen were always kept at cold-room temperature ($2-8^\circ$) during all the experiments described in this paper, unless specifically stated to the contrary. Material precipitated with salt was stored at -10° to -20° . To make up a solution from this material, a portion was gently dispersed by a 'mashing' action in a small volume of buffer of 1% acetic acid, exhaustively dialysed against buffer and cleared on the ultracentrifuge.

The preparation from guinea pig of neutral-salt-soluble collagen containing $[\text{}^{14}\text{C}]\text{glycine}$ is described in the next paper.

Phosphorus content. This was measured by the method of Berenblum & Chain (1938). A sample (5 ml.) containing $2-4\ \mu\text{g.}$ of P was ashed with 2 ml. of conc. HNO_3 and $0.5\ \text{ml.}$ of conc. H_2SO_4 for 4–5 hr.; the residue was hydrolysed with about 5 ml. of water by heating to boiling point for 30 min.

Estimation of radioactivity. A Veall (1948) liquid counter of 10 ml. capacity was used together with standard scaling equipment. About 1000 counts were recorded for each sample, which usually took about 1 hr., and corresponding background counts were taken (9–12/min.). Samples were measured only after dialysis against three changes of buffer, and the very small radioactivity, if any, of the last change of buffer was subtracted from that measured for the sample. Radioactivities of heat gels were measured after dissolving the centrifuge precipitate in 10 ml. of the 10% acetic acid.

Preparation of plasma for estimation of radioactivity. Oxalated blood was collected at the decapitation of the animals and the cells were at once removed by centrifuging. To the supernatant was added trichloroacetic acid, to a final concentration of 6% (w/v), and the precipitated protein was filtered off.

Constituents of collagen fractions. These were kindly measured by Miss Betty Dumsha in the laboratory of Dr Jerome Gross by the methods indicated here. Hydroxyproline: Neumann & Logan (1950); proline: Troll & Lindsay (1955); hexosamine: Boas (1953); hexose: Friedmann (1949); glycine: Christensen, Riggs & Ray (1951) and Kroll (1952).

Ultracentrifuges. The following were used: (1) Spinco preparative model L, with rotors and speeds as stated in the text and operated at 2–6° unless otherwise stated. (2) Svedberg oil-turbine ultracentrifuge installed at Oxford (Philpot, 1938; Cecil & Ogston, 1948) operated at 26–28°. (3) Spinco model E ultracentrifuge operated at 4–9°. Sedimentation coefficients, $S_{20,w}$, corrected to water as solvent at 20°, were calculated by the method of Cecil & Ogston (1948). The partial specific volume of gelatin, 0.71 (Cohn & Edsall, 1943), was used in the calculation and was assumed to vary by 0.005/10°, as given by Svedberg & Pederson (1940) for an average protein. The correction of Biancheria & Kegeles (1954) for adiabatic stretching of the Spinco analytical rotor was applied.

Couette viscometer. The apparatus of Ogston & Stanier (1953) was used, modified so that the torsion head was rotated to constant position of the indicating light beam. Position of the torsion head was read to 0.1° of arc.

Double refraction of flow. The apparatus of Ogston & Stanier (1953), modified for shear rates up to 1000 sec.⁻¹, was used. All measurements were made in duplicate, with alternate directions of rotation.

Refractometer. The differential refractometer of Cecil & Ogston (1951) was used. Protein concentrations were calculated on the basis of an assumed specific refractive increment of 0.00184 refractive-index units/g./100 ml. (Barer, 1954), as insufficient material was available with which to determine a more reliable value.

Spectrophotometer. This was a Hilger Uvispek.

Polarimetry. A Hilger polarimeter, reading to 0.01° of arc, was used with sodium light. The temperature of the 10 cm. cell was controlled to $\pm 0.25^\circ$ by a water thermostat. The specific optical rotation is calculated as the rotation which would be caused by 10 cm. of solution containing 1 g. of material/ml.

'Heat gellation' and 'gel ratio'. To precipitate collagen as a gel by warming, samples were placed in stoppered tubes in a 37° water bath for 1 hr., unless other times or temperatures are stated. The tubes were then either gently transferred to a refrigerator (2–4° in all experiments), or the gel was quickly fragmented with a small glass rod and the precipitate sedimented in a centrifuge rotor warmed to 35°. Tubes were either Lustroid for Spinco rotor 40 (39 000 rev./min. for 20 min.), or 7.5 cm. $\times$ 1.2 cm. glass for a small-angle centrifuge (9000 rev./min. for 10 min.). Medicinal paraffin at 35° was added to fill the residual space of Spinco tubes. Supernatants were withdrawn with a Pasteur pipette. A measure of the amount of material precipitated was defined by the term: gel ratio = 100 $[(n_p - n_s)/n_p]$, where n_p is the refractive increment of the original solution with respect to the buffer against which it had been exhaustively

dialysed, and n_s is the refractive increment of the supernatant against the same buffer. Where specifically stated in the text, other parameters, such as absorption at 275 m μ , were similarly used to define further 'gel ratios'.

