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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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guinea pig by the method of Fessler (1960), exhaustively dialysed against 0.067 M-phosphate buffer, pH 6.8, and the concentration was determined refractometrically (500 $\mu\text{g./ml.}$). Samples (0.5 ml. or less) were placed in Lustroid tubes (approx. $1\frac{1}{2}$ in. $\times$ $\frac{3}{8}$ in. for Spinco rotor no. 40 with adaptor), well stoppered and incubated in a water bath at 27–37° as required, together with liquid paraffin and the Spinco rotor. At the end of incubation tubes were topped up with liquid paraffin, sealed and at once centrifuged (30 000 rev./min. for 10 min.). Most of the liquid paraffin was then removed, and the stoppered tubes were kept in the water bath. Samples (0.1 ml.) were rapidly plated out, the pipette was washed three times with 10% (v/v) acetic acid and the washings were added to the planchets.

Concentration of solutions of fraction B. A dilute solution of fraction B in 0.067 M-phosphate buffer, pH 7.4, was isolated as previously described (Fessler, 1960). About 85% of the water was removed under controlled conditions *in vacuo*, the temperature of the solution being maintained at 7–11° throughout. A fibrous precipitate formed. The suspension was quantitatively transferred to a dialysis bag and practically all the material dissolved at 2–4° after exhaustive dialysis against 0.067 M-phosphate buffer, pH 7.4. The solution was cleared on the ultracentrifuge (30 000 rev./min. for 90 min.).

Determination of 'precipitation time' of neutral-salt-soluble collagen. Solutions of neutral-salt-soluble collagen and of various substances were added to a set of small test tubes (approx. 5 cm. $\times$ 0.5 cm.) chilled in ice. The contents of each tube (0.5 ml. or less) were stirred with a small glass rod. Tubes, together with their stirrers, were quickly placed in a water bath with a glass front. The tubes were stirred in turn. In due course a faint haze would appear in each tube, which could usually be quickly coalesced to a precipitate and then to a clot adhering to the small rod. The time between immersion of a tube and the appearance of a definite precipitate was noted and called 'precipitation time'. Because of a system of randomization, the observer did not know what a tube contained while he determined its 'precipitation time'.

Preparation of material for electron microscopy. A drop of the solution or suspension to be investigated was placed on a specimen grid covered with nitrocellulose film. For a few minutes the grids were supported on a microscope slide above a little water in a closed Petri dish. All materials could be kept chilled in ice, or at 37°. Liquid was blotted off with filter paper and replaced with 1% phosphotungstic acid, and finally the grids were washed twice in water.

RESULTS

Experiments to elucidate the nature of fraction B

Fraction B contains all the material which does not precipitate on warming a solution of neutral-salt-soluble collagen to 37° for about 1 hr., and both chemical and physical analyses had shown it to be similar to the fractions which precipitate as fibres under these conditions (Fessler, 1960). Experiments were made to see whether fraction B could be one or more of the following: (i) a suspension of fine fibrils of fractions A and C; (ii) a saturated solution of collagen in equilibrium with

the fibres of fractions A and C; (iii) a solution of gelatin.

Ultracentrifuging was used in the separation of the fractions and this made contamination of fraction B with fibres unlikely. No fibres were detected in a number of samples of fraction B examined in the electron microscope after removal of the other fractions, provided that solutions placed on the electron-microscope grids were protected from evaporation. Some fibres were found if samples were slowly evaporated on to specimen grids by incubating for several hours in a moist atmosphere at 37°. Large quantities of typical striated collagen fibres were found in the precipitates formed from fraction B upon slow concentration, at 7–11°, of its dilute solutions (see Experimental section). Thus although solutions of fraction B were not initially contaminated with fibres, fibres were formed when the ionic strength of the buffer solutions was increased by evaporation.

