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THE SONIC FRAGMENTATION OF COLLAGEN MACROMOLECULES

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Communicated March 25, 1958

Introduction. It has been shown that collagen macromolecules obtained by extracting collagenous tissues from diverse sources with citrate buffer are essentially monodisperse proteins having in common a high molecular asymmetry.[1-4] For example, soluble calf-skin collagen, termed "procollagen" by Orekhovich, has been shown to be 3100 A in length, 13.5 A in diameter, and to have a molecular weight of 360,000.[4] These macromolecules in solution consist of three polypeptide chains apparently held together with hydrogen bonds in the configuration of Rich and Crick (Collagen I). This highly organized structure disappears in a phaselike transition upon raising the temperature; in the case of soluble calf-skin collagen this thermal denaturation temperature is about 36° C.[4] Various kinds of precipitating conditions cause the collagen molecules to form long fibrils with periodic band patterns.[5] One of these is the one found in native collagen, having a periodicity of about 700 A. The other two have a periodicity approaching the length of the macromolecule, that is, nearly 3000 A.

Having found that deoxyribosenucleic acid, which consists of two molecular chains hydrogen-bonded together, could be broken by sonic irradiation into a series of lower-molecular-weight samples without damaging the native, highly organized structure, we were tempted to see whether collagen molecules could be similarly fragmented.[6] A successful outcome was not clearly predictable because the thermal denaturation temperature is relatively low and local heating in the solution may be sufficient to cause denaturation. The detection of denaturation is easy because soluble calf-skin collagen is characterized by a specific rotation of $-415°$, whereas the denatured collagen (parent gelatin) has a specific rotation of $-135°$.[4]

Preliminary experiments showed that exposure to 9-kilocycle sonic waves caused a continuous fall in intrinsic viscosity accompanied by only a very small change in optical rotation. This clearly indicated that the molecular weight was being decreased by fracturing of the collagen molecules but that their secondary structure

was largely preserved. It therefore seemed of interest to investigate such sonic fragments over a substantial molecular-weight range in order to see whether the rodlike character was strictly preserved and whether fracture might occur at selected points such as that corresponding to the 700 A periodicity. Further stimulus was provided by Hodge and Schmitt's finding that these sonic fragments formed new types of banded structures that reveal new aspects of the molecular morphology of collagen.[7]

Experimental Details.—Soluble calf-skin collagen was prepared by extraction of finely divided calf skin in citrate buffer at pH 3.7, as previously described.[4] The physical properties of the preparation were similar to those previously reported: intrinsic viscosity 12.5 in units of 100 cc/gm and specific rotation, $[\alpha]_D = -415°$.

Eight 30-cc. aliquots of a stock solution of this preparation (0.26 gm/100 cc in 0.15 *M* citrate buffer, pH 3.7) were exposed to 9-kilocycle sonic waves in a 50-watt Raytheon magnetostriction generator for varying lengths of time. Since the extent of degradation depends on the volume and temperature as well as on the time of exposure, the volumes were kept constant and the temperature was maintained at 8°–11° C. by means of circulating cooling water.

The samples produced in this way, termed "sonicates," are listed in Table 1, together with various measurements to be discussed. The eight sonicates prepared

TABLE 1

MOLECULAR CONSTANTS OF COLLAGEN SONICATES

Time of Irradiation (Min.)	$[\alpha]_D$	$[\eta]$	Axial Ratio, a/b	$\beta \times 10^{-6}$	$s^0_{20,w}$	Molecular Weight	K_s*	$K_s/[\eta]$*
0	415	12.50	181	3.44	3.02	374,000	2.65	0.212
10	410	10.75	166	3.38	2.95	336,000	2.50	.232
40	420	8.65	147	3.37	2.87	297,000	2.27	.262
80	...	6.25	123	3.30	2.79	250,000	2.02	.324
120	407	4.95	108	3.25	2.68	217,000	1.82	.368
180	392	4.00	96	3.21	2.64	192,000	1.66	.415
240	398	3.25	85	3.16	2.59	170,000	1.54	.478
360	390	2.45	73	3.11	2.54	149,000	1.42	0.580
440	...	2.20	68	3.08	..	137,000†	...	...

