CONCENTRATION DEPENDENCE OF THE SELF-DIFFUSION OF HUMAN AND *LUMBRICUS TERRESTRIS* HEMOGLOBIN

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ABSTRACT The self-diffusion coefficient of the extracellular hemoglobin of *Lumbricus terrestris* (mol wt 3.7×10^6 daltons) has been measured at protein concentrations ranging from 2 to 25 g/100 ml. The self-diffusion coefficient of human hemoglobin has been measured at concentrations between 10 and 43 g/100 ml. For these measurements, ^{14}C-labeled hemoglobin was made to diffuse from one Millipore filter into three consecutively arranged Millipore filters containing unlabeled hemoglobin. After a suitable time the filters were separated, and the protein diffusion coefficient was determined from the distribution of radioactivity in the four filters with a table given by Kawalki (1894, *Ann. Phys. Chem.* **52**:166–190.). The following results were obtained. The diffusion coefficient of *Lumbricus* hemoglobin is 1.2×10^{-7} cm^2s^{-1} at a protein concentration of 2.1 g/100 ml, and is reduced to about 1/10 of this value when the concentration is 25 g/100 ml (T = 21°C). Between 0 and 16 g/100 ml the logarithm of the diffusion coefficient of *Lumbricus* hemoglobin falls linearly with concentration. Above 16 g/100 ml a marked increase in the concentration dependence of the diffusion coefficient is observed. Extrapolation of the data to zero hemoglobin concentration yields a limiting value of the diffusion coefficient of *Lumbricus* hemoglobin of 1.3×10^{-7} cm^2s^{-1}. The diffusion coefficient of human hemoglobin is 4.5×10^{-7} cm^2s^{-1} at a hemoglobin concentration of 9.7 g/100 ml, and falls to 0.14×10^{-7} cm^2s^{-1} at a hemoglobin concentration of 43.0 g/100 ml. In addition to diffusivities, the viscosities of human and *Lumbricus* hemoglobin solutions were measured in a wide range of protein concentrations.

The concentration dependence of the diffusivity of *Lumbricus* hemoglobin is compared to that of myoglobin, ovalbumin, and tetrameric hemoglobin. Proportionality between the diffusion coefficient and the reciprocal of the viscosity of the protein solution is found for all these proteins. It is also shown that an equation proposed by Anderson (1973) gives an excellent description of the diffusivity of the various proteins up to moderate protein concentrations. Above concentrations of 16 g/100 ml for *Lumbricus* hemoglobin, and 30 g/100 ml for tetrameric hemoglobin, however, protein diffusivity falls much more rapidly with increasing concentration than is predicted by this equation.

INTRODUCTION

It has been recognized in recent years that proteins play an important role in the intracellular transport of small molecules and ions. Intracellular protein diffusion appears to be, for instance, a highly effective mechanism of oxygen, carbon dioxide, and proton

transport (Scholander, 1960; Wittenberg, 1966; Longmuir et al., 1966; Gros and Moll, 1972, 1974). This, of course, has aroused interest in understanding the conditions under which protein diffusion occurs within cells. A first step towards this goal seems to be the study of protein diffusivity in solutions with protein concentrations as high as those found inside cells.

The first measurement of the diffusion coefficient of a protein at high concentration is that of Wang et al. (1954) for ovalbumin. Subsequently, the study of protein diffusion in concentrated solutions has been focused on tetrameric mammalian hemoglobins (Moll, 1966; Keller and Friedlander, 1966; Keller et al., 1971; Riveros-Moreno and Wittenberg, 1972; Veldkamp and Votano, 1976; Alpert and Banks, 1976), and only two other proteins, with physicochemical properties rather similar to those of hemoglobin, were investigated (Moll, 1968; Keller et al., 1971; Riveros-Moreno and Wittenberg, 1972). These studies showed that the diffusion coefficients of proteins decrease markedly as the protein concentrations rise. To understand the molecular mechanisms responsible for this decrease in protein diffusivity, it seemed to be profitable to study the diffusion of a protein whose molecular weight is markedly outside the limited range of molecular weights covered so far. We chose the giant hemoglobin of the earthworm, *Lumbricus terrestris,* which has a molecular weight of 3.7×10^6 and is undissociated in the neutral pH region (Shlom and Vinogradov, 1973; Vinogradov et al., 1977). We report in this paper measurements of the self-diffusion coefficient of this protein at concentrations between 2 and 25 g/100 ml. In addition, we report diffusivity data for human hemoglobin in a concentration range of 10–43 g/100 ml, and compare them with data in the literature.

MATERIALS AND METHODS

Materials

LUMBRICUS TERRESTRIS HEMOGLOBIN Live earthworms were obtained from local commercial sources. They were washed in distilled water and anesthetized with chloroform. After the worms were cut at about the seventh body segment, blood was collected in a flask by applying moderate suction to both sections of the worms. The collecting flask contained 0.15 M NaCl and was kept in ice. The impure hemoglobin solution was centrifuged at 20,000 *g* for 30 min to remove any particulate material. The supernatant was then centrifuged at 180,000 *g* for 90 min in an IEC model B 60 preparative ultracentrifuge (Biotronik, Frankfurt-am-Main, W. Germany). The supernatant was decanted and the sediment was resuspended in 0.15 M NaCl. The hemoglobin solution was then subjected to one more low-speed and three more high-speed centrifugations. After the last high-speed centrifugation, the supernatant was practically protein-free. By dissolving the final pellet in various volumes of 0.15 M NaCl, solutions with hemoglobin concentrations ranging from 0 to 26 g/100 ml were obtained.