In the preparation of fibres from solutions of fraction B, the determining factor was the salt concentration and not the concentration of collagen. Thus most of the fibrous precipitate of fraction B, formed on concentrating its solution at 7–11°, dissolved again after dialysis against the usual 0.067 M-phosphate buffer at pH 7.4. The concentration of such enriched solutions of fraction B was about 700 $\mu\text{g./ml.}$ At this concentration solutions of fraction A form a gel on warming to 37° for 1 hr., and definite precipitates are still formed by solutions of fraction A containing 100 $\mu\text{g./ml.}$ or less. In contrast, no precipitate appeared on warming solutions of fraction B (770 $\mu\text{g./ml.}$) to 37° for 1–24 hr. (Prolonged incubation does not visibly alter the corresponding gels of fraction A.) This finding was quantitatively confirmed. Samples (2 ml.) of fraction B (650 and 765 $\mu\text{g./ml.}$) were kept at 37° for 1 hr., ultracentrifuged at 35° (Spinco rotor no. 40, 20 000 rev./min. for 25 min.) and samples of the supernatants were carefully removed and their concentrations were determined refractometrically (700 and 770 $\mu\text{g./ml.}$ respectively). No decrease in the amount of material in solution had occurred, whereas in corresponding experiments with fraction A 93% of the total material was removed.

One possible method of distinguishing between the soluble collagens and gelatin is the effect of low ionic strength on their solubilities: gelatin is soluble in water, the collagens are not. On dialysis against water, fraction B was found to be quantitatively precipitated. Fraction B (3 ml.) containing 1.93 mg. of collagen when exhaustively dialysed against water formed a voluminous precipitate. This was quantitatively separated on the ultracentrifuge, dissolved in 1% acetic acid and estimated refractometrically after dialysis against

phosphate buffer; 1.93 mg. of collagen was recovered from the precipitate and the supernatant contained less than 0.01 mg. of material.

Interaction of fractions A and B would be possible. To test this, about 0.4 g. of a solution of fraction A (6650 $\mu\text{g./ml.}$) and 1.4 g. of a solution of enriched fraction B (735 $\mu\text{g./ml.}$) were accurately weighed out in duplicate, mixed and the final refractive increment was checked. After warming to 37° for 1 hr., the precipitate was removed in the usual way at 36° on the ultracentrifuge and the refractometric concentration of the supernatant determined. Table 1 shows the results together with corresponding values calculated for the condition that: (i) 100 % of the added fraction A was precipitated; (ii) 92 % of the added fraction A was precipitated; (iii) 92 % or 83 % of the total collagen was precipitated; (iv) the proportion of fraction B which would have to behave as fraction A, to account for the results on the basis of 92 % precipitation of the total of A present, is given.

Solubility measurements on fraction A

The solubility of isolated fraction A was studied in order to determine how large a part of fraction B might represent a solution of fraction A. The effects of temperature and concentration were investigated. Equilibrium conditions were approached from opposite sides, wherever possible, by making use of the change of solubility of neutral-salt-soluble collagen with temperature. For example, in a preliminary study, one set of samples was incubated at 27°. Flocculent precipitates formed and after 20 hr. the concentration of material in solution had decreased from 500 to 160 $\mu\text{g./ml.}$ Concurrently, a duplicate set was first incubated at 37° for 75 min., when firm, opaque gels formed and the concentration of material in solution decreased from 500 to 50 $\mu\text{g./ml.}$ This second set was then placed in the bath at 27° and,

after 20 hr., the concentration of material in solution increased from 50 to 170 $\mu\text{g./ml.}$ Prolonged incubation, up to 42 hr., caused no further change, whereas incubation for 6 hr. or less was insufficient for equilibration.

Fig. 1 shows the apparent solubilities of fraction A of neutral-salt-soluble collagen in the temperature range 27–37°. Incubations of at least 20 hr. were used and the fair agreement between samples directly incubated at a given temperature and corresponding ones first brought to 37° for 75 min., indicates that equilibria were attained. Samples whose final temperature was 37° were incubated only at 37° for 75 min. A separate study confirmed the earlier conclusion (Fessler, 1960) that after 75 min. little further change occurred in the concentration of material in solution at 37°.