* K_s expresses the concentration dependence of sedimentation constants according to the relation $s^0/s = 1 + K_s c$. The ratio $K_s/[\eta]$ generally has a value of about 1.6 for highly flexible, randomly coiled polymers and about 0.6 for the more extended polymers, such as cellulose derivatives. These results show that it has a dependence on axial ratio for rodlike macromolecules.

† This value was determined from the intrinsic viscosity with the use of Fig. 1.

in this fashion involved irradiation up to 440 minutes. The specific rotation of these sonicates fell gradually with time of exposure but did not exceed 10 per cent of the interval between native collagen and parent gelatin.

Results.—The intrinsic viscosity and the sedimentation constant at infinite dilution were determined for each sonicate by making the relevant measurements as a function of concentration in the same citrate buffer solvent. The plots of the reduced specific viscosity and the reciprocal of the sedimentation constant, $s_{20,w}$, against concentration are shown in Figures 1 and 2. The desired quantities, corresponding to the intercepts of these graphs, are listed in Table 1.

It is seen that the intrinsic viscosity, measured at 24.8° C., falls from 12.5 to 2.20, and the sedimentation constant from 3.02 to 2.54 in the range covered by these nine samples. By examining the sedimentation diagrams, it was evident that frag-

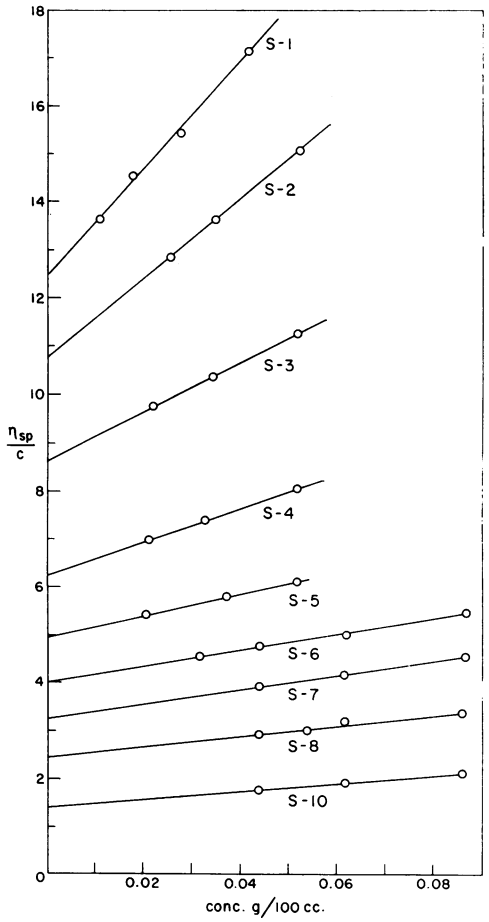

FIG. 1.—The reduced specific viscosity as a function of concentration for the collagen sonicates.

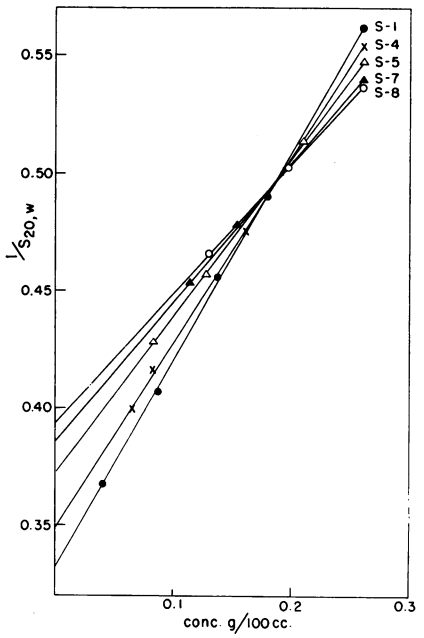

FIG. 2.—The reciprocal sedimentation constants as a function of concentration for the collagen sonicates.

mentation eventually affected all the molecules in the sample. For example, when the sedimentation diagram at 160 minutes (59,780 rpm) of sonicate 7, 8, or 9 was superimposed upon that for the original collagen under identical conditions, there was no overlap, despite the relatively small difference in sedimentation constants.