Separation of neutral-salt-soluble collagen into fractions A, B and C. Clarified and dialysed solutions, containing 0.2–0.3 g. of collagen/100 ml. at pH 7.4 were warmed to 37° for 1 hr. in Lustroid ultracentrifuge tubes, stored in the refrigerator for 62 hr. and the residual gel (fraction C) was fragmented and centrifuged off (rotor 40, 39 000 rev./min., 20 min., 4°). The supernatant was then gelled at 37° for 1 hr., and the gel was immediately fragmented and centrifuged (rotor 40, 39 000 rev./min., 10 min., 33°); the residue was fraction A and the final supernatant fraction B. Residues A and C were gently dispersed in ice-chilled 1% acetic acid and dialysed against 1% acetic acid overnight. An appreciable part of fraction C did not dissolve. After exhaustive dialysis against phosphate buffer, pH 7.4, the solutions were clarified (Spinco rotor 40, 39 000 rev./min., 1 hr.).

RESULTS

Studies on the isolation of neutral-salt-soluble collagen

The neutral-salt-soluble collagen isolated by Jackson & Fessler (1955) was heterogeneous (Fessler, 1957), and so it became necessary to investigate the method of isolation. It is shown below that even material obtained after extended purification along the lines of Jackson & Fessler (1955) is heterogeneous.

Pooled extracts were obtained from the skins of eight young rats which, 2 hr. before being killed, had been given ³²P. The purification procedures of precipitation with salt were repeated on these extracts until the material attained constant properties, as judged by the following criteria: (1) the distribution of material between gel and supernatant formed on warming, in terms of (a) refractive increment ('gel ratio'), (b) absorption at 275 m μ , and (c) radioactivity; (2) (a) the total content of ³²P, and (b) the total absorption at 275 m μ ; (3) the form of the u.v.-absorption curve. Total absorption at 275 m μ decreased somewhat between the last two of the four repetitions of the purification procedure. The results are shown in Figs. 1–3. It is known (Gustavson, 1956) that collagen has an affinity for phosphate ions, and although large volumes of the EDTA-sodium chloride buffer were used in the preparation, there was the possibility that the observed radioactivity was due to phosphate adsorbed on collagen. A large excess of phosphate buffer was added to parts of three samples before heat-gelling, and this should have affected the distribution of radioactivity between heat gel and supernatant, if the radioactivity was due to adsorbed phosphate. As is shown in Fig. 1, such an alteration of the distribution was obtained only in the less purified samples.

A phosphorus determination in one of the supernatants which had been formed after addition of phosphate buffer showed that only little of the added phosphate had been removed by the gel.

The practical conclusion from these experiments and evidence from Fig. 1 show that the purification

Fig. 1. Effect of successive salt precipitations of neutral-salt-soluble collagen on the distribution of material between supernatant and gel formed by warming to 37°. Abscissa represents the number of salt precipitations. Ordinates represent the following ratios: ●, (refractive increment of gel)/(refractive increment of parent solution); *, ($E_{275m\mu}$ of gel)/($E_{275m\mu}$ of parent solution); ○, (refractive increment of supernatant)/(refractive increment of gel); ×, ($E_{275m\mu}$ of supernatant)/($E_{275m\mu}$ of gel); □, (counts/hr. from supernatant)/(counts/hr. from gel); Δ is a repeat of □, but excess of 0.067M-phosphate buffer, pH 6.8, was added before warming to 37°. (Value for gel) = (corresponding value of parent solution) - (value of supernatant).

Fig. 2. Effect of successive salt precipitations on the composition of neutral-salt-soluble collagen prepared from rats injected with ^{32}P ; ▲, variation of specific radioactivity with number of salt precipitations indicated as abscissa; Δ, corresponding specific absorption coefficients at 275 $m\mu$.

Fig. 3. Effect of successive salt precipitations of neutral-salt-soluble collagen on its absorption spectrum. All curves were corrected to $E_{275m\mu}$ of 0.50. Absorptions decreased in step with purification: the top spectrum was obtained after one precipitation. Arrows indicate the location of subsidiary maxima at 257 and 267 $m\mu$.

method of Jackson & Fessler (1955) should be taken to three or four salt precipitations, and that further repetition is unlikely to change the material. The observed radioactivity was too low to allow detailed interpretation, but suggested that the radioactive phosphorus was not due to simple contamination with inorganic phosphate and so warranted some further study. The net zero count obtained with supernatant of sample 3 (Figs. 1, 2) is probably fortuitous, as the solution was rather dilute. The ultraviolet-absorption studies were hindered because they had to be done rapidly at room temperature, before gellation could set in, and so measurements were only taken at 2.5 $m\mu$ intervals. A number of curves similar to those shown in Fig. 3, but omitted from that Figure for clarity, were obtained on similarly prepared material. Various small peaks or shoulders were found in the range 255–270 $m\mu$, and the minimum around 250 $m\mu$ was deeper for samples which had undergone more salt precipitations, or had been ultracentrifuged more exhaustively. The peak at 275 $m\mu$ was always definite.

Studies on the incorporation of radioactive phosphorus

Sets of four rats were given ^{32}P , and killed 2, 10, 18, 36 and 94 hr. after administration. Neutral-salt-soluble collagen was isolated as given in the Experimental section, with EDTA buffer and four sodium chloride precipitations. The final material

was taken up in EDTA buffer and after exhaustive dialysis clarified on the ultracentrifuge. Then it was dialysed against three changes of acetate buffer, pH 3.6, which ensured that no heat-gellation would occur during prolonged measurements at room temperature. Refractive increment, ultraviolet absorption, radioactivity and phosphorus content were measured on each sample. Radioactivity and phosphorus content were also measured on corresponding samples of the supernatant, obtained at the first sodium chloride precipitation of the skin extracts, and of samples of plasma from blood collected at death.

