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# Sedimentation-Equilibrium Studies of the Molecular Weight of Single and Double Chains from Rat-Skin Collagen

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Preparations of $\alpha 1$ and $\alpha 2$ single chains and the covalently linked double chain $\beta_{12}$ ($\alpha 1$-$\alpha 2$) isolated by chromatography from denatured rat-skin collagen were studied by sedimentation equilibrium in *p*H 4.8 potassium acetate buffer at 40° and in 5 M guanidine at 7°. Comparison of the observed distribution of protein with the theoretical distribution calculated from the concentration dependence of molecular weight indicated that $\alpha 1$ was essentially homogeneous. Samples of $\alpha 2$ showed contamination. Although $\beta_{12}$ was unstable at 40°, studies in 5 M guanidine indicated good homogeneity. It was concluded that the molecular weights of $\alpha 1$ and $\alpha 2$ are 98,000 ± 5,000. The molecular weight of $\beta_{12}$ is 196,000 ± 10,000, a value consistent with its structure. Sedimentation coefficients and intrinsic viscosities of the various components are consistent with the random-coil structure. These results support the proposal that the collagen monomer contains three $\alpha$ chains (two $\alpha 1$ and one $\alpha 2$).

Chromatographic studies of denatured collagen have provided strong evidence that newly synthesized molecules consist of three chains of which two, designated $\alpha 1$, are apparently identical while the third, designated $\alpha 2$, has a different amino acid composition. In most collagen samples intramolecular cross-linking produces covalently linked double chains of two types, $\alpha 1$-$\alpha 2$ and $\alpha 1$-$\alpha 1$. The double chains are called $\beta_{12}$ (for $\alpha 1$-$\alpha 2$) and $\beta_{11}$ (for $\alpha 1$-$\alpha 1$( (Piez *et al.*, 1961, 1963).

To support this proposed chain structure and to provide a basis for further structural studies, accurate molecular weights of these various components are necessary. Previous studies, all done with mixtures of single and double chains, have yielded results between 70,000 and 125,000 for single chains and between 160,000 and 290,000 for double chains. Many of these figures were proposed only as approximations. These data have been summarized and discussed by Hannig and Engel (1961). The work reported here was done with samples of $\alpha 1$, $\alpha 2$, and $\beta_{12}$ which have been demonstrated to represent single molecular species by the criteria of chromatography, amino acid composition, sedimentation velocity (Piez *et al.*, 1963) and acrylamide-gel electrophoresis (Nagai *et al.*, 1964). Short-column sedimentation equilibrium was employed under conditions where the chains would be fully denatured as measured by changes in viscosity and optical rotation (see the review by Harrington and Von Hippel, 1961).

## METHODS

*Samples.*—$\alpha 1$, $\alpha 2$, and $\beta_{12}$ were isolated from rat-skin collagen by chromatography on CM-cellulose as previously described (Piez *et al.*, 1963). The collagen was prepared either by 1 M salt or 0.5 M acetic acid extraction. Preparations of $\alpha 1$ routinely showed no evidence of other components by the criteria of chromatography, sedimentation velocity (Piez *et al.*, 1963), and gel electrophoresis (Nagai *et al.*, 1964). The $\alpha 2$ samples usually had small amounts of heavier material (presumably $\beta_{12}$) which could not be removed by rechromatography. The amount present could not usually be seen by sedimentation velocity in the ultracentrifuge but could be visualized by the more sensitive technique of electrophoresis on acrylamide gel (Nagai *et al.*, 1964). The amount of contamination could not be measured quantitatively but was estimated to be about 5%. The samples of $\beta_{12}$ sometimes contained a small amount of lighter material but the degree of purity was difficult to evaluate because of the instability of this component. It has not yet been possible to prepare $\beta_{11}$ in pure enough form to warrant study by the procedures used here.