A set of samples was made of volumes 0.2, 0.33, and 0.5 ml. Each sample contained 100 $\mu\text{g.}$ of fraction A in solution at 4°. The total amount of collagen in solution at 37° was determined for each sample and is shown in Fig. 2. For a constant amount of total material the apparent solubility of fraction A was thus independent of the volume of solvent at 37°.

Sets of samples were made, each of volume 0.5 ml. Samples initially contained 50–250 $\mu\text{g.}$ of fraction A

Table 1. *Fibre formation of a mixture of fraction A and fraction B*

See text for experimental details.

	Concn. of material in supernatant after fibre formation at 37° ($\mu\text{g./ml.}$)		
	Expt. 1	Expt. 2	Mean
Observed	480	500	490
Calculated for fibres to be composed of the following starting materials			
92% of (A + B)	205	205	205
83% of (A + B)	375	385	380
92% of (A)	595	675	635
100% of (A)	475	560	517
92% of (A + 0.2B)	480	—	—
92% of (A + 0.31B)	—	500	—

30

Fig. 1. Effect of temperature on the concentration of collagen in solution. Each sample was initially a solution of 250 $\mu\text{g.}$ of fraction A in 0.5 ml. of 0.067 M-phosphate buffer, pH 6.8, at 2–4°. ○, Results on samples taken directly to the temperature indicated on the abscissa; ●, results on samples first incubated at 37° for 75 min. and then at the temperature indicated on the abscissa. The left-hand ordinates represent the concentration of collagen in solution after incubation for 20 hr. or longer. The right-hand scale of ordinate gives the corresponding percentage of starting material in solution. Each point represents a measurement in duplicate of one sample.

in solution at 4°. The concentrations of collagen in solution at 29.5° and 37° were determined and are shown in Fig. 3. Thus for a constant volume of solvent, the apparent solubility of collagen varied directly with the total amount of collagen present. A line fitted by the method of least squares to the results at 37° and initial concentrations greater than 150 $\mu\text{g./ml.}$ of Fig. 3 has an intercept not significantly greater than zero on the axis. Whether or not the curves of Fig. 3 have a 'shelf' at low initial concentrations could not be determined because of experimental errors, which became

Fig. 2. Variation of total collagen in solution with volume of solution at 37°. Each sample initially contained 100 $\mu\text{g.}$ of fraction A in 0.067 M-phosphate buffer, pH 6.8, at 2-4°.

Fig. 3. Variation of the concentration of collagen in solution at 29.5° and at 37°, with the initial concentration of collagen at 2-4°. ○, Results obtained at 29.5°; ●, results obtained at 37°. Each sample consisted of fraction A in 0.5 ml. of 0.067 M-phosphate buffer, pH 6.8.

appreciable in this range. Definite precipitates were always obtained.

Polarimetric investigations

On warming a solution of neutral-salt-soluble collagen in 0.067 M-phosphate buffer, pH 7.0, from 0° to 25°, the specific optical rotation of -377° did not change; at higher temperatures fibre formation became appreciable. It has been shown (Fessler, 1960) that the optical rotary power of neutral-salt-soluble collagen is not affected by fibre formation at 37°, followed by careful re-solution. Fig. 4 shows the similar results obtained at different temperatures with solutions of neutral-salt-soluble collagen and acid-soluble collagen, both in 0.067 M-acetate buffer, pH 3.7. At 0° the specific optical rotation of neutral-salt-soluble collagen was the same at pH 3.7 as at pH 7.4: -377° ; that of acid-soluble collagen was practically the same: -382° . Warming to 34° or 37° caused a small change of about $+13^\circ$ of arc in both materials and the initial values were restored on cooling. Warming to 39.5° caused a rapid change in both materials, which was hastened at 40°, the final specific rotations at 40° being practically identical (-144° for neutral-salt-soluble collagen and -142° for acid-soluble collagen). This change was partly reversed on