The protein thus prepared appeared to be physically homogeneous, as judged from column chromatography and analytical ultracentrifugation. It was eluted as a single peak from a column of Sepharose 6 B (Pharmacia Fine Chemicals, Uppsala, Sweden), and it exhibited a single symmetrical peak in the ultracentrifuge. The sedimentation coefficient of the earthworm hemoglobin was determined at hemoglobin concentrations of 5 and 10 mg/ml (0.15 M NaCl, pH 7.4, 20°C). By linear extrapolation to infinite dilution an estimate of $s^0_{20,w}$ could be obtained. We found a value of 60.6S, which agrees well with the figures obtained

previously (Svedberg and Eriksson-Quensel, 1936; Shlom and Vinogradov, 1973; David and Daniel, 1974).

The *Lumbricus* hemoglobin was stored in the form of little droplets in liquid nitrogen, where it proved stable for several months.

HUMAN HEMOGLOBIN 2–3 wk-old acid citrate dextrose blood was obtained from the blood bank. Red cells were thrice washed in saline, and lysed by addition of three volumes of distilled water. Subsequently, NaCl was added to give an ionic strength of 0.15, and the ghosts were removed by 30 min of centrifugation at 20,000*g*. The hemoglobin solutions were then concentrated in an Amicon ultrafiltration apparatus (Amicon Corp., Lexington, Mass.) (with a PM 10 membrane) under 5 atm CO.

REACTION OF HEMOGLOBIN WITH CYANATE All diffusion experiments were carried out with ^{14}C-labeled and unlabeled carbamylated hemoglobin. Carbamylation of earthworm hemoglobin was performed at 25°C with a total concentration of KNCO (Merck Co., Darmstadt) of 2.1 mM in a 5.2 g/100 ml earthworm hemoglobin solution. The reaction was allowed to proceed for 5 hr, while pH was maintained at 6.50. The reaction mixture was then stored for 15 hr at 5°C. The reaction was stopped by two high-speed centrifugations with replacement of the supernatants by 0.15 M NaCl. Subsequently, the solution was exhaustively dialyzed against several changes of 0.15 M NaCl. Labeled and unlabeled hemoglobin was prepared under exactly identical conditions except that in order to obtain labeled hemoglobin, 15% of the total KNCO added was substituted by KN^{14}CO (Amersham-Buchler, Braunschweig, W. Germany; sp act 51 mCi/mmol). The procedure for the carbamylation of human hemoglobin was identical. At the end of the reaction time, the solutions of human hemoglobin were placed in a dialysis bath containing 60 vol of 0.15 M NaCl. Several changes of NaCl solution followed.

Cyanate reacts irreversibly with amino groups of proteins (Stark and Smyth, 1963; Stark, 1965). At a pH value of 6.5 it should preferably bind to α-amino groups, but also ϵ-amino groups will be modified to some extent (Williams et al., 1975). The specific activity of our preparation indicates that about 20 mol of cyanate were incorporated per mole of (whole) *Lumbricus* hemoglobin, and about 8 mol of cyanate per mole of human hemoglobin. Determinations of the sedimentation coefficient of the carbamylated earthworm hemoglobin gave a value of $s^0_{20,w}$ identical to that obtained for the unmodified protein.

SODIUM-22 ^{22}NaCl was used for calibration of the filters and was purchased from Amersham-Buchler (Braunschweig). It had a specific activity of 200 mCi/mg Na. The solution used for the diffusion experiments with ^{22}Na was 0.09375 M KCl containing 2.13×10^{-7} M ^{22}Na.

Analytical Methods

HEMOGLOBIN CONCENTRATION The concentration of *Lumbricus* hemoglobin was determined spectrophotometrically after conversion to the cyanmet derivative. For absorbances read at 540 nm absorptivities of 4.42/1% and per centimeter, or $11.2 \cdot 10^3$ cm^2/mmol of heme, were used (Shlom and Vinogradov, 1973). For human hemoglobin an extinction coefficient of $11 \cdot 10^3$ cm^2/mmol was applied.

CARBON-14 Defined volumes (up to 0.2 ml) of labeled hemoglobin solutions were brought onto filter paper. The filter paper was allowed to dry, then folded and wrapped in a second filter paper, and pressed to give a compact tablet weighing ~ 0.5 g. The tablets were burned in an automatic tritium-carbon-oxidizer (model 305, Packard Instrument Co., Downer's Grove, Ill.). The oxidizer produced 20 ml of solution containing the ^{14}C of the original sample in the form of $^{14}CO_2$ bound to ethanolamine, as well as scintillator. Radioactivity of this solution was determined with a Tri-Carb liquid scintillation spectrometer (model 3380, Packard Instrument Co.). A linear relationship between the counts per minute

obtained with the scintillation spectrometer and the amount of labeled hemoglobin fed into the oxidizer was established for radioactivities ranging from 1,000 to 100,000 cpm.

SODIUM-22 The radioactivity was generally determined with the above spectrometer in samples containing 0.2 ml of 0.09375 M KCl with varying tracer amounts of ^{22}Na and 10 ml of Unisolve 1 scintillator (Werner Zinsser, Frankfurt-am-Main, W. Germany).

Diffusion Coefficients

The principle used here to estimate the diffusion coefficient of earthworm hemoglobin has been described by Kawalki (1894). Four layers of equal thickness and area are arranged consecutively. The diffusing substance is originally confined to the first layer. Let this substance begin to diffuse into the other three layers at time $t = 0$. The ratio of the amount of substance present in layer n, a_n, over the total amount of substance present in the four layers, a_{tot}, will then depend on the diffusion coefficient, D, on the diffusion time, t, and on the thickness of the layers, l:

$$a_n/a_{tot} = f_n(4Dt/l^2) \ (n = 1, 2, 3, 4). \tag{1}$$

Numerical values of the functions, f_n, were obtained by Stefan (1879) and Kawalki (1894). They have been tabulated by Jost (1960), and are graphically shown in Fig. 1. They allow the calculation of the diffusion coefficient when the concentrations in the four layers have been measured and when t and l are known.