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*Sedimentation Equilibrium.*—A four-place An-J rotor designed for equilibrium studies was used in a Spinco Model E ultracentrifuge with Rayleigh optics. The methods followed were essentially those of Richards and Schachman (1959). Two 12-mm double-sector cells with wedge centerpieces and a standard 30-mm double-sector cell contained the samples. A counterbalance was placed opposite the 30-mm cell. Two solvents were employed, 0.15 M potassium acetate, *p*H 4.8, and 5 M guanidine hydrochloride, *p*H 7.0. The samples were dissolved to give concentrations of about 0.5%, warming to 45° for a few minutes to effect solution in the case of the acetate buffer. The solutions were dialyzed against solvent at 5° for 24 hours. Solvent from outside the dialysis sack was used for the preparation of dilutions and to fill the reference side of the ultracentrifuge cell. Concentrations were measured in a capillary-type double-sector synthetic-boundary cell and expressed as the number of interference fringes. By calibration of the ultracentrifuge optics with a standard sucrose solution and employing a value of 0.187 for the refractive increment of collagen (Boedtker and Doty, 1956) it was found that 4.10 fringes were equivalent to 1 mg/ml of denatured collagen in the acetate buffer at 40°. Dilutions were made by volume displacement with a Hamilton microsyringe. In most experiments six concentrations were studied in two runs of three each. The two lowest concentrations were placed in the 30-mm cell for increased sensitivity. Liquid columns were approximately 3 mm long unless otherwise indicated. FC-43 was used to provide a false bottom. The runs were performed at 40.0° for samples in the acetate buffer and at 7.0° for samples in 5 M guanidine. The rotor was initially overspeeded at about one and one-half times the speed selected for equilibrium for approximately twice the time required for the disappearance of the plateau region, about 1–6 hours. Preliminary experiments in 5 M guanidine established that speeds of 9945 rpm for $\alpha 1$ and $\alpha 2$ and 6995 rpm for $\beta_{12}$ were appropriate. In acetate buffer at 40° the corresponding speeds used were 6995 and 5227 rpm. These speeds were selected to give a concentration at the bottom of the cell approximately three times greater than at the top. The number of fringes across the cell was always greater than 10 and was usually about 20. Measurements at various times demonstrated that equilibrium could be reached in 1000 minutes at 40° in buffer and 2000 minutes at 7° in 5 M guanidine. The Rayleigh patterns were measured in a Nikon comparator and molecular weights were calculated as described by Richards and Schachman (1959). Absolute concentrations in the cell were determined by applying the conservation-of-mass principle. This was applicable since in no case was there loss of protein as evidenced by thickening of the lower meniscus or a continually changing fringe pattern. Computations were performed with the aid of a Minneapolis Honeywell 800 computor. A value of 0.705 was used for the partial specific volume. There is general agreement on this value as indicated by the following results reported in the literature: 0.705 for ichthyocol gelatin at 31° (Gallop, 1955), 0.695 for ichthyocol gelatin in 2 M KCNS (Boedtker and Doty, 1956), 0.710 for codskin gelatin at 20° (Young and Lorimer, 1961), and 0.706 in citrate and 0.686 in phosphate for calfskin collagen (Rice *et al.*, 1964). The value calculated from the amino acid composition is 0.706.

*Sedimentation Velocity.*—Sedimentation coefficients were measured in the *p*H 4.8 acetate buffer at 40°. Concentrations were determined by polarimetry at 313 mμ. The specific rotation at this wavelength, measured on denatured collagen solutions standardized by the micro-Kjeldahl technique, was −825 at 45° in the acetate buffer. Protein concentrations between about 1 and 6 mg/ml were studied. The ultracentrifuge was operated at 59,780 rpm and schlieren optics were used. Measurements and calculations were made by standard procedures.

*Intrinsic Viscosity.*—Viscosities were measured at 40° in the *p*H 4.8 acetate buffer employing coiled capillary viscometers which had flow times for water of 2–3 minutes. Protein concentrations were measured by polarimetry.

## Results

*Calculation of Theoretical Protein Distribution.*—Because a large dependence of molecular weight on concentration was encountered in both buffer and 5 M guanidine solutions, the usual criterion of homogeneity linearity of the relationship between ln $c$ and $x^2$, did not apply. Therefore the theoretical distribution of protein, assuming homogeneity, was calculated as follows:

If the concentration dependence of molecular weight is expressed by the equation

$$\frac{1}{M} = \frac{1}{M_0} + Bc \qquad (1)$$

where $M$ is the apparent molecular weight at concentration $c$, $M_0$ is the molecular weight at $c = 0$, and $B$ is a constant analogous to the second virial coefficient, there can be substituted

$$\frac{d \ln c}{dx^2} = \left(\frac{d \ln c}{dx^2}\right)_0 \frac{1}{1 + B'c}$$

where $x$ is the distance from the center of rotation and $B' = BM_0$. Substituting $dc/c$ for $d \ln c$ and rearranging gives

$$\left(\frac{1}{c} + B'\right)dc = \left(\frac{d \ln c}{dx^2}\right)_0 dx^2$$

and integration gives

$$\ln c + B'c = \left(\frac{d \ln c}{dx^2}\right)_0 x^2 + K \qquad (2)$$

where $K$ is the integration constant. Here $B'$ was calculated from the slope and intercept of the plot of $1/M$ versus $c$. For any set of data at one concentration, the constants $(d \ln c/dx^2)_0$ and $K$ were calculated by the use of simultaneous equations employing the measured values of $c$ and $x$ at the top and bottom of the liquid column. It was then possible to calculate the distribution of protein between the menisci as described by equation (2). This is shown in the figures as a solid line.

*Estimation of Sensitivity to Heterogeneity.*—Since the samples were not necessarily homogeneous, it was important to have an estimation of the sensitivity of the sedimentation-equilibrium method as applied here. The theoretical distribution of protein, assuming no concentration dependence, was calculated for several model systems employing equation (3′) of Yphantis (1964). The results (Fig. 1) demonstrate that as little as a few per cent of impurities, either half as heavy or twice as heavy as the major component, would cause a visible deviation from the distribution calculated for a single component. While these calculated curves may be used for comparison with data from experiments in buffer at low protein concentrations where the correction for concentration dependence is relatively small,

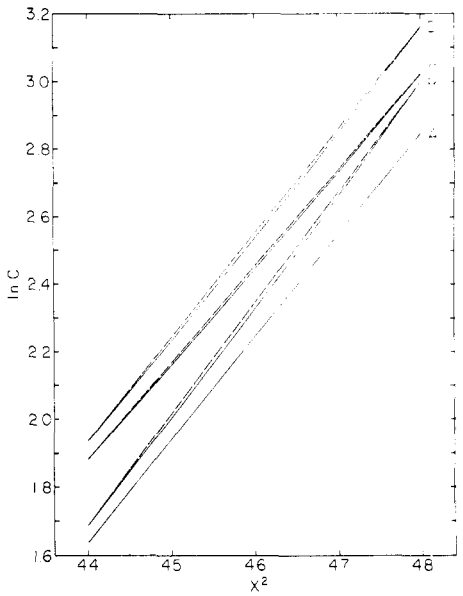


FIG. 1.—Theoretical distribution of protein. At equilibrium (solid lines) for a homogeneous sample of molecular weight 100,000 (A) and mixtures containing in addition 10% 200,000 (B), 10% 50,000 (C), and 5% each of 200,000 and 50,000 (D). Curves B, C, and D are displaced upward for clearer presentation. The dashed lines are straight lines between the menisci. The following conditions were assumed: liquid column, 2.8 mm; $C_0$ = 10 fringes; speed, 6995 rpm; temperature 40°; $\bar{v}$ = 0.705; $\rho$ = 100.

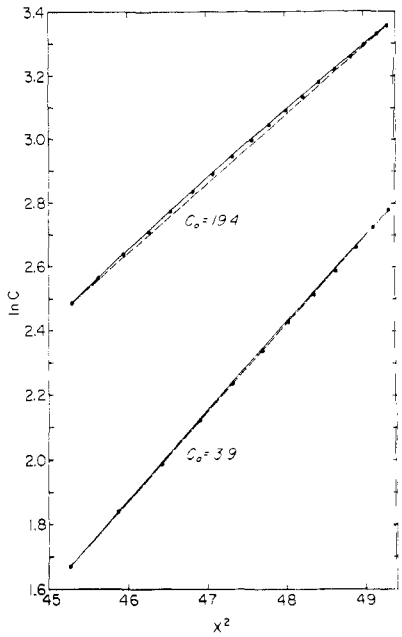


FIG. 2.—Distribution of $\alpha$1 in acetate buffer at 40° after 1200 minutes at 6995 rpm at two different concentrations. The solid lines show the theoretical distribution for a homogeneous preparation calculated from the concentration dependence of molecular weight. The dashed lines are straight lines between the menisci. The solid circles are the experimental points. The data at the lower initial concentration were obtained in a 30-mm cell giving apparent concentrations 2.5 times greater than actual. Concentrations are in fringes.

they are not necessarily applicable to the experiments in 5 M guanidine, as discussed later.

