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<sup>1</sup>M. F. Schwartz, *J. Acoust. Soc. Amer.* 43, 1178-1179(L) (1968).

<sup>2</sup>M. F. Schwartz, *J. Acoust. Soc. Amer.* 44, 1736-1737(L) (1968).

<sup>3</sup>F. Ingemann, *J. Acoust. Soc. Amer.* 44, 1142-1144(L) (1968).  
<sup>4</sup>F. S. Brodnitz, *Vocal Rehabilitation* (Whiting Press, Rochester, Minnesota, 1959), pp. 16-59.

<sup>5</sup>C. Van Riper and J. V. Irwin, *Voice and Articulation* (Prentice-Hall, Englewood Cliffs, N. J., 1958), Chap. 8.

<sup>6</sup>B. Weinberg and M. Zlatin, *J. Speech Hearing Res.* 13, 418-425 (1970).

<sup>7</sup>B. Weinberg and S. Bennett, "Talker Sex Recognition of Esophageal Voices," *J. Speech Hearing Res.* (to be published).

<sup>8</sup>B. Weinberg and S. Bennett, "Vowel Formant Frequencies, Voice Fundamental Frequency, and Listener Judgments of Speaker Sex," *Speech Res. Lab., Indiana Univ. Med. Ctr.* (1970) (unpublished).

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## Ultrasonic Examination of the Hemoglobin Dissociation Process in Aqueous Solutions of Guanidine Hydrochloride\*

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The ultrasonic absorption was determined, in aqueous solutions of hemoglobin undergoing dissociation as a function of the strong electrolyte guanidine hydrochloride (GuHCl), up to 5.4M (molar), over the frequency range 1-50 MHz. With increasing GuHCl concentration to 0.7M, the Hb molecule dissociates into half-molecules without loss of tertiary structure, and at GuHCl concentrations greater than 2.5M, the Hb molecule dissociates further into its four individual polypeptide chains with complete loss of tertiary structure. The ultrasonic absorption coefficient appears to be insensitive to the dissociation process, though a small contribution appears at the lower frequencies, owing to the expansion of the polypeptide molecule. A maximum in the absorption occurs near 2M GuHCl concentration, which does not correspond to dissociation or expansion of the Hb molecule, and it is thought to be associated with the proton transfer reaction.

The role of the conformation of proteins in the absorption of ultrasonic energy in biological media has received some attention.<sup>1-3</sup> The ultrasonic absorption spectra of macromolecules which assume random coil conformation in aqueous solution, viz., dextran<sup>1</sup> and polyethylene glycol,<sup>4</sup> exhibit magnitudes considerably less than those of the globular proteins which exist in compact rigid structures. This magnitude difference has been attributed to the secondary and tertiary structures possessed by the proteins, which dextran and polyethylene glycol

do not exhibit. In addition, the protein gelatin, which does not possess a secondary or tertiary structure, has a lesser absorption magnitude than those proteins with such structuring.<sup>5</sup> Thus, it appears profitable to examine the ultrasonic absorption spectra of a globular protein possessing a higher-ordered structure as it is denatured into its random-coil conformation. The protein hemoglobin was selected, since its conformational changes have been well characterized as a function of the protein denaturant guanidine hydrochloride, GuHCl.<sup>6</sup>

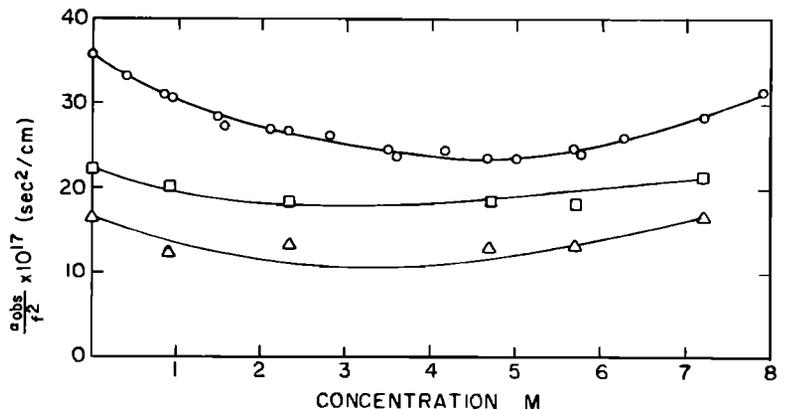


FIG. 1. Concentration dependence of observed (total) ultrasonic absorption of aqueous solutions of guanidine hydrochloride ( $f=8.87-50.5$  MHz). ○, 10°C; □, 22.2°C; △, 37.1°C.