The results are given in Fig. 4: 100 mg. of neutral-salt-soluble collagen contained 9.2 μ g. of phosphorus (mean of four samples; range 8.6–9.6 μ g. of phosphorus).

Heterogeneity of neutral-salt-soluble collagen as shown by its behaviour on warming

When a solution of neutral-salt-soluble collagen is warmed, most, but not all, of the material is

Fig. 4. Specific radioactivity of phosphorus from samples of neutral-salt-soluble collagen prepared at different times after administration of ^{32}P to rats. x, Neutral-salt-soluble collagen; O, residue of skin extracts after removal of neutral-salt-soluble collagen; Δ , plasma obtained at time of death.

precipitated, i.e. the 'gel ratio' is less than 100%. From Fig. 1 it is evident that the gel ratio is not changed by purification beyond a certain point. The concentrations of materials which were gellable in different samples, obtained both from rabbit and rat, are plotted in Fig. 5 against the concentrations of the initial solutions. A straight line fitted to these points by the method of least squares indicates a common gel ratio of 84% (83.7 ± 1.5). Slight variations of experimental procedures do not appear to alter this gel ratio substantially. Thus the pH of samples represented in Fig. 5 ranged from 6.7 to 7.4, the temperature from 28° to 37°, the method of centrifuging from ultracentrifuging at 35° to quick manipulation on a bench centrifuge at room temperature. Rabbit material prepared particularly carefully had a slightly higher gel ratio, about 88%. The material which remains in solution is not dialysable, and has been named fraction B.

The time course of the precipitation of collagen as gel at 37° is shown in Fig. 6, in which the concentration of ultracentrifuge supernatant is plotted against length of time for which the sample was incubated at 37°. Similar curves were obtained for neutral-salt-soluble collagen from rats and from guinea pigs. The standard time of warming was chosen as 1 hr., because little more material precipitated upon longer incubation (Fig. 6). The first stages of the process were followed at 22°, when the rate of precipitation is much slower, and indicated an S shape for the overall precipitation curve.

When a heat gel of neutral-salt-soluble collagen is left undisturbed in the refrigerator, the gel becomes less opaque and less rigid. This suggests that partial reversal of the heat-gellation phenomena occurs, and to investigate this the experimental

Fig. 5. Variation of the concentration of material which can be gelled with the concentration of the parent solution of neutral-salt-soluble collagen.

scheme of Fig. 7 was devised. The techniques described in the section on Methods were used. A set of 0.6 ml. samples was taken from a solution of known concentration and warmed to 37° for 1 hr. in Lustroid Spinco tubes. One tube was centrifuged at 35° and the concentration of supernatant measured; the other tubes were placed in the refrigerator. After known intervals of about 2, 4 or 6 days' cold storage, the remaining gels were centrifuged off and the refractometric concentrations of the supernatants determined. The results can be plotted as curves such as Fig. 8 (1). The total refractive increment corresponding to a point on curve (1) (Fig. 8) is composed of (a) refractive increment of material which did not gel on warming, (b) refractive increment of material which has dissolved from the gel, (c) any other change in refractive index of the mixture. In order to determine the concentration of redissolved material, the supernatants were warmed to 37° for 1 hr. and then centrifuged, warm, in a small-angle centrifuge. The refractive increments of the final supernatants were measured and could be plotted as curves such as Fig. 8 (2). The differences between corresponding points of curves (1) and (2) in Fig. 8 represent the concentrations of material which had gelled on warming to 37° and had subsequently dissolved on cooling: these differences are plotted as curve (3) in Fig. 8. The ordinate scale of Fig. 8 is a concentration scale, and different units of concentration may be chosen: the left scale is in g./100 ml., the right scale is in per cent, with 100% equal to the concentration of total gellable material in the original solution. If all the material which gelled originally had redissolved again, then curve (3) (Fig. 8) should rise to 100%; in fact, a plateau is reached at about 70%, consistent with the residual

Fig. 6. Time course of the precipitation of neutral-salt-soluble collagen in 0.067 M-phosphate buffer. The large curve shows results obtained at pH 7.4 and 37°. The insert shows the initial stages of precipitation at pH 6.8 and 22°.

gel which was always found in these tubes. This suggests that the gellable material consists of two fractions: one which redissolves on cooling, named fraction A, and one which does not redissolve, named fraction C; the results of duplicate experiments are plotted in Fig. 8. Experimental difficulties are illustrated both in the scatter of points and in the rise of curve (2) with time. The errors were principally due to repeated transfers and ultracentrifuging of small volumes of solution; the refractive index would also be affected by quite small changes of water content, such as would occur on storage in Lustroid tubes and in centrifuging under medicinal paraffin. Results closely similar to those of Fig. 8 were obtained with guinea-pig neutral-salt-soluble collagen containing

Fig. 7. Scheme of alternate warming and cooling of neutral-salt-soluble collagen. Stage 1 represents division of initial solution into several samples of 0.6 ml. and one sample (*) which contains enough solution for recycling experiments. Gels formed on warming are indicated as shaded areas. Density of shading represents the opacity of a gel, which decreases on cold storage. At stage 2 each sample is divided into a supernatant solution and a sedimented gel of collagen, the size of each shaded area representing the relative amount of gel. Most of the supernatant corresponding to sample (*) of stage 1 is made the initial solution for a further experimental cycle. Solutions are analysed for gellable collagen by the scheme enclosed with broken lines.