*Sedimentation Equilibrium in Buffer.*—In buffer at 40° plots of ln $c$ versus $x^2$ showed that $\alpha$1 behaved in a nonideal fashion. At a concentration of about 0.5

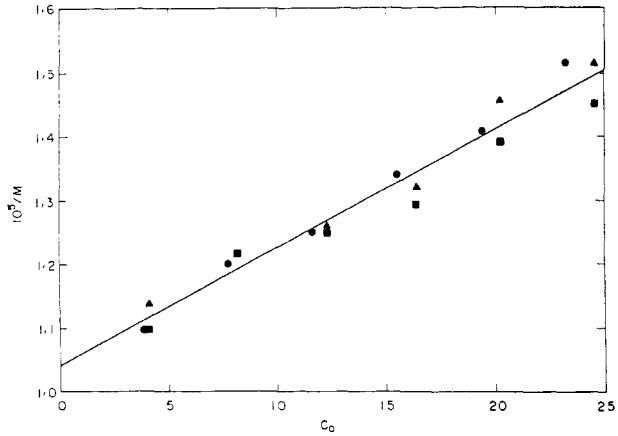


FIG. 3.—Concentration dependence of the molecular weights of $\alpha$1 and $\alpha$2 in acetate buffer at $p$H 4.8, 40°. Two sets of data for $\alpha$2 were obtained from measurements after two different times at 6995 rpm, 2010 minutes (■) and 3000 minutes (▲), in the same experiment. Measurements on $\alpha$1 (●) were made after 1200 minutes at 6995 rpm.

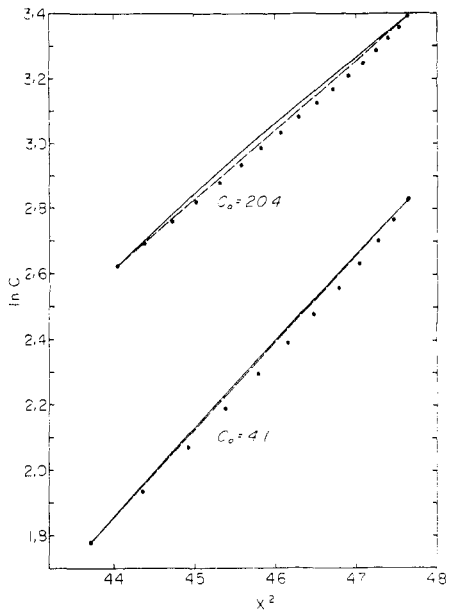


FIG. 4.—Distribution of $\alpha$2 in acetate buffer at 40° after 2010 minutes at 6995 rpm. See legend to Fig. 2.

mg/ml the experimental points described a curve which was convex upward but became essentially linear at about 0.1 mg/ml (Fig. 2). Comparison with the theoretical distribution calculated as described above showed that the experimental points were very close to the curves for a homogeneous sample (Fig. 2). This was true at all concentrations. Comparison of this data with model calculations (Fig. 1) indicates that only a very small percentage of the sample was appreciably heavier or lighter than the major component.

Extrapolation of the reciprocal of the apparent molecular weight to zero concentration gave a molecular weight for $\alpha$1 of 97,900 (Fig. 3; Table I). The value of $B$ (equation 1) was $1.76 \times 10^{-7}$ fringe$^{-1}$.

Unlike $\alpha$1, samples of $\alpha$2 consistently showed a considerable degree of heterogeneity (Fig. 4). Although approximately 5% contamination with the double-chain component $\beta_{12}$ was expected, comparison of the data with model calculations (Fig. 1) indicates that this may have been an underestimate. Here $\alpha$2 may also be subject to a greater degree of contamination than $\alpha$1 since it is the last component in the chro-