Fig. 4. Effect of various temperatures on the specific optical rotations of neutral-salt-soluble collagen and acid-soluble collagen in 0.067 M-acetate buffer, pH 3.7. The specific optical rotation of a sample shown at a given point in the upper curves was measured when the sample was at a temperature shown by the corresponding ordinate given in the lower curves. ●—●, Neutral-salt-soluble collagen; ×—×, acid-soluble collagen.

cooling and after overnight storage at $2-4^{\circ}$ the corresponding rotations were -297° and -309° . Further warming and cooling reproduced the changes between about -143° and -305° , but the initial higher specific rotation could not be restored. The final solutions were dialysed against 0.067M -phosphate buffer, pH 7.0, in the cold. On warming to 37° these solutions remained clear, and the concentration of material in solution was not altered by subsequent centrifuging at 35° (Spinco rotor no. 40, 20 000 rev./min. for 20 min.).

The specific optical rotation in 0.067M -phosphate buffer, pH 7.4, at 5° of fraction A was -369° and of fraction C -363° , not differing significantly from that of the parent material. Under these conditions separate preparations of fraction B gave -353° and -332° , which on warming changed to -247° and -245° respectively at 37° . All solutions of fraction B were dilute and so there may be considerable error in the values of the stated specific rotations of fraction B. The variation of rotation with temperature was, however, quite definite for fraction B, both at pH 3.7 and pH 7.0, and the ratio (rotary power at 4°)/(rotary power at 37°) was found to be: 1.28, 1.35, 1.43; mean 1.36. The variation of rotation with temperature of fraction B is sufficient to account for the reversible small change of rotation, some 13° of arc (Fig. 4), which occurred on warming unfractionated neutral-salt-soluble collagen to 37° .

Relationship of fractions A, B and C as indicated by incorporation of [^{14}C]glycine

A relationship between newly formed collagen and the soluble collagens has been shown to be likely by Harkness, Marko, Muir & Neuberger (1954), Gross (1958), Jackson (1957) and Orekhovitch (1952); and, in contrast, mature collagen appears normally to be insoluble, or soluble only with difficulty. By comparison, the different solubility properties of fractions A, B and C could reflect different stages of maturation of neutral-salt-soluble collagen. To investigate this possibility [^{14}C]glycine was injected into guinea pigs and 6 hr. later neutral-salt-soluble collagen was isolated from their skins, separated into fractions A, B and C, and the specific radioactivities of the constituent glycine were determined; counts/min. were plotted against concentration of DNP-glycine (spectrophotometer reading) and values for all samples and their dilutions fell on a common straight line. The standard deviation of the slope of this line was in one experiment 2% of the slope of the line, and in the duplicate set 5%. Thus no difference was found in the specific radioactivities of glycine isolated from fractions A, B and C of neutral-salt-soluble collagen 6 hr. after injection of [^{14}C]glycine.

Some observations on the rate of precipitation of neutral-salt-soluble collagen

'Precipitation time' varied inversely as the hydrogen-ion concentration, as shown in Fig. 5. The proportionality coefficient was found to be different in two samples. Below 10° a solution at pH 7.4 had not formed a precipitate after several months, a solution at pH 6.8 would give one after several weeks and one of pH 6.4–6.6 within a few

Fig. 5. Variation of 'precipitation time' of neutral-salt-soluble collagen with hydrogen-ion concentration. Abscissae denote reciprocals of 'precipitation times'. Left-hand scale of ordinate denotes hydrogen-ion concentrations for sample (X) of collagen preparation III, and correspondingly the right-hand scale of ordinate for sample (O) of collagen preparation VI. Measurements were made at 30° in 0.067M -phosphate buffer.

Fig. 6. Variation of 'precipitation time' with temperature. Ordinates are reciprocals of the absolute temperatures, abscissae are logarithms of the reciprocals of 'precipitation times'. Measurements were made in 0.067M -phosphate buffer, pH 6.8, in the temperature range $20-31^{\circ}$.