[^{14}C]glycine and by using radioactivity in place of refractive index as a measure of concentration.

This method of analysis by change of phase with temperature was repeated, varying the sample, the initial concentration of material and the pH of the phosphate buffer. The results are collected in Table 1. The concentration of fractions A and C

were determined on three or four plateau points, equivalent to curve (3) (Fig. 8), and the values after storage for about 100 hr. in the refrigerator are given; after 150 hr. there was sometimes a further slight rise in the curve corresponding to some more gel dissolving.

The behaviour of fraction A in the absence of fraction C was studied by recycling solutions through the scheme of Fig. 7. As before, samples of the solution were heat-gelled and allowed to redissolve by standing in the refrigerator. A sample (8 ml.) was used for the portion that was removed after 2 days from the refrigerator and centrifuged in the cold. The supernatant formed the starting material for a further complete analysis by the scheme of Fig. 7, and the whole process was repeated once more in one experiment. The results are given in Fig. 9 and are plotted as a percentage of gellable material redissolved against time, i.e. analogous to curve (3) of Fig. 8; for each curve the 100% unit of concentration is the total gellable material in the starting solution of that cycle. The gels formed in the second and third cycles of the scheme of Fig. 7 seemed to disappear completely on standing in the refrigerator, and the curves of Fig. 9 are in agreement with this. Recovery of gellable material must in part have been affected by losses during the repeated handling. The total evidence for the existence of the three separate fractions A, B and C will be evaluated in the Discussion section.

Fig. 8. Time course of the re-solution of collagen fibres at 2–4°. Solid and open symbols are results from duplicate experiments. Solutions of neutral-salt-soluble collagen, having concentration $\times$, were warmed to 37° for 1 hr. and then kept at 2–4°. Curve (1) shows the increase in the concentration of total material in solution with time. The concentration of material in solution was decreased to the values of curve (2) on warming to 37° for 1 hr. corresponding isolated solutions represented on curve (1). Curve (3) is a plot of the difference between corresponding values of curves (1) and (2). Left-hand ordinate is the refractometric concentration measured. Right-hand ordinate is on a % scale, with 100% as the concentration of all the material which precipitated on initially warming to 37° for 1 hr.

Solution of neutral-salt-soluble collagen in acetic acid and acetate buffer

Gels of neutral-salt-soluble collagen can be dissolved in dilute acetic acid and this was made use of in separating the fractions of neutral-salt-soluble collagen. The following experiments showed that re-solution via dilute acetic acid did not alter (1) the amount of material gelled at 37° (at pH 7), (2) the specific optical rotation and (3) that true solution occurred as judged by ultracentrifuging.

Table 1. *Composition of different samples of neutral-salt-soluble collagen*

Samples were dialysed against 0.067 M-phosphate buffer of stated pH and their concentrations were adjusted to the tabulated initial values. Compositions were then determined by the method given in the text. For preparation of fractions A, B and C see text.

Sample	pH	Initial	Concentration (g./100 ml.)			Percentage composition		
			Fraction			Fraction		
			A	B	C	A	B	C
I	7.4	0.463	0.233	0.093	0.137	50	20	30
II	7.2	0.327	0.154	0.033	0.140	47	10	43
III	7.0	0.346	0.177	0.029	0.139	51	9	40
IV	7.0	0.238	0.110	0.044	0.084	46	18	35
V	7.0	0.192	0.093	0.047	0.058	49	24	30
VI	7.0	0.163	0.071	0.040	0.053	43	24	32
VII	6.8	0.314	0.180	0.048	0.086	57	15	27
VIII	6.65	0.334	0.183	0.034	0.116	55	10	35
IX	6.5	0.314	0.161	0.037	0.117	51	12	37

(1) A portion (0.72 ml.) of a known solution of neutral-salt-soluble collagen in phosphate buffer, pH 7.0, was placed in a dialysis sac, and this in a small volume of buffer in a test tube. The whole was warmed to 37° for 75 min. and then cooled to 4°, care being taken throughout not to disturb the sac. This was now gently transferred to cold 1% acetic acid and after dialysis for 2 hr. the contents were fluid and clear. After exhaustive dialysis against phosphate buffer, the contents of the sac were quantitatively collected and weighed. The clear solution was ultracentrifuged (rotor 40, 39 000 rev./min., 40 min.) and its gel ratio determined in the usual way, as was that of a control.

The calculated total gellable material placed into the sac was 1.40 mg. and that recovered was 1.39 mg.

(2) The previous experiment was repeated with a larger volume of solution; the initial and final solutions were dialysed to pH 7.4 and their specific optical rotations measured: start -376°, end -378°.