Moll (1966) has adopted this principle to measure the diffusion coefficient of mammalian hemoglobin, using Millipore filters (Millipore Corp., Bedford, Mass.) to prepare thin layers

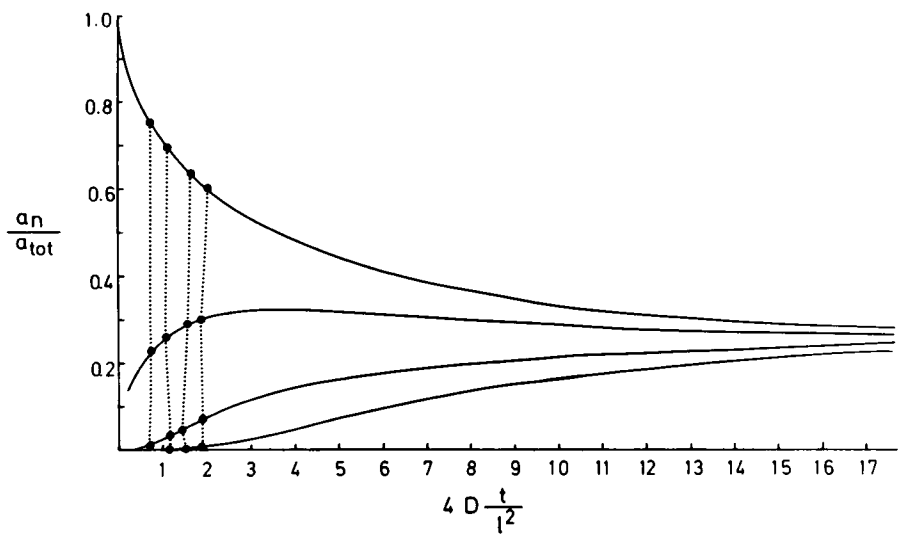

FIGURE 1 The relative amount of radioactive hemoglobin contained in the n^{th} layer, a_n/a_{tot}, as a function of $4\ Dt/l^2$, where D is the hemoglobin diffusion coefficient, t is diffusion time, and l is the thickness of one of the layers. The curves are drawn after the numerical values given by Kawalki (1894). The labeled hemoglobin is originally confined to the first layer (top curve) and diffuses into the other three layers when the four layers are brought into contact. The points connected by dotted lines represent values of a_n/a_{tot} obtained for the four layers from single diffusion experiments. The four examples shown demonstrate that the distribution of radioactivity among the layers deviated little from the ideal.

of hemoglobin solution. We have used this method in the present study, as it has two important advantages over other methods developed to measure protein diffusion coefficients in concentrated solutions (Wang et al., 1954; Keller et al., 1971; Riveros-Moreno and Wittenberg, 1972): (*a*) The diffusion time is short due to short diffusion paths. Even for a protein as large as earthworm hemoglobin, diffusion times of only 20–120 min are required. No degradation of the protein is likely to occur during this time. (*b*) Only small amounts of protein are required. A set of eight diffusion experiments can be done with about 7 ml of protein solution. A disadvantage of the method, when compared to the capillary method of Wang et al. (1954), is the uncertainty of the actual length of the diffusion path, which is generally longer than the thickness of the Millipore filter. This makes calibration of the filters with a substance of known diffusivity necessary.

The self-diffusion coefficients of hemoglobin were evaluated from the diffusion of labeled carbamylated hemoglobin against unlabeled carbamylated hemoglobin. The unlabeled hemoglobin was also carbamylated to ensure that both diffusing hemoglobins carry the same average electrical charge.

PROCEDURE A labeled and an unlabeled hemoglobin solution were adjusted to identical hemoglobin concentrations. In both solutions pH was adjusted to 7.40 (7.20 in the case of human hemoglobin) and ionic strength to 0.15. Millipore filters of type HA (thickness 140–170 μm, pore size 0.45 μm, diameter 4.7 cm, porosity 79%) were soaked in the solutions for 15 hr at 5°C. Hemoglobin concentrations of the solutions were then once again checked. The filters were removed from the solutions, and each filter was put in between two 25-μm thick Teflon membranes. By firmly rolling a cylindrical bar over the Teflon, the excess solution was removed from the filter surfaces. The average thicknesses of the filters, together with those of the Teflon membranes, were measured with a screw micrometer. Four filters with thicknesses identical within 2 μm, three of them soaked in unlabeled solution and one soaked in labeled solution, were selected for a diffusion experiment. The four filters were put on one another, while we carefully avoided air bubbles between the surfaces. The top filter (containing the labeled hemoglobin) and the bottom filter were covered with Teflon membranes to prevent desiccation. The four layers were left in contact for a suitable time (20 min–2 hr) and then separated within 20 s. The hemoglobin was eluted from each of the filters in beakers containing 2 ml of 0.15 M NaCl under continuous shaking. After 8 min, aliquots were taken from the eluate and the radioactivity contained in each filter was determined. From the ratio of the activity found in the single filters over the sum of activities found in all four filters, values of $4\ Dt/l^2$ were read from Fig. 1 or from Kawalki's tables (Jost, 1960). Thus, four estimates of the protein diffusion coefficient were obtained from one diffusion experiment. The measurements were done at room temperature 21° ± 1°C).

ELUTION OF HEMOGLOBIN FROM MILLIPORE FILTERS An elution time of 8 min was considered sufficient as the hemoglobin concentration in the eluant was found to reach a maximal value after 6 min and then show no further increase for more than ½ h. The final hemoglobin concentration in the eluant indicated that only 70% of the total filter volume is occupied by protein solution, somewhat less than the 79% fluid-containing volume asserted by the manufacturer. The discrepancy may be due to a small part of the filter space being accessible to water but not to the protein. The fraction of the total filter volume available to the hemoglobin was independent of the hemoglobin concentration of the solution in which the filters were soaked. This was found to hold for concentrations between 1 and > 21 g/100 g, which makes it unlikely that reversible binding of hemoglobin to the filter structures occurs to an appreciable extent in this concentration range.