TABLE I
MEASURED VALUES OF THE MOLECULAR WEIGHT OF SINGLE- AND DOUBLE-CHAIN COMPONENTS FROM RAT-SKIN COLLAGEN

| Component | Acetate Buffer, pH 4.8, 40° | 5 M Guanidine, 7° |
|---|---|---|
| $\alpha 1$ | 97,900 | 101,900 |
| | | 95,500 |
| $\alpha 2$ | 95,400 | 108,000 |
| | 114,000 | 110,000 |
| | | 108,000 |
| $\beta_{12}$ | 196,000 | 169,300 |
| | | 192,500 |
| | | 223,100 |

matographic effluent while $\alpha 1$ is the first (Piez *et al.*, 1961, 1963). There is pronounced tailing of all the components and degradation products may also chromatograph with $\alpha 2$. The extrapolated weight-average molecular weight of this sample was 95,400 (Fig. 3; Table I). Another sample gave a value of 114,000 (Table I).

Both $\alpha 1$ and $\alpha 2$ were relatively stable for extended periods under the conditions employed for sedimentation equilibrium. Measurements of the molecular weight at several times after equilibrium showed a loss of molecular weight no greater than a few per cent per day (Fig. 3).

Sedimentation-equilibrium experiments with $\beta_{12}$ at 40° gave variable results and the ln $c$ versus $x^2$ plots indicated marked heterogeneity. To investigate this phenomenon the weight-average molecular weight was followed as a function of time in the ultracentrifuge. Liquid columns of 1.5 mm were employed so that equilibrium would be reached rapidly. Apparent weight-average molecular weights were calculated from the difference in concentration at the top and bottom of the liquid column (Richards and Schachman, 1959). Six concentrations of the same sample were followed and extrapolations were done at each time point to correct for concentration dependence. After about 400 minutes, which was required to attain a steady state, the change in the logarithm of the molecular weight became essentially constant, describing a decrease in molecular weight of about 1% per hour. Extrapolation to zero time gave a molecular weight of 196,000 (Fig. 5; Table I).

There was an obvious interest in determining whether the degradation of $\beta_{12}$ were a reversal of the cross-linking process to yield single chains or were non-specific. Samples kept at 40° for 0, 4, 8, and 24 hours were examined by sedimentation velocity which readily distinguishes $\alpha$ and $\beta$. Schlieren patterns showed a progressive loss of $\beta_{12}$ but without the appearance of another discrete boundary. The degradation products were markedly heterogeneous but did not include measurable amounts of $\alpha 1$ and $\alpha 2$.

*Molecular Weights in 5 M Guanidine.*—As another approach to the problem of instability of $\beta_{12}$ and to provide additional evidence that the components under study were completely disaggregated, molecular weights were measured in 5 M guanidine at 7°. Under these conditions $\beta_{12}$, as well as $\alpha 1$ and $\alpha 2$, was stable for extended periods.

Since sedimentation-equilibrium studies in solvents such as 5 M guanidine involve both technical and theoretical problems, the methods were first applied to $\alpha 1$ and $\alpha 2$, which had been characterized in buffer solution. The measurement of protein concentration

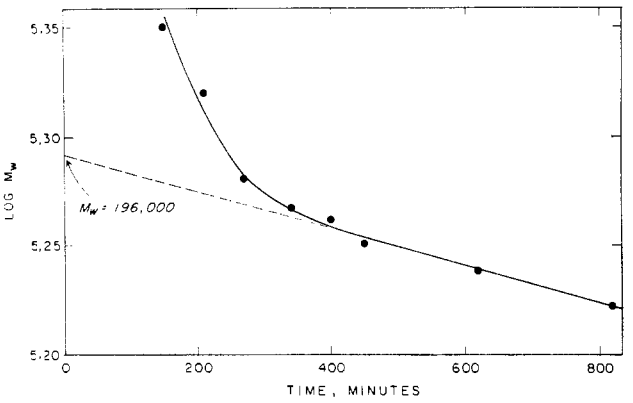


FIG. 5.—Dependence of the weight-average molecular weight of $\beta_{12}$ on time in acetate buffer at pH 4.8, 40°. The high values at early times resulted from initial overspeeding. Each point was corrected for concentration dependence by extrapolation of six concentrations to zero concentration.