(3) In the cold a solution of neutral-salt-soluble collagen was exhaustively dialysed against acetate buffer, pH 3.7. In the analytical ultracentrifuge a sample showed only the usual single collagen peak. The remaining solution was centrifuged (rotor 40, 39 000 rev./min., 1 hr., 4°) and no material was thereby removed: initial concentration 2.20 mg./ml., final concentration 2.19 mg./ml.

Fig. 9. Percentage re-solution at 2-4° of fibres of neutral-salt-soluble collagen cycled repeatedly through the scheme of Fig. 7. First cycle: O, x; second cycle: □, ▽; third cycle: ●. The value of the point shown in parentheses is uncertain.

Fig. 10. Variation of reciprocal of sedimentation coefficient of neutral-salt-soluble collagen with concentration at 4-9° (O) and 26-28° (x). Bars indicate the standard deviations of values (preparation P.1).

Table 2. Summary of physical measurements on neutral-salt-soluble collagen

Native materials were separate preparations which had not been fractionated. 'Re-dissolved' material had been subjected to the fractionation procedure without separating the fractions. Concentrations are in g./100 ml.

$[\eta]_c$ = intrinsic viscosity = $\text{Lt}_{c \rightarrow 0} \left(\frac{[\eta_0] - 1}{c} \right)$, where $\eta_0 = \text{Lt}_{G \rightarrow 0} (\eta_R)_G$, $(\eta_R)_G$ = relative viscosity at shear rate G ; $J = a/b$, where a = semimajor and b = semiminor axis of prolate ellipsoidal particle of effective specific volume V' , particle weight M and rotary diffusion coefficient θ . For preparation of fractions A, B and C see text.

Preparation	$10^{13} S_{20,w}^0$	$10^{-13} \left[\frac{d(1/S_{20,w})}{dc} \right]$	$[\eta]_c$	θ (sec. ⁻¹)	J	V' (ml./g.)	$10^{-5} M$	$2a$ (Å)	$2b$ (Å)
Native									
P.1	3.23	1.68	47	220	42	36	9.8	3400	80
P.2	2.76	1.03	28	—	43	19	5.5	—	—
P.3	2.76	0.81	57	360	83	14	6.2	3100	40
Fraction A									
P.3.A	2.87	1.08	20	440	35	21	4.4	2600	75
Fraction B									
P.3.B	2.97	0.33	60	—	152	5	5.9	—	—
Fraction C									
P.3.C	2.84	0.99	33	—	50	19	5.1	—	—
'Re-dissolved'									
P.3.K	2.86	0.94	36	—	55	18	5.1	—	—

Physical properties of neutral-salt-soluble collagen and of separated fractions A, B and C

Sedimentation, Couette viscosity and streaming birefringence measurements were made on three separate preparations (P. 1, P. 2, P. 3), of neutral-salt-soluble collagen, on the fractions A, B, C into which one of these (P. 3) was divided, and on a control sample (P. 3.K) in which P. 3 was subjected to a process used in fractionation, namely gelling at 37° for 1 hr. and subsequent re-solution via 1% acetic acid. Preparation P. 1 had not been clarified by ultracentrifuging. Samples were kept at 4° throughout, except during sedimentation in the Svedberg ultracentrifuge and during refractometry at room temperature. Each preparation was usually investigated at five concentrations (0.01–0.09 g./100 ml.) and the following properties were always found.

Sedimentation. Schlieren diagrams consisted of a single sharpened peak. Sedimentation behaviour was the same in the cold (4–9°, Spinco) and at room temperature (26–28°, Svedberg) when compared for one preparation, P. 1, as is shown, after the usual correction to water at 20°, in Fig. 10. Sedimentation coefficients at zero concentration $S_{20,w}^0$ were obtained by extrapolation of similar linear plots of $1/S_{20,w}$ against concentration and are given in Table 2.

Viscosity. Relative viscosity $(\eta_R)_G$ varied with shear rate G , an example being given in Fig. 11. At greater dilutions $(\eta_R)_G$ was independent of shear rate at all but the lowest shear rates. Relative viscosities at zero shear rates η_0 were obtained by extrapolation of $1/(\eta_R)_G$ against G . The specific viscosities $[\eta_0 - 1]/c$ were found to be independent of concentration, at concentrations less than 0.08 g./100 ml. The intrinsic viscosities

$$[\eta]_c = \left\{ \text{Lt}_{c \rightarrow 0} \frac{[\eta_0 - 1]}{c} \right\}$$

are given in Table 2.

Flow birefringence. At a given shear rate, G , the angle of isocline, χ , varied with concentration of the sample, and was linearly extrapolated to zero concentration. The rotary diffusion coefficient θ was calculated for a long rod from χ and G by the treatment of Scheraga, Edsall & Gadd (1951). The variation of θ with G is shown for some samples in Fig. 12.

Calculation of molecular parameters. Particle weight, hydration, axial ratio and length were calculated by the 'porous-plug' treatment of Ogston (1953) and are given in Table 2. With the axial ratio obtained in this way, the length of the particles was also calculated from flow-birefringence measurements by the method of Scheraga *et al.* (1951). For these calculations a partial specific volume of 0.71 and a specific refractive

Fig. 11. Variation of relative viscosity (η_R) of solutions of neutral-salt-soluble collagen with shear rate at different concentrations (g./100 ml.): *, 0.08; O, 0.06; Δ, 0.04; □, 0.015.