The two systems utilized in the measurements of the ultrasonic absorption have been extensively described elsewhere.<sup>7,8</sup> Basically, the high-frequency system, for measurements greater than 8 MHz, is an automated version of that of Pellam and Galt,<sup>9</sup> and the low-frequency system, which minimizes diffraction effects, is a comparison technique initially described by Carstensen *et al.*<sup>10</sup> Both systems maintain the temperature of the liquid under investigation to within an accuracy of  $\pm 0.05^\circ\text{C}$  as checked against an NBS calibrated thermometer. The absolute accuracy of the ultrasonic absorption measurements, for both systems, is within  $\pm 3\%$ .

Aqueous solutions of uncrystallized bovine hemoglobin (Nutritional Biochemicals Corp., Control No. 3099) were prepared by placing the proper amount of powder on top of a measured volume of singly deionized and distilled water and stored at  $7^\circ\text{C}$  until mixing was complete, usually 2–5 h. The solutions were centrifuged at 20 000 g's for 2 h to remove the heavier particles, and the supernatant was filtered twice through type-A glass fiber filters (Gellman Instrum. Co.) which removed particles larger than  $0.3\ \mu$  in diameter. Eight molar aqueous guanidine hydrochloride (GuHCl) solutions were prepared (Eastman Organic Chemicals, Lot No. 882K, and Nutritional Biochemicals Corp., Control No. 4124). They were filtered once through a type-A glass fiber filter and the concentration determined by evaporating over air, to near dryness (as it is highly hydroscopic), and dried in a vacuum desiccator over indicating

anhydrous  $\text{CaSO}_4$  for one week. The concentration determination of the GuHCl–Hb solutions was obtained first by determining accurately the Hb solution concentration before GuHCl was added, and then, while adding the GuHCl, measuring both the increase in volume due to GuHCl and its dry weight. The concentration accuracies are estimated to be from  $\pm 0.3\%$  to  $\pm 3\%$ , with the accuracy decreasing as the GuHCl concentration increased.

The conformational changes of hemoglobin which result from GuHCl solutions have been characterized by a number of investigators.<sup>5,11</sup> Figure 1 shows the frequency-free absorption characteristics of the aqueous GuHCl solutions as a function of concentration at  $10.0^\circ$ ,  $22.2^\circ$ , and  $37.1^\circ\text{C}$ . No relaxation processes were observed over the frequency range 8–50 MHz, and the absorption magnitude was found to be less than that of water throughout the temperature range considered.

In Figs. 2 and 3, the ordinate is the frequency-free excess Hb solution absorption per unit Hb concentration, defined as

$$A = (\alpha_{\text{obs}} - \alpha_{\text{GuHCl}}) / c_{\text{Hb}} f^2, \quad (1)$$

where  $\alpha_{\text{obs}}$  is the measured Hb solution ultrasonic absorption (corrected for diffraction) at frequency  $f$  and hemoglobin concentration  $c_{\text{Hb}}$ , and  $\alpha_{\text{GuHCl}}$  is the absorption of the solvent, taken from Fig. 1. In both figures the peaks in the absorption parameter  $A$  occur around a GuHCl concentration of  $2M$ . Also, for frequencies 1.7, 3.1, 5.1, 8.9, and 14.8 MHz,  $A$  is greater at zero GuHCl concentration than at the higher concentrations employed, *viz.*, around  $5M$ .

Kirshner and Tanford<sup>12</sup> have shown that negligible differences resulted between bovine and human hemoglobin in aqueous  $\text{NaCl}$ ,  $\text{MgCl}_2$ , and  $(\text{NH}_4)_2\text{SO}_4$  solutions up to  $3M$  concentration in the temperature range  $10$ – $25^\circ\text{C}$ . On the other hand, marked differences between human and horse hemoglobin have been observed.<sup>13,14</sup> Therefore, it is assumed in this study that bovine and human hemoglobin molecules behave similarly under varying conditions of ionic environment. This assumption is necessary, as much work has been reported on the elucidation of the physical and chemical properties of human hemoglobin, while little information is available for bovine hemoglobin.