DISTRIBUTION OF RADIOACTIVITY AMONG THE FOUR LAYERS Ideally, the distribution of radioactivity should be such that the values of $4\ Dt/l^2$ (Eq. 1) obtained for the four layers of a given diffusion experiment are identical. Stefan (1879) has pointed out that the

degree of consistency of the four values of D derived from one experiment provides some control over the presence of artifacts such as incomplete contact between layer surfaces, or convection caused by the manipulation of the filters, when these are put one on another. In Fig. 1 examples of the distribution of radioactivity amoung the layers are shown for four representative diffusion experiments. It can be seen that the deviations from theoretical distributions are small. The standard deviations calculated from the four values of D obtained in the single diffusion experiments averaged to 6%.

CALIBRATION OF MILLIPORE FILTERS It has been observed by several authors (Moll, 1966; Wittenberg, 1970; Gros and Moll, 1971, 1974; Kreuzer and Hoofd, 1972) that the diffusion path across Millipore filters is longer than the thickness of the filters. This was generally attributed to tortuosity of the channels through which diffusion occurs. As the square of the diffusion path enters into the calculation of the diffusion coefficient (Eq. 1), precise knowledge of the actual diffusion path is particularly important for the present method. We measured therefore the diffusion coefficient of $^{22}Na^+$ and compared it to the value determined with the capillary method by Wang (1952). The diffusivity of Na^+ is about 100 times that of *Lumbricus* hemoglobin. To be able to use diffusion times similar to those for the hemoglobin diffusion experiments, each of the four layers was made to consist of 10 Millipore filters instead of one. For the diffusion of a tracer amount of $^{22}Na^+$ in 0.09375 M potassium chloride solution at 25.0°C a diffusion coefficient of 0.96×10^{-5} cm^2s^{-1} (SE ± 1.0%, $n = 18$) was found. Wang (1952) obtained for identical conditions but in the absence of filters a value 1.28×10^{-5} cm^2s^{-1}. Thus, the tortuosity of the filter channels seems to cause a decrease of the diffusion coefficient by 34%, i.e. an increase in the diffusion path by 16%. This latter figure tallies reasonably with figures estimated previously from the effect of Millipore filters on the diffusivity of CO_2 in aqueous solutions (Gros and Moll, 1971, 1974). Consequently, all diffusion coefficients reported in the following were calculated using as diffusion path, l, the 1.16-fold of the measured filter thickness.

RESULTS

Diffusivity of Lumbricus *Hemoglobin at Various Protein Concentrations*

The measured self-diffusion coefficients of *Lumbricus terrestris* hemoglobin in 0.15 M NaCl at pH 7.4 and 21°C are summarized in Table I. Each value listed is the average of 9–20 determinations. The standard errors of the diffusion coefficients average ~ 3%. Table I shows that the self-diffusion coefficient decreases with increasing protein concentration from a value of 11.7×10^{-8} cm^2s^{-1} at 2.12 g/100 ml to a value of 1.28×10^{-8} cm^2s^{-1} at 25 g/100 ml, i.e. to about 1/10. This suggests an even stronger concentration dependence of the diffusion coefficient of *Lumbricus* hemoglobin than has been found for tetrameric mammalian hemoglobins in this concentration range. The diffusion coefficient of mammalian hemoglobin is reduced to $\frac{1}{4}$ of its value at infinite dilution when the hemoglobin concentration is 25 g/100 ml, and reaches 1/10 only when the concentration is increased to 33 g/100 ml (Moll, 1966; Riveros-Moreno and Wittenberg, 1972).

Diffusion Coefficient of Lumbricus *Hemoglobin at Infinite Dilution*

The limiting diffusion coefficient in infinitely dilute solutions, D_0, given in Table I was obtained by linear extrapolation to zero hemoglobin concentration in a semilogarithmic plot (see Fig. 2). A value of 1.3×10^{-7} cm^2s^{-1} is found. This value is

TABLE I

DIFFUSION COEFFICIENTS OF *LUMBRICUS TERRESTRIS* HEMOGLOBIN AT VARIOUS HEMOGLOBIN CONCENTRATIONS

Hemoglobin concentration	Self-diffusion coefficient	SE	n	Diffusion time
g/100 ml	$cm^2 s^{-1}$	%		*min*
0	13×10^{-8}			
2.12	11.7×10^{-8}	1.5	12	20
4.16	10.4×10^{-8}	3.4	15	20
7.32	8.8×10^{-8}	2.5	20	25
15.1	6.0×10^{-8}	2.2	15	30
19.1	3.56×10^{-8}	1.8	18	45
22.4	1.99×10^{-8}	2.5	9	80
25.0	1.28×10^{-8}	6.3	15	120

Given are mean values of the diffusion coefficients, standard errors as percentage of the mean, number of determinations, n, and diffusion time t (see Eq. 1). Temperature 21°C.

about 20% lower than the value of $D_0 = 1.6 \times 10^{-7}$ $cm^2 s^{-1}$ reported by Shlom and Vinogradov (1973). Using Einstein's equation (1906),

$$D_0 = kT/6\pi\eta r, \tag{2}$$

where k is Boltzmann's constant, T temperature, η solvent viscosity, and r the molecular radius of the protein, one can calculate an effective r from D_0. With $D_0 = 1.3 \times 10^{-7}$ $cm^2 s^{-1}$ a radius of 165 Å, and with $D_0 = 1.6 \times 10^{-7}$ $cm^2 s^{-1}$ a radius of 134 Å is obtained. Both radii are within the range of molecular dimensions derived from electron microscopic studies of *Lumbricus* hemoglobin (Roche et al., 1960; Levin, 1963; Ben-Shaul, 1974). Thus neither of the two values of D_0 can be favored on this basis. However, inserting $D_0 = 1.6 \times 10^{-7}$ $cm^2 s^{-1}$ into the Svedberg equation led to an underestimation of the molecular weight of *Lumbricus* hemoglobin (Shlom and Vinogradov, 1973; Vinogradov et al., 1977). To obtain a molecular weight identical to that determined from sedimentation equilibrium (David and Daniel, 1974; Wood et al., 1976; Vinogradov et al., 1977), D_0 would have to be about 1.4×10^{-7} $cm^2 s^{-1}$. It appears therefore that the value obtained by extrapolation from the present data represents a reasonable estimate of D_0.