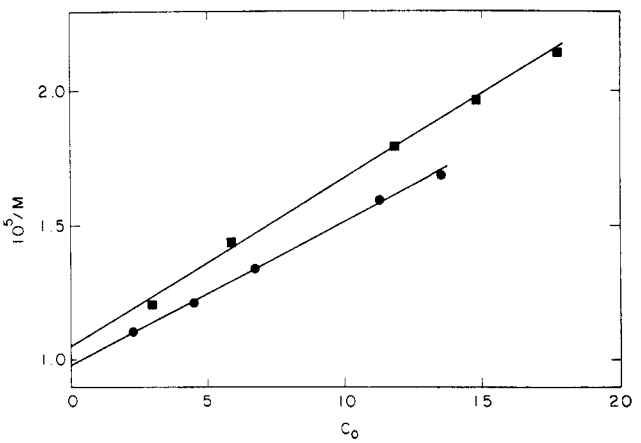


FIG. 6.—Concentration dependence of the molecular weight of $\alpha 1$ in 5 M guanidine at 7°. Measurements were made on two separate samples after 2620 minutes (●) and 2520 minutes (■) at 9945 rpm.

in the synthetic-boundary cell required particular care. If the solution were not carefully dialyzed against the solvent or if either were allowed to evaporate even a small amount, the resulting difference in guanidine concentration would contribute to the apparent protein concentration. The theoretical problems are associated with the possible effect of guanidine binding and the very large concentration dependence of molecular weight. These are considered later.

In spite of these difficulties, the apparent molecular weight of $\alpha 1$ in 5 M guanidine was found to be the same as in buffer within experimental error. Values of 101,900 and 95,500 were obtained with two separate samples (Fig. 6; Table I). The plots of ln $c$ versus $x^2$ showed nonideality to essentially the same degree as described below in detail for $\beta_{12}$. The very large dependence of molecular weight on concentration is indicated by a value of $B$ (equation 1) of $5.2 \times 10^{-1}$ fringe$^{-1}$. This is about three times as large as obtained from the experiments in buffer at 40°. The same value was obtained for $\alpha 2$ and $\beta_{12}$.

Several samples of $\alpha 2$ gave molecular weights near 110,000 (Table I). Heterogeneity was evident from the plots of ln $c$ versus $x^2$ as upward concavity of the curve. This was readily apparent in spite of the opposing effect of nonideality. This is in agreement with the results obtained in buffer.

Since measurements of molecular weight in guanidine gave acceptable results for $\alpha 1$ and $\alpha 2$, the procedure was

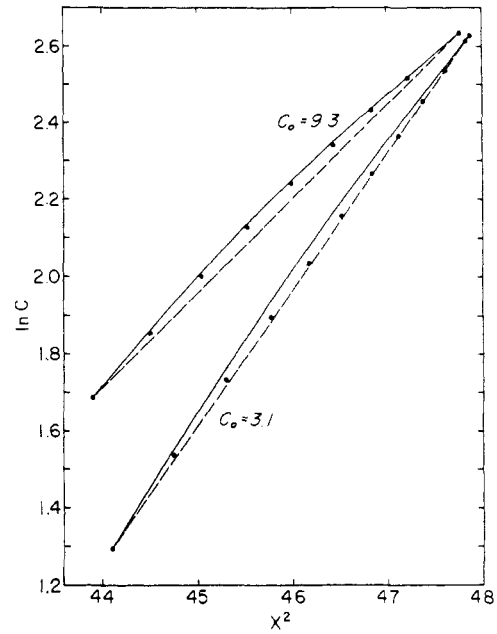


FIG. 7.—Distribution of $\beta_{12}$ in 5 M guanidine at 7° after 3100 minutes at 6995 rpm. See legend to Fig.2.

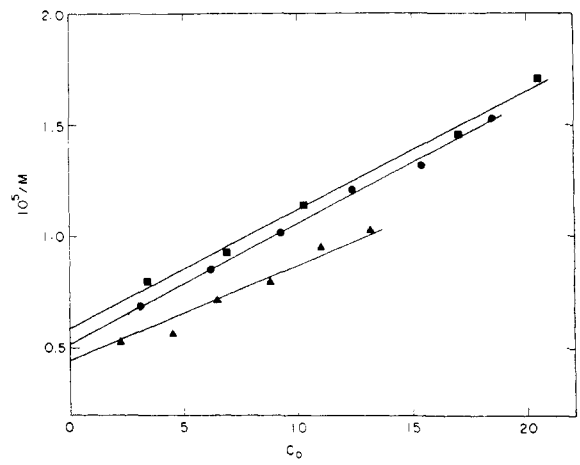


FIG. 8.—Concentration dependence of the molecular weight of $\beta_{12}$ in 5 M guanidine at 7°. Experiments with three separate samples are shown in which measurements were made after 4060 minutes (■), 3100 minutes (●), and 2560 minutes (▲) at 6995 rpm.