Table 2. *Effect of some buffer ions on 'precipitation time'*

To 0.05 ml. of a solution of neutral-salt-soluble collagen in 0.067 M-phosphate buffer, pH 6.8, 0.02 ml. volumes of the following 0.1 M solutions, adjusted to pH 6.8 (glass electrode), were added and the 'precipitation time' was measured at 29.4°.

Substance	'Precipitation time' (min.)
Phosphate	5.7
α -Picoline	2.7
Glyoxaline	3.1
Collidine	3.3

Similar experiment with another sample of neutral-salt-soluble collagen; pH 7.3 throughout.

Substance	'Precipitation time' (min.)
Phosphate	6.5
Tris	2.0

days. A solution at pH 6.8 would form a precipitate within several hours at room temperature and the variation of 'precipitation time' from 20° to 31° is given in Fig. 6. At 37° precipitates were obtained within a few minutes. The slower the rate of precipitation, the more flocculent and easily teased apart was the material and the larger were the fibres formed. Artificial partial alignment of the particles by shearing between concentric cylinders at 180 sec.⁻¹, for 6 hr. at 4°, did not cause precipitation. 'Precipitation time' was increased by dilution. At constant final concentration of neutral-salt-soluble collagen, 'precipitation time' was not affected by addition of: (i) the precipitate obtained by warming another sample of neutral-salt-soluble collagen to 37°; (ii) the supernatant of (i); (iii) a dialysed solution of the material left in the skin extracts after removal of the soluble collagen. Table 2 shows the effect of some buffer ions on 'precipitation time'. 'Precipitation times' varied from sample to sample and also depended on the observer. All solutions whose 'precipitation times' were to be compared were included in any one experiment. Repeat experiments led to identical conclusions, though corresponding 'precipitation times' were not necessarily identical; e.g. results given in Fig. 5.

DISCUSSION

Solubility properties of fraction A

The equilibria which were reached were probably independent of the diameter of the precipitated fibres, because slowly formed flocculent precipitates tend to consist of larger fibres than those present in rapidly formed gels, and the nature of the precipitate did not affect the equilibria. This gives some support for assuming the fibrous pre-

cipitate to be a single thermodynamic phase. Only with this assumption can the Phase Rule be applied to the finding that the solubility varies with the total amount of fraction A present (Figs. 2 and 3) and it follows that fraction A contains more than one component.

The considerable variation of apparent solubility with temperature (Fig. 1) invites comparison with temperature-dependent properties of gelatin (Ferry, 1948; Robinson, 1953), and this is done in Fig. 7. Specific optical rotations of gelatin obtained from Fig. 4 are plotted at different temperatures, together with the sum, $-[300x + 144(1-x)]$, where x equals the fraction of collagen in solutions at that temperature (from Fig. 1, right-hand scale of ordinate), -300 is the specific optical rotation of gelatin at 0° and -144 that at 40° (from Fig. 4). The similarity suggests that the two phenomena are expressions of one property of the collagen peptide chain.

One interpretation of the solubility properties of fraction A is that there are two components, a soluble 'cold' form and an insoluble 'warm' form, with distribution of material between the two forms

Fig. 7. Comparison of the temperature-dependent behaviour of fraction A of neutral-salt-soluble collagen and of the corresponding gelatin. The specific optical rotations observed for gelatin are denoted by Δ . Specific optical rotations calculated for gelatin by the method given in the text are shown as $\circ$ and are based on the variation of solubility of fraction A with temperature and the specific optical rotations of gelatin at 0° and 40°.

being regulated only by temperature. Florey (1956) showed theoretically that small variations in interaction energies between solvent and long rod-like particles critically affect the distribution of material among phases. Thus only a small change of the interactions between collagen and solvent with temperature could account for the solubility properties of fraction A. Florey, however, considered only an uncharged solute.