Diffusion Coefficient of Human Hemoglobin

In Table II values of the diffusion coefficient of human hemoglobin obtained at various hemoglobin concentrations (0.15 M NaCl, pH 7.2, 20°C) are compiled. Each value is the average of about 15 determinations. The standard errors average 2% of the mean, i.e. the standard deviations amount to about 8%. This figure compares with standard deviations of 3–6% obtained with a capillary method by Wang et al. (1954), and an average standard deviation of 5% obtained from hemoglobin tracer diffusion measurements across Millipore filters by Keller et al. (1971). No standard deviations

TABLE II
DIFFUSION COEFFICIENTS OF HUMAN HEMOGLOBIN AT VARIOUS HEMOGLOBIN CONCENTRATIONS

Hemoglobin concentration	Self-diffusion coefficient	SE	n	Diffusion time
g/100 ml	$cm^2 s^{-1}$	%		*min*
9.7	45.3×10^{-8}	1.8	14	15*
21.9	22.3×10^{-8}	1.1	15	15
31.5	9.53×10^{-8}	1.6	15	20
36.5	4.50×10^{-8}	2.9	15	35
43.3	1.38×10^{-8}	2.8	15	90

Given are mean values of the diffusion coefficients, standard errors as a percentage of the mean, number of determinations, n, and diffusion time t. The diffusion coefficients are corrected for 20°C with a temperature dependence of 2.3%/°C (Keller et al., 1971).
*In this case each layer consisted of two Millipore filters.

have been given by Riveros-Moreno and Wittenberg (1972), but from their Fig. 4 a scatter of the data points of up to 50% is apparent. Thus, the reproducibility achieved with the present method is similar to the reproducibilities of previously described methods. Table II shows a pattern of concentration dependence of protein diffusion that agrees qualitatively with that reported by various authors for hemoglobin and other proteins: the dependence on protein concentration is much more pronounced in high than in low concentration ranges. Doubling the hemoglobin concentration from 9.7 to 21.9 g/100 ml leads to a reduction of the diffusion coefficient to about 50%, while doubling the concentration from 21.9 to 43.3 g/100 ml results in a reduction of the diffusion coefficient to a value as low as 6% of the value at 21.9 g/100 ml.

No Effect of pH on the Diffusivity of Human Hemoglobin

Scatchard and Pigliacampi (1962) have shown that the osmotic coefficient of hemoglobin increases when the hemoglobin net charge increases. To test whether the diffusivity of hemoglobin is also affected by its charge, we measured the diffusion coefficient at different pH values. In Table III the diffusion coefficients of human hemoglobin measured at pH 6.5 and pH 7.9, and protein concentrations of about 35 g/100 ml, are compared with the diffusion coefficients found at pH 7.2 and identical protein concentrations. While the net charge of unmodified hemoglobin is approximately zero at pH 7.2, it is about +7 at pH 6.5 and −7 at pH 7.9. It is apparent from Table III that charge and pH have no significant effect on the diffusivity of human hemoglobin.

DISCUSSION

In Fig. 2 the concentration dependence of the self-diffusion coefficient of *Lumbricus* hemoglobin is compared to the concentration dependence reported by various authors for the diffusion coefficient of myoglobin, tetrameric hemoglobin, and ovalbumin.

TABLE III

EFFECT OF pH ON THE DIFFUSIVITY OF HUMAN HEMOGLOBIN

Hemoglobin concentration	pH	Self-diffusion coefficient	SE	n
g/100 ml		$cm^2 s^{-1}$	%	
34.8	6.5	6.2×10^{-8}	1.5	12
	7.2	6.0×10^{-8}		
34.9	7.9	5.8×10^{-8}	2.0	15
	7.2	5.9×10^{-8}		

Given are mean values of the diffusion coefficients, standard errors as percentage of the mean, number of determinations n, pH of the hemoglobin solution, and hemoglobin concentration. For comparison, diffusivities for identical hemoglobin concentrations, but with pH = 7.2, were read from the curve of Fig. 2. Temperature 20°C.

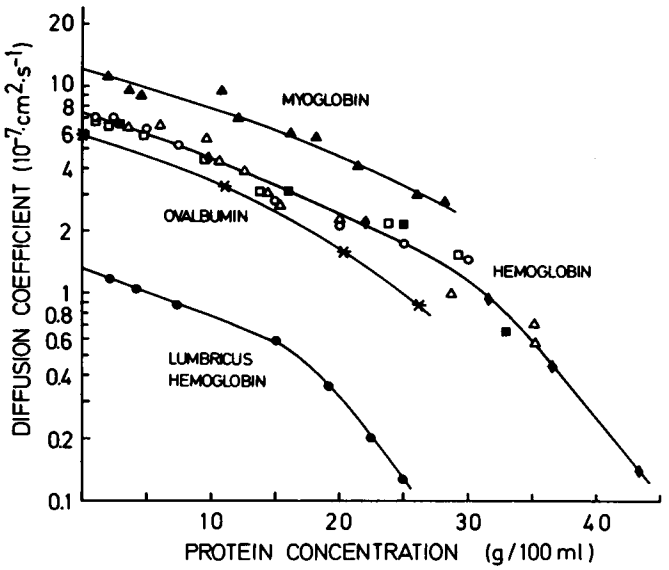

FIGURE 2 Semilogarithmic plot of the protein diffusion coefficient versus protein concentration. The figure gives a comparison of the concentration dependence of the diffusion coefficients of *Lumbricus terrestris* hemoglobin (●, data of Table I), ovalbumin (*, data of Wang et al., 1954), tetrameric mammalian hemoglobin (■, data of Moll, 1966; □, mutual diffusion coefficients of Keller et al., 1971; ○, tracer diffusion coefficients of Keller et al., 1971; △, data of Riveros-Moreno and Wittenberg, 1972; ◆, present data), and myoglobin (▲, data of Riveros-Moreno and Wittenberg, 1972). Temperatures are 21°C for *Lumbricus* hemoglobin, 10°C for ovalbumin, and 20°C for myoglobin. The diffusion coefficients of tetrameric hemoglobin were all corrected to 20°C with the temperature coefficient of 2.3%/°C given by Keller et al. (1971). The data of Moll (1966), obtained with Millipore filters, were multiplied by the calibration factor 1.33.