Molecular Weight and Hydrodynamic Properties of the Collagen Sonicates.—In view of the negligible amount of denaturation produced in the sonicates, it appears very likely that the sonicates are homologous pieces of the native collagen molecules that retain the rigid, rodlike structure. If this is true, the molecules will behave hydrodynamically as ellipsoids of revolution, and the molecular weight can be computed from the viscosity and sedimentation values, using the Scheraga-Mandelkern equation;[8]

$$M = \frac{s^0 n^{1/3}}{[\beta]} \frac{\eta_0 N}{(1 - \bar{v}\rho)}^{2/3}. \quad (1)$$

In this equation η_0 (= 1.095 centipoise) is the viscosity of the solvent; N is Avogadro's number; $\bar{v}$ is the partial specific volume of collagen, which we have found to be 0.73; ρ (1.015) is the solvent density; and β is a parameter whose value is determined by the axial ratio of the hydrodynamically equivalent ellipsoid. This axial ratio is in turn determined by the intrinsic viscosity.

In Table 1 the axial ratio, a/b, is listed with each value of the intrinsic viscosity and next to this the value of β.[8] Substitution into the above equation then yields the molecular weight; this is listed in the next column.

The dependence of intrinsic viscosity and sedimentation constant on the molecular weight can now be examined. The results are shown in the form of double logarithmic plots in Figures 3 and 4. In the case of intrinsic viscosity (Fig. 3)

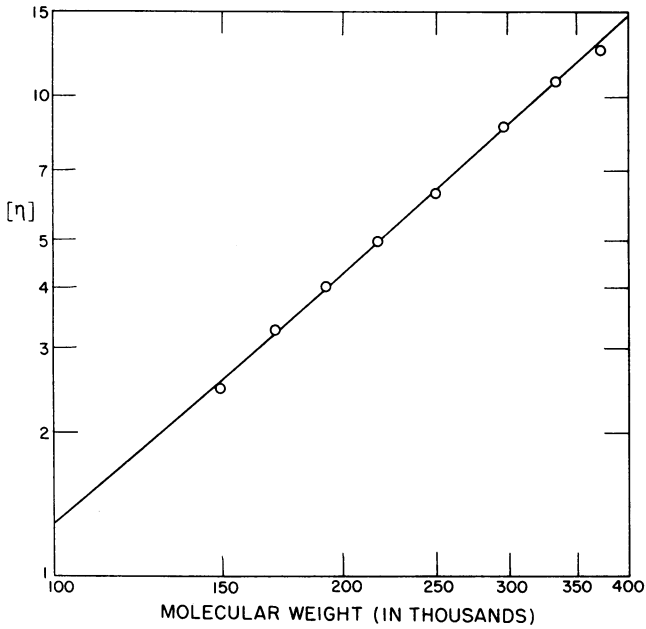

FIG. 3.—A double logarithmic plot of intrinsic viscosity against molecular weight. The line is a plot of Simha's equation with $2b$ = 16.7A and v = 0.73.

the dependence is very high. It can be represented empirically by the equation

$$[\eta] = 1.23 \times 10^{-9} M^{1.80} \quad (2)$$

This value of the exponent is that of the upper limit for ellipsoids having a constant minor axis or for cylinders of constant diameter, according to Simha's equation. The fit is excellent and confirms our assumption concerning the homologous, rod-like nature of the collagen sonicates.[10] The value used for the minor axis is quite acceptable. To convert it to the diameter of a cylinder of equivalent length and density, it must be multiplied by $(2/3)^{1/2}$: this yields 13.6 A. This value is in exact agreement with that previously found for collagen: it is slightly greater than the 12.0 A value derived from equatorial reflections in X-ray diffraction, as would be expected for a solvated molecule.