Nature of fraction B

Fraction B could be a mixture of fraction A and gelatin. As fraction B constitutes 16% of the total neutral-salt-soluble collagen (Fessler, 1960) and 8% of fraction A is in solution at 37°, about half of fraction B would be fraction A. From the warming of mixtures of fractions A and B (Table 1), 25% of fraction B is calculated as behaving like fraction A. The specific optical rotations of fraction B at 4° and 37° may be compounded from those of gelatin (−300° at 4°, −144° at 40°) and of the other neutral-salt-soluble-collagen fractions (mean −370°, independently of temperature). This indicates 55% of fraction B to be gelatin.

The behaviour of fraction B at 37° and neutral pH is not in accord with this view: fibres should be formed from that part which is fraction A, but no fibres appear in 0.067 M-phosphate buffer. Further, on dialysis against water fraction B was quantitatively precipitated, and, whereas this would be possible if some of fraction B were gelatin, it would not be likely.

Temperature-dependent properties of neutral-salt-soluble collagen

The re-dissolving of fibres of fraction A on lowering the temperature from 37° shows that collagen can be in a stable state in solution. Conversely, fibres form a stable state at 37°, as further warming of fibre-solution systems does not decrease the amount of material in the solid state (unless gelatin is formed). That reversible equilibria can be established is proven by the experiments on fraction A (Fig. 1). At acid pH the fibrous state is probably unstable, as no precipitate appeared on warming solutions of pH 4.5 or less.

The rate of change of collagen from solution to fibre depends on concentration, temperature, pH and the nature of other materials present in solution. The effect of concentration was not in accord with any simple kinetic scheme. From Fig. 6 an activation energy of approx. 80 kcal./mole/degree was calculated by the Arrhenius Equation, which is in the range of values found for protein denaturation. The effects of increasing hydrogen-ion concentration (see Results section and Fig. 5) show that either these ions act catalytically, or the state of

ionization of the collagen particle is a rate-determining factor, or both. The influence of a variety of materials added to solutions of collagen has been studied, while this work was in progress, by Gross & Kirk (1958), and Bensusan & Hoyt (1958).

The positively charged, nitrogenous, buffer ions which accelerated the rate of precipitation of neutral-salt-soluble collagen could either act catalytically, or could alter the charge on collagen particles. Interaction of collagen must occur with 2-amino-2-hydroxymethylpropane-1:3-diol (tris) ions, as on dialysis at 2–4° against this buffer (pH 7.3, 0.067 M) neutral-salt-soluble collagen was precipitated quantitatively from solution, as it was on warming to 37°. Binding of buffer ions by collagen is well known (Gustavson, 1956; Jackson & Neuberger, 1957). These effects of nitrogenous buffer ions and of hydrogen ions may together indicate that the rate of precipitation is increased with decreasing negative charge on the collagen particle. When there is a net positive charge on collagen, as at pH 3.7 (Jackson & Neuberger, 1957), no fibres are formed on warming a solution. Probably, fibre formation is facilitated when the net charge of the collagen particle is small. In accord with this is the transient precipitate formed when solutions of neutral-salt-soluble collagen are dialysed at 2–4° from 0.067 M-phosphate buffer, pH 7.0, into 0.067 M acetate buffer, pH 3.7, or vice versa.

The dissolution of fibres on cooling was not studied in detail. This process is accelerated at 2–4° from 48 hr. (Fessler, 1960) to a few hours by dialysing at the same time against 1% acetic acid (or 0.067 M-acetate buffer, pH 3.7), thus supporting the previous conclusion that a net positive charge on the particles is contrary to fibre stability.

That gelatin had rapidly been formed at 39.5°, pH 3.7, was shown by the subsequent mutarotation (cf. Ferry, 1948) and the inability of the residual material to form fibres at neutral pH. Although in solution no change was observed with temperature in the optical rotary power of that collagen which could form fibres, it cannot be decided whether the actual fibre formation corresponds to a change in specific optical rotation or not.

Relationship of fractions A, B and C

Although fractions A, B and C had the same rate of incorporation of [¹⁴C]glycine over a 6 hr. interval, the fractions could have differed at other times. This, however, is unlikely, for the chances are small that an interval was used such that the specific activity-time curves of materials standing in sequential relationship might overlap sufficiently for separate specific activities to be indistinguishable. Thus no difference was found in the age of the three fractions.