Log D is plotted versus the protein concentration (wt/vol). It can be seen that the curves obtained for the four proteins follow parallel courses in the concentration range between 0 and 16 g/100 ml. That is, the relative decrease of diffusivity with protein concentration is the same for all proteins in this concentration range. As the molecular weights of these proteins vary between 1.7×10^4 for myoglobin and 3.7×10^6 for *Lumbricus* hemoglobin, it appears that the obstructing effect of proteins on protein diffusion is solely determined by their wt/vol concentration, i.e. by the volume fraction of the solution occupied by protein, and is independent of the size of the protein molecules. It will be shown below that this behavior of protein diffusivity indeed is predicted by theory (Eq. 4).

Above a concentration of 16 g/100 ml this simple picture seems not to hold any more. At 16 g/100 ml the slope of log D versus concentration for *Lumbricus* hemoglobin shows a sharp increase by a factor of three, while the slopes seen for the other proteins remain essentially unchanged. Then, at a concentration of about 30 g/100 ml, a similarly sharp increase in the slope of log D versus concentration occurs in the case of tetrameric hemoglobin. The slopes obtained for ovalbumin and myoglobin, on the other hand, change very little in the entire range studied (0–30 g/100 ml). We conclude that a drastic increase in the concentration dependence of protein diffusivity, probably due to an increased protein-protein interaction, occurs at a concentration of 16 g/100 ml for *Lumbricus* hemoglobin, and at 30 g/100 ml for tetrameric hemoglobin.

It may be seen in Fig. 2 that the diffusion coefficients of human hemoglobin obtained with the present method agree well with the values reported by other authors. It is noteworthy that all the diffusion coefficients of tetrameric hemoglobin compiled in Fig. 2 tally reasonably well, although they were obtained by widely differing methods. The result seems to be the same whether the diffusion coefficients are determined from tracer flow with zero net flow of protein (Moll, 1966; Keller et al., 1971; Riveros-Moreno and Wittenberg, 1972; this study) or from a net flow of unlabeled protein (Keller et al., 1971), whether diffusivity is determined in the absence (Moll, 1966) or in the presence of filters (Moll, 1966; Riveros-Moreno and Wittenberg, 1972; Keller et al., 1971; this study). The concentration dependence of hemoglobin diffusivity, as obtained by these methods and shown in Fig. 2, is in sharp contrast to the concentration dependence derived from light-scattering spectroscopy (Veldkamp and Votano, 1976; Alpert and Banks, 1976). While this latter method gives correct values of the diffusion coefficient at infinite dilution, it gives values about 4 times higher than those of Fig. 2 at a hemoglobin concentration of 30 g/100 ml.

Prediction of the Concentration Dependence of Protein Diffusion

Various attempts have been made to describe theoretically the concentration dependence of the diffusion of macromolecules. A first heuristic attempt is due to Wang et al. (1954). These authors concluded from measurements of the self-diffusion coefficient of ovalbumin that

$$D/D_0 = \eta_0/\eta \tag{3}$$

where D and η are diffusion coefficient and solution viscosity at a given protein concentration, D_0 and η_0 are the corresponding quantities at infinite dilution. This implies that the expression $D \cdot \eta/(D_0 \cdot \eta_0)$ should have a constant value of 1 independent of the protein concentration. In Fig. 3, $D \cdot \eta/(D_0 \cdot \eta_0)$ is plotted versus protein concentration for ovalbumin, tetrameric hemoglobin, and *Lumbricus terrestris* hemoglobin. The values of D are those shown in Fig. 2, and the values of D_0 were obtained by extrapolation to zero concentration as indicated in Fig. 2. The viscosities were taken from Chick and Lubrzynska (1914) for ovalbumin, and from own measurements for human and *Lumbricus* hemoglobin (see Appendix). Myoglobin was not included in Fig. 3 since no viscosity data are available. The regression line calculated from the data points of Fig. 3 has an intercept not significantly different from 1, 0.96 ± 0.04, and a slope not significantly different from 0, 0.002 ± 0.002 g^{-1} $\times$ 100 ml. Although there is considerable scatter in the diffusion data, we may thus conclude that Eq. 3 provides a good description of the concentration dependence of the diffusivity of a variety of proteins. This means that the diffusion coefficient at any protein concentration can be predicted from Einstein's equation (Eq. 2), when the macroscopic solution viscosity rather than the solvent viscosity is used. An interesting implication of this is that the viscosity experienced by a macromolecule appears to be the same as the viscosity effective in capillary flow. This does not hold for smaller molecules, whose diffusivity shows a less than proportional decrease with increasing viscosity (see for example Wang et al., 1954; Broersma, 1958; Gros and Moll, 1971).