Fig. 12. Variation of rotary diffusion coefficient θ with shear rate G of neutral-salt-soluble collagen: sample P.1 (●), sample P.3 (×) and the fractions A (▲) and C (+) into which sample P. 3 was separated.

increment of 1.84×10^{-3} refractive-index units/g./100 ml. were assumed, as experimental difficulties precluded obtaining more accurate values. The assumed partial specific volume is that of gelatin (Cohn & Edsall, 1943), and the specific-refractive increment value is representative of proteins (Barer, 1954).

Composition of separated fractions A, B and C. Proline, hydroxyproline, glycine, hexosamine and hexose contents were measured by Miss Betty Dumsha in the laboratory of Dr Jerome Gross on the separated fractions of two samples. No significant differences were found between the fractions, no hexosamine and only traces of hexose were found. Hydrolysates of fraction B contained material which interfered with the glycine estimation by the method of Christensen *et al.* (1951), and therefore glycine was isolated and estimated as the dinitrobenzene derivative (Kroll, 1952).

DISCUSSION

The author's principal interest was in the ability of an inherently fibrous material, collagen, to change from solution to fibre by warming from 4° to 37°, and the partial, though incomplete, reversal of this change on cooling. The initial investigations (Jackson & Fessler, 1955) were made with material isolated by the salt-precipitation method of Jackson and it was therefore necessary to study this method in greater detail. The possibility that phosphohydroxylysine, found in calf by Gordon (1948), could be a constituent of such material was investigated at the same time.

Isolation of neutral-salt-soluble collagen and its composition. From the results given in Figs. 1 and 2 it is concluded that repeated salting-out of neutral-salt-soluble collagen gives a product of constant properties after three such precipitations. Contamination with nucleic acid-like material (u.v. curve, Fig. 3) is thereby successively decreased to the limits of detection, hexosamine-containing materials are eliminated and hexose is decreased to trace amounts. Jackson, Leach & Jacobs (1958) have made a complete amino acid analysis on material similarly isolated, and the composition was comparable with other isolated collagens (Kendrew, 1954). The present analyses were made only to detect any relative differences between the separated fractions, and no such differences were found. The high specific optical rotation of -377° is similar to that of other isolated soluble collagens (Cohn, 1955). How far the isolation procedure has affected the original state of aggregation of the material *in vivo* is not known, though the possible disruptive effects of high ionic strength and acid pH, used in other extractions, were avoided. The salted-out precipitates were amorphous under the

electron microscope. Some irreversible aggregation could have been caused during salting out and storage, as subsequent resolution was at times incomplete.

Phosphorus content and rate of incorporation of ^{32}P . A small but definite phosphorus content of material isolated by salt precipitation is indicated from the results (Figs. 1 and 2) and was determined. This phosphorus could be due to (1) adsorbed inorganic phosphate, (2) phosphorus constituent of some trace contaminant, in particular a nucleic acid, (3) phosphorus as a true constituent of these collagens. Swamping experiments with 'cold' phosphate buffer make simple adsorption unlikely, though it cannot be excluded. Contamination with nucleic acid is a very possible explanation, the measured phosphorus content being within the error of the final u.v.-absorption curves, which indicated absence of nucleic acids. When, however, the material was gelled at 37°, the radioactivity was distributed between solution and solid in the same proportion as the bulk collagenous material (measured by refractive increment). The relationship between time after administration of radioactive phosphate and the specific activity of the phosphorus in the corresponding sample of purified collagen (Fig. 4) was similar to that obtained by Harkness *et al.* (1954) for the incorporation of [^{14}C]glycine into such collagen. Lastly, the curves of Fig. 4 show that simple, random contamination of the extracted collagen did not occur, as the specific activity of the skin residues was at times lower than that of collagen, though contamination by a small amount of highly active material was certainly not ruled out. If phosphorus were a true constituent of neutral-salt-soluble collagen, there would, according to the results, be one atom of phosphorus per particle of 340 000 molecular weight units. Such a particle would contain seven hydroxylysine residues (Kendrew, 1954), but only one of these could be phosphorylated. Phosphohydroxylysine is thus not likely to be a constituent, or a constituent of any importance, of collagen.

Heterogeneity of neutral-salt-soluble collagen as shown by its behaviour on warming and cooling. The results given in Figs. 5-9 and Table 2 lead to the conclusion that in solutions of neutral-salt-soluble collagen three fractions could be distinguished: fraction A, which formed fibres at 37°, that completely dissolved again on cooling; fraction B, which did not precipitate at 37°; fraction C, which irreversibly formed fibres at 37°. (If fibres of fraction C do redissolve at 4°, they do so extremely slowly.) Complex solubility phenomena are likely in a system of rod-like particles that aggregate in an integrated manner into long fibres, and so the concept of separate fractions should be viewed with scepticism. Within the range in which the material