Fractions B and C could be artifacts of preparation or of the first warming to 37°. This appears unlikely in view of (1) there being no difference between materials which had been salt-precipitated three and four times, (2) repeated warming and cooling produces neither fraction B nor fraction C. In view of the diverse effects of substances on the rate of fibre formation (Gross & Kirk, 1958) traces of impurities might affect the equilibria between fibre and solution. At higher ionic strengths such interference would be decreased and this would fit in with the fibre formation of fraction B under these conditions.

General

There are small, yet definite, differences between the neutral-salt-soluble collagen studied and soluble collagen extracted at acid pH. Thus the present material was obtained by repeatedly extracting skins with phosphate buffer at neutral pH. After no more collagen could be dissolved this way, extraction with 0.067 M-citrate, pH 3.6, gave an ample yield of collagen. Subsequent dialysis of this citrate solution of collagen against the phosphate buffer used for previous extractions precipitated the collagen. Conversely some apparent solutions of neutral-salt-soluble collagen in 0.067 M-citrate buffer, pH 3.6, proved to be suspensions in the ultracentrifuge (Fessler, 1960). Further, the rates of incorporation of [¹⁴C]glycine *in vivo* into collagens extracted in these two ways differ (Harkness *et al.* 1954; Jackson, 1957). Neutral-salt-soluble collagen and citrate-extracted collagen have closely similar structures, as they are indistinguishable both by optical rotation and the temperature at which rapid conversion into gelatin occurs. The amino acid compositions of these two collagen preparations have been compared by Jackson, Leach & Jacobs (1958). Differences in solubility may not be important, especially as collagen is known to adsorb ions (e.g. Gustavson, 1956; Jackson & Neuberger, 1957). On the other hand, small differences in the interaction energies between collagen particles and solvent may, in theory (Florey, 1956), profoundly affect the distribution between phases and therefore be critical in fibrogenesis. Different impurities in the two preparations could further affect the systems.

The similarity between the effects of temperature upon the solubility behaviour of fraction A and the specific optical rotation of gelatin may reflect an inherent property of the collagen polypeptide chain, in one case constrained and in the other free. Changes in the hydrogen-bonding of gelatin are known to occur with changes of temperature (e.g. Robinson, 1953), and corresponding

changes may be principally responsible for the temperature-dependent-solubility behaviour of fraction A.

SUMMARY

1. Fibres formed from concentrated solutions of fraction B of neutral-salt-soluble collagen by salting-out had the typical striated appearance of collagen fibres in the electron microscope.

2. No fibres were formed on warming enriched solutions of fraction B in 0.067 M-phosphate buffer, pH 7.4, although the collagen concentration was several times as great as concentrations at which fraction A forms fibres under these conditions.

3. Solubility properties of fraction A varied considerably with temperature in the range 27–37°, and were not those of a single Phase-Rule component.

4. At pH 3.7 the specific optical rotation of fraction B varied with temperature (0–37°), whereas those of fractions A and C did not.

5. The polarimetric behaviour of solutions of neutral and acid-soluble collagen were identical on varying the temperature at pH 3.7. Conversion into gelatin was rapid at 39.5°.

6. The glycine of fractions A, B and C had identical specific radioactivities in samples isolated from guinea pigs 6 hr. after injection of [¹⁴C]glycine.

7. The rate of precipitation of neutral-salt-soluble collagen varied directly with hydrogen-ion concentration in the range pH 6.5–7.4. The activation energy was about 80 kcal./mole/degree at pH 6.8.

8. The temperature-dependent-solubility behaviour of neutral-salt-soluble collagen and the nature of fractions A, B and C are discussed.