While Eq. 3 is useful in estimating protein diffusion coefficients when viscosity data are available, it does not aid in elucidating the physical mechanism underlying the decrease of diffusivity (and possibly the increase in viscosity as well), which occurs

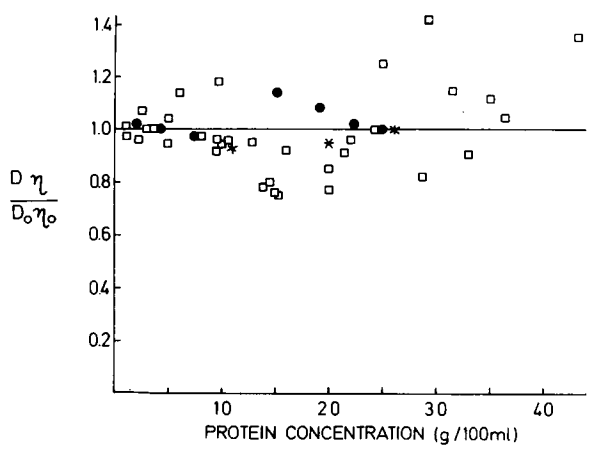

FIGURE 3 Normalized protein diffusivity times normalized viscosity of the protein solution, $(D/D_0)\cdot(\eta/\eta_0)$, as a function of protein concentration. The data points are the same as those shown in Fig. 2: • *Lumbricus* hemoglobin, □ tetrameric hemoglobin, * ovalbumin. The line represents the prediction of Eq. 3. Calculation of a regression equation from all data points of Fig. 3 yields: $D\eta/D_0\eta_0 = 0.96 \pm 0.002 \cdot c$; the correlation coefficient is $r = 0.138$.

when the protein concentration rises. Nor does it allow us to predict the concentration dependence of protein diffusion without viscosity data for the specific protein considered. Theories aimed at the solution of these problems have been developed by Phillies (1975), Alpert and Banks (1976), and Veldkamp and Votano (1976), none of which fits the concentration dependencies shown in Fig. 2. An equation proposed by Anderson (1973), on the other hand, provides an excellent prediction of protein diffusivity up to moderate concentrations, as shall be shown in the following. Anderson's equation is based on the treatment of hindered sedimentation given by Maude and Whitmore (1958). Anderson presents arguments that their result for the concentration dependence of sedimentation velocity can be applied to describe the concentration dependence of protein diffusion. Using numerical values derived by Batchelor (1972) he arrives at the following semi-empirical equation:

$$D/D_0 = (1 - c \cdot \bar{v})^{6.5} \tag{4}$$

where c is the protein concentration (wt/vol), and $\bar{v}$ is the partial specific volume (0.740 ml/g for *Lumbricus* hemoglobin, 0.749 for tetrameric hemoglobin, and 0.741 for myoglobin). Although in the original derivation of Maude and Whitmore (1958) the volume fraction occupied by the hydrated protein, ϕ, is employed, we use here, as does Anderson, the volume fraction of the dry protein, $c \cdot \bar{v}$, since the hydration of protein molecules is a largely undetermined quantity.

The validity of this equation was tested with the diffusivity data points shown in Fig. 2 for *Lumbricus* hemoglobin, tetrameric hemoglobin, and myoglobin (ovalbumin was not included, because measurements at only three concentrations are available, and D_0 is derived from a rather uncertain calculation). Values of D/D_0 were determined as described above. Fig. 4 shows a plot of $\log(D/D_0)$ versus $\log(1 - c \cdot \bar{v})$ obtained in this way. It can be seen that all diffusivity data follow a straight line between 0 and 16 g/100 ml [$\log(1 - c \cdot \bar{v}) = 0$ to -0.055]. Above 16 g/100 ml, like what is seen in Fig. 2, the diffusion coefficients of *Lumbricus* hemoglobin show an abrupt increase in their dependence on protein volume fraction, while tetrameric hemoglobin and myoglobin still follow the same straight line. Above a protein concentration of 30 g/100 ml [$\log(1 - c \cdot \bar{v}) = -0.11$] a similar pronounced increase in protein concentration dependence then occurs for tetrameric hemoglobin. When the data for *Lumbricus* hemoglobin up to 16 g/100 ml and the diffusivities of all other proteins up to 30 g/100 ml are used for a regression analysis, a good linear correlation ($r = 0.97$) with the regression equation

$$\log(D/D_0) \approx 6.65 \log(1 - c \cdot \bar{v}), \tag{5}$$

is found. The standard deviation of the slope is ± 0.25. The excellent agreement with Anderson's equation (Eq. 4) is obvious. We conclude that this equation can predict the diffusion coefficients of *Lumbricus* hemoglobin up to 16 g/100 ml and those of myoglobin and hemoglobin up to 30 g/100 ml.

The dotted lines of Fig. 4 show that above distinct concentration limits the slopes