could be studied, however, the relationship between the fractions was independent of absolute concentration, pH and time, provided that sufficient time for equilibration had been allowed (e.g. Figs. 5 and 6 and Table 1); constancy of relationship was indicated when concentrations were expressed in relative percentage form. Moreover, if the sequence of warming and cooling was repeated according to the scheme of Fig. 7, reproducible results were obtained, as shown in Fig. 9; that is, once the initial portion of fraction C (irreversible fibre formation) was removed, all subsequently made fibres dissolved on cooling and the proportion of material which did not form fibres (fraction B) also remained constant. Further, such quantitative behaviour was shown independently of the volumes of solution used, nature of containing vessels, gross appearance of the gels and, within limits, of temperature and time of warming. Thus it is unlikely that the observed fractions were simply artifacts inherent in the polymerization process which occurred at 37°. Certainly the first warming to 37° could have wrought some irreversible change. The matter will be further discussed in a following paper (Fessler, 1960), in which further evidence is considered. The principal conclusions are that (1) on the basis of warming and cooling the material appears to be heterogeneous, and (2) that a part of neutral-salt-soluble collagen reversibly forms fibres, according to temperature (previously reported, Fessler, 1957). Gross (1958*b*) extended the time of incubation at 37° to as much as 24 hr. and found that on subsequent cooling the re-solution of his collagen was greatly decreased in neutral buffers. He made the suggestion that upon prolonged maintenance of collagen at 37° the constituent particles of fibres become arrayed in a progressively more ordered crystalline state, making subsequent re-solution more difficult. Although this may possibly be so, the present findings cannot be fully interpreted along these lines: successive incubations at 37° for 1 hr. of a given sample should yield equal proportions of material which does not dissolve on cooling, whereas this occurred only on the first incubation (fraction C, see scheme of Figs. 7 and 9). Both rate and equilibrium processes are involved: to obtain full re-solution of the fibres of fraction A the material had to be kept for at least 48 hr. at 4° (Fessler, 1957) whereas Gross (1958*c*) principally used overnight storage in the cold.

Physical properties of neutral-salt-soluble collagen and re-solution of precipitates in dilute acetic acid. The variations of rotary diffusion coefficient (θ) with shear rate (G), shown in Fig. 12, indicate heterogeneity of all the materials studied, with the possible exception of fraction A. The single peak of the ultracentrifuge schlieren diagrams, together with the evidence discussed above, lead one to

regard this heterogeneity as due to a range of particles of basically one material, rather than due to radically different materials. Boedtker & Doty (1956) found with acid-soluble fish collagens that homogeneous material occurred only in the supernatant of an extensively ultracentrifuged solution, all other material being discarded. Such extensive ultracentrifuging would have narrowed the sampling more than was desired in the present study. The particle parameters given in Table 2 therefore represent some form of average and hence little was to be gained by any more refined treatment of calculation; similarly, the assumptions made in choosing partial specific volume and specific refractive increment cannot have introduced any appreciable error.

The conclusions drawn from the results given in Table 2 are that the neutral-salt-soluble collagens are highly elongated particles, of axial ratios of the order of 50, a few thousand Ångström units long and with a particle weight of some 500 000. Given the estimated length, the magnitude of the hydrodynamically hydrated volume suggests a rod-like particle. Contamination of any one sample with a relatively small amount of a higher aggregate of such nature would have increased the relative viscosities measured at low shear rates and decreased the apparent rotary diffusion coefficients, and as a result the stated particle weight, length and axial ratio would be somewhat higher than the representative values. Considerable particle-particle interaction was indicated both by the shape of the sharpened schlieren peak in the sedimentation diagram and by the variation of the angle of isocline at a given shear rate with concentration. The effects of such interactions should have been minimized by the extrapolation of all values to zero concentration. Differences in the parameters calculated for the different samples are on the whole not likely to be significant. The largest particle weight of 9.8×10^5 was found for the crudest preparation P.1, which had been obtained before a preparative ultracentrifuge was available.

The difference between P.3 and P.3.K, which was made from P.3 by gellation (without fractionation) and re-solution in 1% acetic acid, suggests that dilute acetic acid may have some dispersing action on possible aggregates. Under the specified conditions, treatment with dilute acetic acid did not cause degradation to gelatin, as both the amount of gellable material and the specific optical rotation were not thereby changed. Although ultracentrifuge studies showed that true solution of these neutral-salt-soluble collagens occurred in 0.067 M-acetate buffer, pH 3.6, this was found not to occur in 0.067 M-citrate buffer of similar pH, which gave a dispersed material that was in part readily

removed by ultracentrifuging. This stands in contrast with the acid-soluble collagens that have been studied in such citrate buffers. It may be significant that not all of the heat gel of fraction C could ever be dissolved in 1% acetic acid, and further that some of the material which did dissolve was subsequently precipitated on exhaustive dialysis against phosphate buffer. The results on measurements of fraction C can thus only be representative of a part of it. In contrast, all of fraction A was recovered by such re-resolution procedure. The particle weight and length calculated for fraction B may easily be too large, as only dilute solutions of this fraction were available and little contamination with higher aggregates would have appreciably affected the results.

No striking differences between the three fractions were thus observed by the physical methods employed.

Further discussion is in the corresponding section of the following paper. It must be emphasized that the results and conclusions of this work may only be applicable to the particular materials used, as unfortunately we know little about the relationships between the various collagens which different investigators have studied in solution. The generic name neutral-salt-soluble collagen does not imply identity; indeed the materials used here have different solubility properties from those isolated by Gross *et al.* (1955) from calf skin.