applied to $\beta_{12}$. The plots of ln $c$ versus $x^2$ showed nonideality which was evident even at concentrations below 1 mg/ml (Fig. 7). Calculation of the distribution of protein from the concentration dependence of molecular weight was carried out as described. Comparison of the theoretical curves with the experimental points showed a reasonably good fit at concentrations above 2 mg/ml but the points fell considerably below the curve at lower concentrations (Fig. 7). Although the deviation can be explained in part by heterogeneity, it would be expected that there would be a similar deviation at all concentrations as was observed in buffer at 40°. That the deviation was greater at low concentrations suggests that the treatment of this experimental system as described is not adequate. In any event, it is obviously difficult to independently assess the degree of homogeneity of $\beta_{12}$ from sedimentation-equilibrium experiments in 5 M guanidine. However, since similar results were obtained for $\alpha 1$ it may be assumed that $\beta_{12}$ has a similar degree of homogeneity.

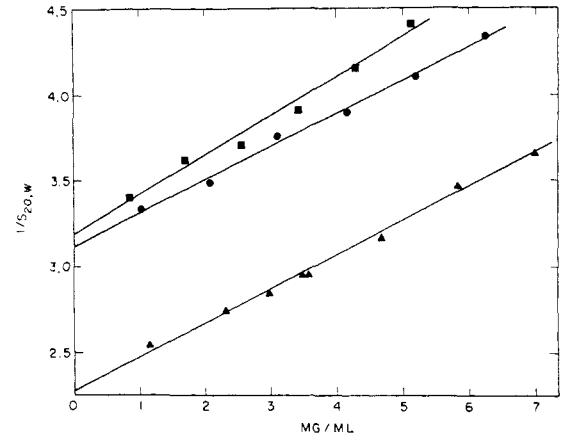


FIG. 9.—Concentration dependence of the sedimentation coefficients of $\alpha 1$ (●), $\alpha 2$ (■), and $\beta_{12}$ (▲).

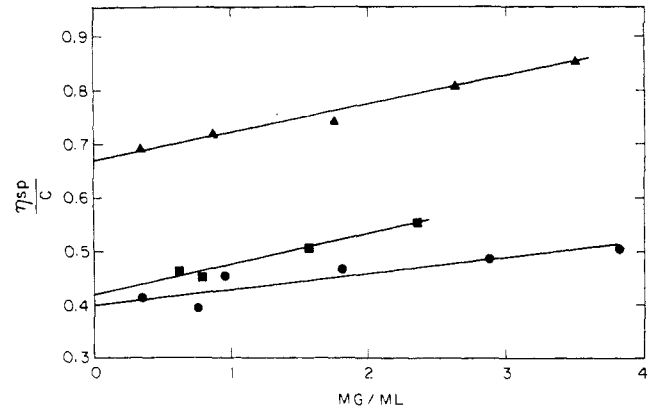


FIG. 10.—Concentration dependence of the reduced viscosities (dl/g) of $\alpha 1$ (●), $\alpha 2$ (■), and $\beta_{12}$ (▲).

The extrapolated molecular weights (Fig. 8, Table I) showed considerable scatter but averaged about 195,000, in good agreement with the result obtained with buffer as the solvent.

In studies done in 5 M guanidine the possible contribution of protein-bound guanidine to the apparent molecular weight must be considered. Cassasa and Eisenberg (1961) have demonstrated that the effect disappears in the usual extrapolation to zero concentration if an analog to the partial specific volume is obtained by measurements in the solvent employing an absolute measure of the protein concentration. This was not necessary in the present studies, since for both $\alpha 1$ and $\alpha 2$ the same molecular weights were obtained whether measured in guanidine or in buffer. Apparently guanidine binding was negligible.