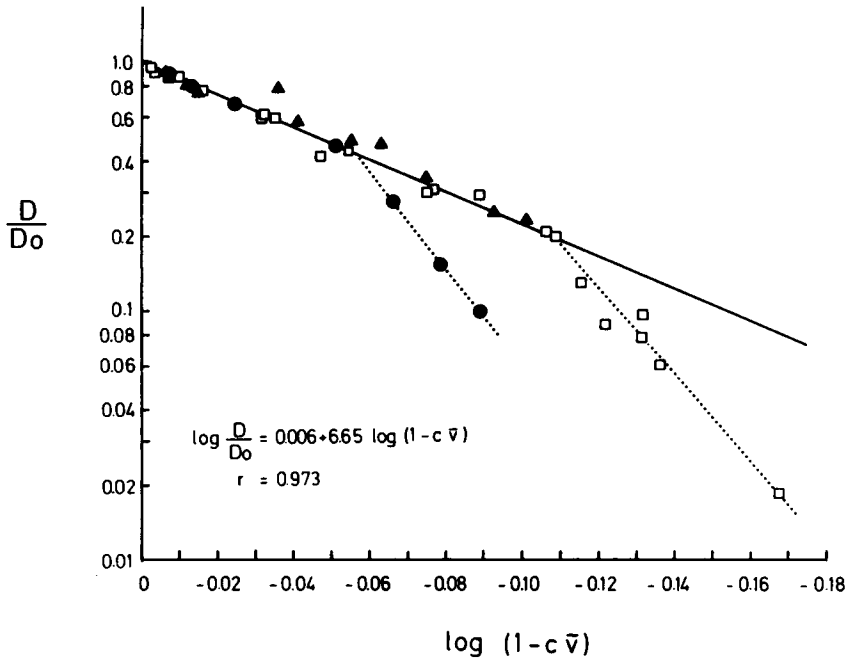

FIGURE 4 Plot of the logarithm of the normalized protein diffusion coefficient, (D/D_0), versus log $(1 - \bar{v} \cdot c)$, where c is the wt/vol protein concentration. The data points are the same as in Fig. 2: • *Lumbricus* hemoglobin, □ tetrameric hemoglobin, ▲ myoglobin. The solid line is the regression line obtained from the diffusion coefficients of *Lumbricus* hemoglobin at protein concentrations between 0 and 16 g/100 ml and from the diffusion coefficients of hemoglobin and myoglobin at concentrations between 0 and 30 g/100 ml; the regression equation and the correlation coefficient are inserted. For the sake of clarity, not all diffusion coefficients seen in Fig. 2 for tetrameric hemoglobin are shown, but all values are included in the regression analysis. The dotted lines indicate the increased concentration dependence of protein diffusivity, found for *Lumbricus* hemoglobin above a concentration of 16 g/100 ml, and for tetrameric hemoglobin above 30 g/100 ml.

of $\log(D/D_0)$ versus $\log(1 - c \cdot \bar{v})$, and with them the concentration dependencies of protein diffusion, deviate from the prediction of Eq. 4 and are increased by a factor of three. This increase in concentration dependence occurs at the lowest concentration (16 g/100 ml) for the largest molecule, *Lumbricus* hemoglobin (mol wt 3.7×10^6), occurs at a higher concentration (30 g/100 ml) for the second largest molecule, tetrameric hemoglobin (mol wt 6.5×10^4), and is not seen in the entire concentration range of Fig. 4 for the smallest molecule, myoglobin (mol wt 1.7×10^4). This seems to suggest some role of molecular size in this phenomenon. Above certain concentration limits, the concentration dependence of protein diffusion apparently cannot be described purely in terms of protein concentration (wt/vol) or volume fraction, but also depends on molecular parameters, such as the dimensions of the protein molecules.

APPENDIX

Concentration Dependence of the Viscosity of Human and Lumbricus *Hemoglobin Solutions*

In order to determine the relation between hemoglobin self-diffusion coefficient and solution viscosity (see Fig. 3), the viscosities of earthworm and human hemoglobin solutions were determined at various protein concentrations. The measurements were performed in an Ostwald-type viscometer at 21°C in the case of earthworm hemoglobin, and at 20°C in the case of human hemoglobin. Absolute values of viscosity were calculated with 0.979 cP for the viscosity of water at 21°C, and 1.002 cP at 20°C. The results are given in Table IV. For *Lumbricus* hemoglobin, they are described by the following polynomial regression equation ($R^2 = 0.9999$):

$$\log \eta = 8.84 \cdot 10^{-4} + 2.292 \cdot 10^{-2} c - 1.085 \cdot 10^{-3} c^2 + 1.013 \cdot 10^{-4} c^3 - 1.199 \cdot 10^{-6} c^4, \quad (6)$$

where η is the viscosity in centipoise, and c is the hemoglobin concentration in grams per 100 ml. For human hemoglobin the following equation was found ($R^2 = 0.99996$):

TABLE IV

VISCOSITIES OF SOLUTIONS OF *LUMBRICUS TERRESTRIS* AND HUMAN HEMOGLOBIN

Protein	Hemoglobin concentration	Viscosity
	g/100 ml	*cP*
Lumbricus Hemoglobin	4.94	1.25
	9.10	1.55
	14.37	2.26
	19.6	4.18
	25.0	10.24
Human Hemoglobin	0*	1.020
	5.18	1.20
	9.87	1.48
	15.5	1.99
	20.1	2.63
	26.0	4.53
	29.4	6.81
	35.6	14.37
	40.8	37.00
	42.8	65.78

Conditions for earthworm hemoglobin solutions were: pH 7.4, 0.15 M NaCl, 21.0°C, and for solutions of human hemoglobin: pH 7.2, 0.15 M NaCl, 20.0°C. The viscosities are based on values of 0.979 and 1.002 cP for the viscosities of water at 21 and 20°C, respectively. The densities, ρ, used for the calculation were measured pycnometrically. They are described by the regression equations $\rho = 1.000 + 0.00254 \cdot c$ for earthworm hemoglobin, and $\rho = 1.0050 + 0.00250 \cdot c$ for human hemoglobin, where c is the protein concentration in g/100 ml.

*0.15 M NaCl solution

$$\log \eta = 8.8989 \cdot 10^{-3} - 5.5997 \cdot 10^{-4} c + 4.5916 \cdot 10^{-3} c^2$$
$$- 4.7573 \cdot 10^{-4} c^3 + 2.3926 \cdot 10^{-5} c^4 - 5.4013 \cdot 10^{-7} c^5 + 4.5564 \cdot 10^{-9} c^6. \quad (7)$$

The viscosities obtained for human hemoglobin solutions agree within a few percent with the values reported by Veldkamp and Votano (1976) for concentrations ranging from 1 to 21 g/100 ml. They can also be well represented by Eq. 5 of Ross and Minton (1977) with $k/\nu = 0.41$.

It may be seen from Tables I and IV that at a *Lumbricus* hemoglobin concentration of 25 g/100 ml the viscosity is increased to the 10-fold of its value at infinite dilution, while the diffusivity is decreased to 1/10. In the case of human hemoglobin, the viscosity amounts to the 10-fold and the diffusivity to 1/10 of their limiting values at a concentration of 33 g/100 ml.

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