SUMMARY

1. Neutral-salt-soluble collagen was extracted from the skins of young rabbits and rats and purified by repeated salting-out. The purification process was studied.

2. This neutral-salt-soluble collagen was separated into three parts: (a) fraction A, which reversibly formed fibres at 37°, the fibres dissolving again on cooling, (b) fraction B, which did not form fibres on warming to 37°, and (c) fraction C, which on warming to 37° formed fibres that did not dissolve on subsequent cooling.

3. The proportions of these fractions were found to be independent of concentration and pH over a limited range.

4. Evidence for the existence of three fractions was obtained from a study of the effects of repeated warming and cooling.

5. The original material and the separated fractions were investigated by sedimentation, viscosity and streaming birefringence analyses. No significant differences were found between the fractions. A representative particle was estimated as a highly elongated rod, several thousand Ångström units long, with a particle weight of about 500 000.

6. No differences in the hydroxyproline, proline or glycine contents of the separate fractions were found. Hexose and hexosamine contents were negligible.

7. Neutral-salt-soluble collagen was found to contain not more than one atom of phosphorus in a particle of 340 000 molecular-weight units. The phosphorus is probably present as an impurity.

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Some Properties of Neutral-Salt-Soluble Collagen. 2

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It was shown in the preceding paper (Fessler, 1960) that a purified preparation of neutral-salt-soluble collagen could be separated into three closely similar fractions. The significant difference between these fractions was their solubility behaviour at 4° and 37°: fraction B remained in solution at both temperatures, fractions A and C were in solution at 4° and formed typical collagen fibres at 37°, the fibres of fraction A completely dissolved again on subsequent cooling but those of fraction C did not. The physical and possible physiological relationships between these fractions have been further explored and the nature of the temperature-dependent process of fibre formation studied.

EXPERIMENTAL

Unless specifically stated, the experimental techniques were those previously described (Fessler, 1960).

Preparation from guinea pig of neutral-salt-soluble collagen labelled with [¹⁴C]glycine. Suckling animals, which had steadily been gaining weight to a final weight of 160–225 g., were each given 100 µc of [¹⁴C]glycine intraperitoneally and killed 6–7 hr. later by heart puncture. The hair was clipped off and skins, free of muscle layers, were removed and minced in a chilled grinder. The mince was shaken overnight with twice its weight of buffer (0.12 M-KCl–0.016 M-K₂HPO₄–0.004 M-KH₂PO₄, pH 7.4) and the suspension was centrifuged (Spinco rotor 21, 15 000 rev./min., 2 hr.). The supernatant was kept and the skin residues were re-extracted by the same process. Neutral-salt-soluble collagen was isolated from the pooled supernatants by repeated salting-out as previously described (Fessler, 1960).

Contamination of the purified neutral-salt-soluble collagen by [¹⁴C]glycine was ruled out by the following experiment. To a solution of 0.6 mg. of neutral-salt-soluble collagen, equivalent to 1.6×10^4 counts/min., was added

10 mg. of glycine and the mixture was dialysed against phosphate buffer. The maximum total radioactivity in the external medium was 70 counts/min., and more than 80 % of the glycine expected at equilibrium was recovered from the external medium [the glycine was isolated by chromatography on Dowex 50 and estimated by the method of Yemm & Cocking (1955)].

Neutral-salt-soluble collagen from rabbit. This was prepared as described by Fessler (1960).

Acid-soluble collagen from rabbit. The skin residues left after five extractions of neutral-salt-soluble collagen with 0.067 M-phosphate buffer, pH 7.0 (Fessler, 1960), were extracted with 0.067 M-citrate buffer, pH 3.7. Collagen was precipitated from the clarified extract by adding NaCl to 5% (w/v). The precipitate was redissolved in citrate buffer and precipitated by further addition of NaCl. This was repeated twice. Finally, the material was precipitated by dialysis of the citrate solution against tap water, followed by distilled water.

Radioactivity of protein. This was measured as the time required for 1000 or 10 000 counts to be recorded by a gas-flow counter on 0.1 or 0.2 ml. of sample evenly dried as an 'infinitely thin' layer on a planchet. The linear relationship between counts/min. and concentration, obtained on a set of five serially diluted samples, had a standard deviation of 2.5%.

Specific radioactivity of the glycine of collagen. Equal parts of collagen solution and conc. HCl were mixed and, after hydrolysis at 135° for 3 hr., water and HCl were removed *in vacuo*. The amino acids in duplicate samples of hydrolysate, corresponding to 300 µg. of collagen each, were converted into 2:4-dinitrophenyl (DNP) derivatives and DNP-glycine was isolated by the method of Kroll (1952); the final solution was made in N-NH₃ solution in place of the bicarbonate solution used by Kroll. Absorption at 365 mµ and radioactivity were measured on a set of several dilutions of each ammoniacal solution. For plating out, carageenin (Seapant Chemical Corp., New Bedford, Mass., U.S.A.) was added to 0.01 % final concentration and 0.9 ml. of the mixture was evaporated on planchets in duplicate.

Solubility measurements on fraction A. Fraction A was isolated from ¹⁴C-labelled neutral-salt-soluble collagen of

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