The dependence of sedimentation constant on molecular weight is small. Empirically it can be represented by (dashed line in Fig. 4)

$$s^0_{20,w} = 0.232\ M^{0.20}. \tag{3}$$

This likewise approximates the lower limiting value of the exponent for ellipsoids of constant minor axis. Perrin's equation[11] expresses the relation between s^0 and the axial ratio; for axial ratios greater than 10 it takes the following form:

$$s^0 = \frac{(1 - \bar{v}\rho)}{\eta_0 \bar{v}} \frac{2}{9} b^2 \ln 2\frac{a}{b}. \tag{4}$$

The axial ratio is related to the molecular weight of the collagen sonicates by

$$M = (4\ \pi/3)(a/b)b^3(N/v) = 2020\ (a/b). \tag{5}$$

Substitution for the constants in Perrin's equation leads to

$$s^0 = 1.16 \log 2\ (a/b) = 1.16 \log\ (M/1010). \tag{6}$$

The solid line drawn in Figure 4 results from equation (6). The slope of 1.22 in the empirical line is a little larger than 1.16 in the theoretical one. This discrepancy, however, is within experimental error. Thus it is seen that the sedimentation constants, as well as the intrinsic viscosities, vary with molecular weight in the manner expected of homologous, rodlike molecules with variable length.[12]

The Molecular-Weight Limit in Sonic Fragmentation.—If the rate of molecular scission, that is, sonic rupture of the collagen molecules, were directly dependent on the time of irradiation, the molecular weight would fall with time in such a manner that the reciprocal of the molecular weight would be linear with time. When the data in Table 1 are plotted in this manner, one observes such a linear relation for the first 120 minutes, but thereafter the rate of increase of the reciprocal molecular weight diminishes. This behavior is widely observed in the sonic degradation of polymers. Several analyses of this behavior have been based on the assumption that there is a lower limit of molecular weight below which sonic irradiation will not produce scission. Schmid's[13] formulation of the problem is convenient for our purposes. He assumes that the rate of scission is proportional to the difference between the molecular length at a given time and the limiting molecular length beyond which

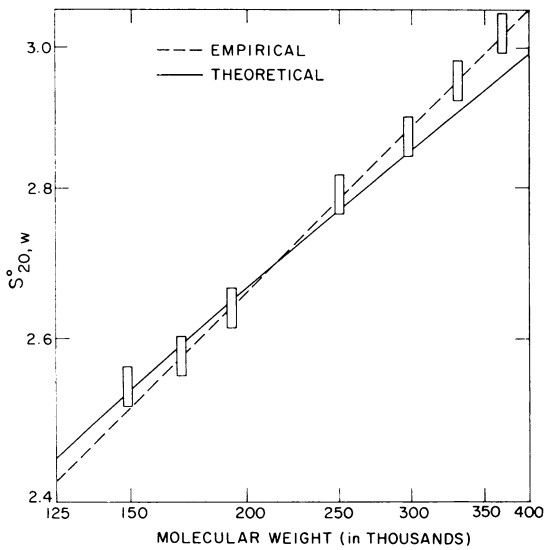

FIG. 4.—A double logarithmic plot of sedimentation constants at infinite dilution against molecular weight. The solid line is a plot of Perrin's equation; the dashed line is a plot of empirical values.

scission will not occur. Since length is proportional to molecular weight, we may denote M_0 as the initial molecular weight, M_t as the molecular weight at time t, and M_f as the final or limiting molecular weight. His analysis then takes the form

$$\frac{M_f}{M_t} + \ln\left(1 - \frac{M_f}{M_t}\right) = -\frac{KM_f^2}{cm^2}t + \frac{M_f}{M_0} + \ln\left(1 - \frac{M_f}{M_0}\right), \qquad (7)$$

where c is the initial concentration of the polymer in moles per liter and m is the molecular weight of a monomeric unit.