*Sedimentation Coefficients.*—Samples of $\alpha 1$, $\alpha 2$, and $\beta_{12}$ were examined by sedimentation velocity in acetate buffer at 40°. The sedimentation coefficients obtained at a series of concentrations, corrected to standard conditions, are shown in Figure 9. Extrapolation to zero concentration gave values of 3.16 S for $\alpha 1$, 3.08 S for $\alpha 2$, and 4.20 S for $\beta_{12}$. The corresponding uncorrected values were 4.61 S, 4.50 S, and 6.13 S. The small difference between $\alpha 1$ and $\alpha 2$ may be within experimental error.

*Intrinsic Viscosities.*—Reduced viscosities of $\alpha 1$, $\alpha 2$, and $\beta_{12}$ were measured at a series of concentrations at 40° in acetate buffer. The results appear in Figure 10. The intrinsic viscosities, determined by extrapolation to zero concentrations, were 0.40 dl/g for $\alpha 1$, 0.42 dl/g for $\alpha 2$, and 0.67 dl/g for $\beta_{12}$. In view of the

TABLE II
PHYSICOCHEMICAL PROPERTIES OF SINGLE- AND DOUBLE-CHAIN COMPONENTS FROM RAT-SKIN COLLAGEN

| Component | Molecular Weight | $s^\circ_{20,w}$ | $[\eta]$ (dl/g) | $\beta^a$ |
|---|---|---|---|---|
| $\alpha 1$ | 98,000 ± 5,000 | 3.16 S | 0.40 | 2.24 |
| $\alpha 2$ | 98,000 ± 5,000 | 3.08 S | 0.42 | 2.23 |
| $\beta_{12}$ | 196,000 ± 10,000 | 4.28 S | 0.67 | 2.31 |

[a] Scheraga-Mandelkern.

contamination of $\alpha 2$ with $\beta_{12}$, the small difference between $\alpha 1$ and $\alpha 2$ has questionable significance.

## DISCUSSION

The physicochemical data are summarized in Table II. The molecular weights listed are the "best" values obtained as follows: (1) The value for $\alpha 1$, 98,000, is an average of the values in Table I. It can be accepted with considerable confidence since $\alpha 1$ is readily obtained free of other components and the sedimentation-equilibrium studies indicate reasonably good homogeneity. (2) The measured molecular weight of $\alpha 2$ (Table I) is probably high, since most samples probably contained at least 5% $\beta_{12}$. The actual molecular weight is difficult to estimate since other contaminants may also be present. However, $\alpha 1$ and $\alpha 2$ are likely to be closely related since they have similar amino acid compositions. For these reasons it is assumed that they have the same molecular weight, although a small difference cannot be ruled out. (3) By virtue of its constitution, $\beta_{12}$ should have a molecular weight equal to the sum of the single chains, 196,000. Since the measured values are very close to this number, it is accepted. The error in the molecular weights was estimated from the range of values obtained (Table I) and uncertainty in the partial specific volume (see Methods) to be about 5%.

The data in Table II can be combined (using, however, the uncorrected sedimentation coefficients) as described by the Scheraga-Mandelkern equation (see, for example, the discussion by Scheraga, 1961) to give the $\beta$ value, a number which is related to the hydrodynamic properties of the molecule. These values, shown in Table II, are all close to 2.3 for the three components. This number is consistent with the random-coil structure which would be expected under the conditions employed.

It has been proposed that the native collagen molecule from a variety of vertebrate species contains one $\alpha 2$ and two $\alpha 1$ chains which are to some extent intramolecularly cross-linked, the degree of cross-linking depending on the source. The evidence was derived from chromatographic behavior, amino acid composition, and stoichiometry (Piez *et al.*, 1961, 1963). A structure composed of three similar chains is also the simplest explanation of the three-stranded arrangement derived from X-ray diffraction. If this is correct, the molecular weight of the collagen molecule would be the sum of the individual chains. From the data obtained in these studies this value would be 294,000. This is significantly lower than the usually quoted value of 345,000 obtained by Boedkter and Doty (1956) in carefully conducted experiments. However, Rice *et al.* (1964) have recently reinvestigated this problem using a variety of methods including sedimentation equilibrium and concluded that a lower value, at most about 310,000, is better. A similar molecular weight, 286,000, would be calculated from the generally accepted values for the length of the molecule (3000 A), the residue spacing (2.86 A), the average residue weight (91), and the assumption of three similar chains

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