At zero GuHCl concentration, the hemoglobin molecule is in aqueous solution in its native conformation, *i.e.*, a compact rigid molecule made up of four subunits noncovalently linked.<sup>6</sup> Each of these subunits, two termed  $\alpha$  polypeptide chains and two termed  $\beta$  polypeptide chains, is conjugated to an iron-containing moiety. There are no disulfide cross links which would prevent each of the four polypeptide chains from assuming a completely random coil configuration. As the GuHCl concentration increases to  $0.7M$ , the sedimentation coefficient decreases and the diffusion coefficient increases. Between  $0.7$  and  $2.5M$ , the sedimentation

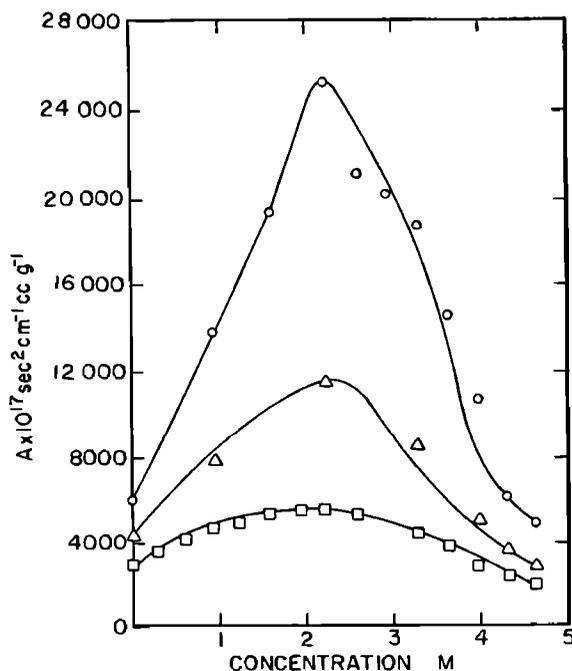


FIG. 2. Guanidine hydrochloride titration of bovine hemoglobin at  $10^\circ\text{C}$  over the frequency range 1–5 MHz;  $\circ$ , 1.7 MHz;  $\Delta$ , 3.1 MHz;  $\square$ , 5.1 MHz.

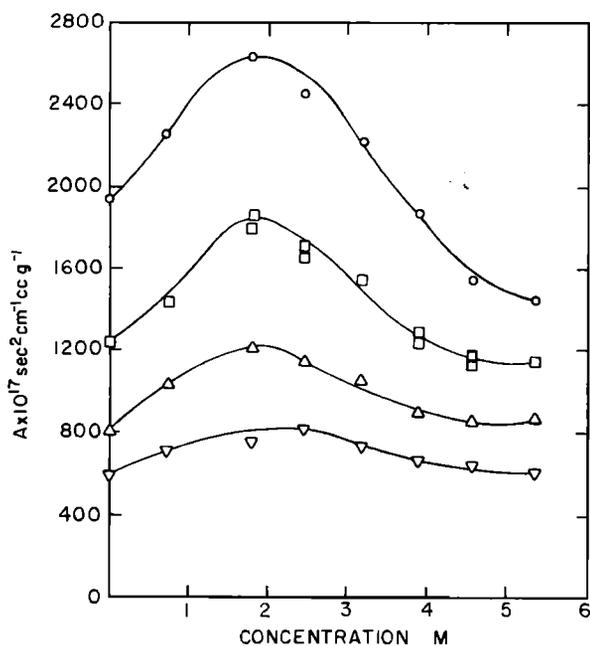


FIG. 3. Guanidine hydrochloride titration of bovine hemoglobin at 10°C over the frequency range 8–50 MHz. ○, 8.9 MHz; □, 14.8 MHz; △, 26.7 MHz; ▽, 50.4 MHz.

coefficient remains constant. Physically, the hemoglobin molecule has dissociated in half without any structural changes occurring to the individual polypeptide chains. These half-molecules each contain an  $\alpha$  polypeptide chain and a  $\beta$  polypeptide chain. As the GuHCl concentration is increased beyond 2.5M, the sedimentation coefficient decreases to a limiting value around 5M and the intrinsic viscosity of the solution indicates that the protein has greatly expanded. Thus the half-molecules have broken in half again, yielding the individual  $\alpha$  and  $\beta$  polypeptide chains, and these chains have assumed the random coil formation.