To explore the feasibility of this explanation of our data, we have plotted the left side of equation (7) against time for various values of M_0 relative to M_f. When M_f is taken as $1/4\ M_0$, a linear plot, shown in Figure 5, is obtained. Choices of

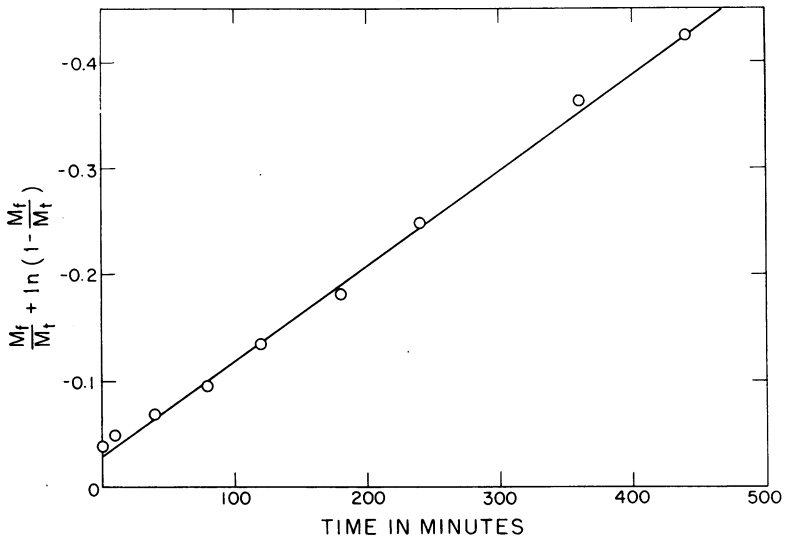

FIG. 5.—A plot of time-dependence of the molecular weight upon exposure to sonic waves in terms of the Schmid equation.

$1/2\ M_0$ or $1/8\ M_0$ show clear deviations from linearity in opposite directions. This indicates that our data are at least compatible with the idea that one-fourth the original collagen molecule is the limiting molecular weight produced by scission. To test this possibility further, a sample was prepared by very long sonication, 1,140 minutes. From its intrinsic viscosity (1.405) and the plot of Simha's equation in Figure 3, its molecular weight was estimated to be 105,000. On the other hand, the value of M_t at 1,140 minutes' sonication predicted by equation (7) with $M_f/M_0 = 1/4$ and the slope in Figure 5 ($= -(KM_f^2/\text{cm}^2) = 0.912 \times 10^{-3}$/minute) was 108,000.

Thus it appears that the time-dependence of the molecular weight of collagen exposed to sonic irradiation can be explained in terms of scission being most probable at three evenly spaced regions of the molecule. Since this would correspond to the 700 A periodicity observed in native collagen fibrils, it is tempting to suggest that the molecular regions giving rise to the 700 A spacing are the points where the collagen molecule is most easily broken. However, at this stage this must be considered only as a plausible suggestion. Electron-microscopy and flow-birefringence

studies will provide a critical test. Meanwhile, it is relevant to point out that the molecular lengths of 45 and 55 per cent of the over-all length of the original collagen molecule, found by Hodge and Schmitt[7] in their electron-microscopic study of collagen sonicates is consistent with this suggestion that there is a preferential point of scission located at (approximately) the mid-point as well as at one-quarter and three-quarters of the distance along the original collagen macromolecule.

Summary.—The exposure of soluble calf-skin collagen to 9-kilocycle sonic irradiation at low temperature causes fragmentation of the long, rodlike collagen macromolecules into shorter pieces that retain the three-stranded, helical structure. A series of samples produced by varying the time of exposure from 10 to 440 minutes were found to have molecular weights in the range of 335,000–140,000. The intrinsic viscosity of these samples depended on the 1.80 power of the molecular weight, and the sedimentation constant on the 0.20 power. This dependence was compatible with the behavior expected of homologous, rodlike molecules of 13.6 A diameter (solvated). The time-dependence of the molecular-weight change is compatible with preferential fracture of the collagen macromolecule into halves and quarters.

* This investigation was supported by a research grant (C-2170) from the National Cancer Institute, U. S. Public Health Service.

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