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The Phospholipids of Human-Blood Plasma and their Exchange with the Cells

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When whole human blood is incubated *in vitro* with radioactive acetate, radioactivity is incorporated into the phospholipids (James, Lovelock & Webb, 1957; Rowe, 1959). Moreover, there is an exchange of the radioactive phospholipids between the cells and the plasma (Lovelock, James & Rowe, 1960). Further information, both on the composition and structure of the plasma phospholipids and their exchange with the cells, is required. This paper reports the analysis of the radioactive phospholipids of a plasma fraction obtained from blood which had been incubated with sodium [$Me-^{14}C$]acetate. The plasma had been treated with dextran sulphate and the precipitated β -lipoproteins removed before analysis (Oncley, Walton & Cornwell, 1957).

METHODS

The incubation of blood and other experimental procedures are essentially those described by Rowe (1959).

Preparation of the plasma fraction. Whole blood (140 ml.) which had been incubated with sodium [$Me-^{14}C$]acetate (30 μ C; specific activity 13.7 mc/mg.) and [^{32}P]orthophosphate (1.6 mc; carrier-free) for 6.5 hr. was centrifuged at 600 *g* for 5 min. The cells were removed and calcium chloride (2 ml., 0.25 M) and dextran sulphate (6 ml.; 1%, w/v) were added to the stirred plasma. After 20 min. the plasma was centrifuged again at 600 *g* to remove the precipitated β -lipoprotein (Oncley *et al.* 1957). A previous experiment established that under these conditions all of the red and white cells, and most of the platelets, were removed from the plasma by the first centrifuging and that the remaining platelets were removed with the precipitated β -lipoprotein.

Extraction of phospholipids. The supernatant plasma was added to 2 l. of ethanol-ether (2:1, v/v). The mixture was allowed to stand at room temperature for 24 hr. and then stored at 2° in the dark for 3 weeks. The solids were removed by filtration and the filtrate was concentrated to

dryness under reduced pressure in a rotary evaporator. The residue was extracted twice with 10 ml. of acetone and then successively with 10, 20 and 10 ml. of ethanol. All extractions were performed at room temperature. The small amount of insoluble residue in the ethanol extract was removed by centrifuging. The β -lipoprotein-dextran sulphate complex was added to ethanol-ether (500 ml.) and treated in the same manner.

Chromatography of phospholipids. Ethanol extract (35 ml.) from the β -lipoprotein-free plasma containing 74.9 μ moles of phosphorus was distilled to dryness under reduced pressure and the residue suspended in 150 ml. of chloroform-methanol (7:1, v/v). The suspension was passed through a column of silica gel (2.5 cm. diam. $\times$ 33 cm.). The silica gel was prepared by the method of Lea, Rhodes & Stoll (1955) and the column constructed as described by Hannahan, Dittmer & Warashina (1957). The column was then washed successively with chloroform-methanol mixtures of volume ratios 7:1 (235 ml.), 4:1 (270 ml.), 3:2 (295 ml.) and 1:4 (290 ml.). The flow rate was approx. 0.9 ml./min. Eluate fractions of approx. 4 ml. were collected.

Estimation of radioactivity. Each eluate fraction was diluted to 5 ml. with its own solvent mixture and 0.2 ml. evaporated to dryness on a polythene planchet. The radioactivity of each fraction was estimated by counting with an end-window Geiger-Müller tube. The fractions were combined according to their radioactive peaks, as shown in Fig. 1. The combined fractions were concentrated and then diluted to 10 ml. or 25 ml. with chloroform and methanol. Portions (0.20 ml.) containing 0.18–2.31 μ mole of phosphorus were concentrated to dryness on planchets and their radioactivities estimated. The radioactivity due to ^{32}P and ^{14}C in the unfractionated phospholipids (Table 1) was estimated in the same way with 0.10 ml. of solution. The planchet was then shielded with a foil of calibrated phosphor-bronze and the radioactivity due to ^{32}P estimated (Rowe, 1959).

Identification of the fractions. The fractions were chromatographed on silica-impregnated paper with 20% methanol in chloroform (Lea *et al.* 1955). The components were detected by immersion of the chromatogram in iodine