From Figs. 2 and 3, it is seen that the conformational changes of the Hb molecule do not produce concomitant changes in the ultrasonic absorption data, which would suggest that  $A$  is responsive to the dissociation process, within the GuHCl range 0–0.7M. However, there does appear to be a small contribution to the ultrasonic absorption due to conformational changes as seen by the difference in the magnitude of the absorption at zero concentration of GuHCl, where the Hb molecule is compact and rigid, and beyond 5M concentration, where it has assumed the random coil conformation. This is most notable in the frequency range 1–15 MHz.

The peak in the absorption parameter  $A$ , occurring around 2M GuHCl, can be attributed to the proton transfer reaction. It has been shown<sup>15</sup> that the guanidinium ion ( $\text{GuH}^+$ ) can partake in a proton exchange reaction with water under appropriate conditions in which a second solute, Hb in this case, forms strong hydrogen bonds with either water or  $\text{GuH}^+$ , and it is suggested that this proper mixture is achieved around 2M GuHCl at which the proton exchange reaction is maximized. This particular mechanism has already been invoked to explain the ultrasonic absorption in aqueous polypeptide and protein solutions.<sup>7,16–18</sup>

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<sup>1</sup> S. A. Hawley, L. W. Kessler, and F. Dunn, *J. Acoust. Soc. Amer.* **38**, 521 (1965).

<sup>2</sup> L. W. Kessler and F. Dunn, *J. Phys. Chem.* **73**, 4256 (1969).

<sup>3</sup> J. Lang, C. Tondre, and R. Zana, *J. Phys. Chem.* **7**, 374 (1971).

<sup>4</sup> L. W. Kessler, W. D. O'Brien, Jr., and F. Dunn, *J. Phys. Chem.* **74**, 4096 (1970).

<sup>5</sup> H. Pauly and H. P. Schwan, *J. Acoust. Soc. Amer.* **50**, 692–701 (1971).

<sup>6</sup> K. Kawahara, A. G. Kirschner, and C. Tanford, *Biochemistry* **4**, 1203 (1965).

<sup>7</sup> W. D. O'Brien, Jr., PhD thesis, Univ. of Illinois, Urbana, Ill. (1970).

<sup>8</sup> L. W. Kessler, S. A. Hawley, and F. Dunn, *Acustica* **23**, 105 (1971).

<sup>9</sup> J. R. Pellam and J. K. Galt, *J. Chem. Phys.* **14**, 608 (1946).

<sup>10</sup> E. L. Carstensen, K. Li, and H. P. Schwan, *J. Acoust. Soc. Amer.* **25**, 286 (1953).

<sup>11</sup> K. Kurihara and K. Shibata, *Arch. Biochem. Biophys.* **88**, 298 (1960).

<sup>12</sup> A. G. Kirschner and C. Tanford, *Biochemistry* **3**, 291 (1964).

<sup>13</sup> A. Rossi Fanelli, E. Antonini, and A. Caputo, *Adv. Prot. Chem.* **19**, 73 (1964).

<sup>14</sup> H. Polet and J. Steinhardt, *Biochemistry* **8**, 857 (1969).

<sup>15</sup> K. C. Tewari, N. C. Li, and R. J. Kurland, *J. Phys. Chem.* **73**, 2853 (1969).

<sup>16</sup> W. D. O'Brien, Jr., and F. Dunn, to be published in *J. Phys. Chem.*

<sup>17</sup> M. Hussey, PhD thesis, Univ. Pennsylvania, Philadelphia, Pa. (1970).

<sup>18</sup> M. Hussey and P. D. Edmonds, *J. Acoust. Soc. Amer.* **49**, 1309 (1971).