THE RATE OF UPTAKE OF CARBON MONOXIDE AND OF NITRIC OXIDE BY NORMAL HUMAN ERYTHROCYTES AND EXPERIMENTALLY PRODUCED SPHEROCYTES*

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ABSTRACT

The uptake of gases such as oxygen, carbon monoxide, or nitric oxide by the erythrocyte involves: (*a*) diffusion across the cellular membrane, (*b*) intraerythrocytic diffusion, and (*c*) chemical combination with hemoglobin. The aim of this investigation was to obtain data which would permit an analysis of each of these factors in limiting the rate of gas uptake.

The initial over-all rate of uptake of gases which combine chemically with hemoglobin to produce a color change can be measured by a modified version of the Hartridge-Roughton-Millikan constant flow, rapid reaction apparatus. If nitric oxide is the reactant gas, only (*a*) and (*b*) are measured since the chemical combination of this gas with hemoglobin is extremely rapid. Our studies have shown that human biconcave discoidal erythrocytes at 38 and 48°C., have the same initial rate of carbon monoxide and nitric oxide uptake as the same cells converted into spherocytes of equal volume. Similarly there was no difference between discs and cells sphered with a 30 per cent increase in volume. Shrunken erythrocytes showed a marked decrease in rate of gas uptake. This suggests that surface area and maximum linear distance for intracellular diffusion of this magnitude do not measurably retard gas uptake. In the shrunken cells, a change in the orientation and concentration of intraerythrocytic hemoglobin and/or of the membrane components may have impeded gas diffusion.

Oxygenation of pulmonary capillary blood involves several processes. Oxygen molecules present within the alveoli must first traverse the alveolar-

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capillary membranes to enter the capillary blood, then diffuse through a layer of plasma, pass through the red blood cell membrane, and diffuse throughout the interior of the cell. With gases such as oxygen, carbon monoxide, and nitric oxide, the final stage of gas uptake by blood is chemical combination with hemoglobin.

It has been assumed generally that only the alveolar-capillary membranes offer resistance of any magnitude to the passage of gases such as oxygen and carbon monoxide. Recent studies by Kruhøffer (9) and by Forster and his associates (16), however, indicate that the processes beyond alveolar-capillary membrane diffusion may impose as much resistance to gas uptake as do the membranes themselves. This emphasizes the importance of learning more about the final processes of gas exchange including the passage of gas molecules across the red blood cell membrane, the movement of these molecules within the red blood cell, and the rates of chemical combination of gases with hemoglobin. On these points there has been a little speculation, and even less experimentation. The speculation has been challenging:— Ponder, acting on suggestions made earlier by Hartridge (7), proposed that the ratio of cellular surface to cellular volume was a critical factor in the respiratory function of the erythrocyte; he stated: "in order to obtain the same rapidity of gas diffusion (as in biconcave discs) for the same volume distributed in spheres, we should require approximately nine spheres each one-ninth the volume" (13). Perutz, focussing his attention upon the intracorpuscular hemoglobin, believes that an increase in concentration above that which normally exists may impose severe limitations to gas uptake; he has stated: "from the point of view of its functional efficiency as an oxygen carrier, the red cell should combine maximum oxygen capacity with high speed of reaction and diffusion of oxygen through the cell . . . 34 per cent is the highest concentration which is compatible with these conditions . . . any further increase in concentration would lead to mutual hindrance in the rotation of the molecules, and would be likely to affect the reaction rates" (12). Roughton, on the basis of experimental observations upon extracorpuscular dilute hemoglobin solutions and on red blood cell suspensions, combined with some mathematical computations and speculation, believes that the red blood cell membrane may be a critical factor in the rate of gas uptake (4).

Our contribution to the study of factors determining rate of gas uptake by erythrocytes has been a direct experimental approach. Stated simply, we measured the rates of gas uptake by normal human erythrocytes, then altered their size or shape, and remeasured the rate of gas uptake. In the course of our experiments, we changed the cellular surface area in relation to cellular volume, intra-erythrocytic diffusion distances, intracorpuscular hemoglobin concentration, and probably the physical and chemical characteristics of the cellular membrane. Our findings do not define the factors offering

the greatest limitation, but we believe that our data permit the exclusion of certain factors once thought to be of critical importance in the uptake by blood of gases which react with hemoglobin. Much work remains to be done on the non-excluded factors.

Methods

The velocity of gas uptake was measured with a modified version of the Hartridge-Roughton-Millikan rapid reaction, constant flow apparatus. This has been described in detail by Forster, Roughton, Kreuzer, and Briscoe (4), and we shall discuss here only our modifications.

Initially we measured the rate of uptake of carbon monoxide by human red blood cell suspensions; the uptake of this gas is determined by three processes: the rate of passage of carbon monoxide across the erythrocytic membrane, intraerythrocytic diffusion rates, and the rates of chemical association of carbon monoxide with hemoglobin. In a later phase of the study we measured the rate of uptake of nitric oxide by red blood cells. The reaction between hemoglobin and nitric oxide is a fascinating one, partly because the affinity of this gas for hemoglobin is tremendous (1800 times that of carbon monoxide and approximately 400,000 times that of oxygen), but more so because the rate of association of nitric oxide with hemoglobin is so extremely fast that, under the conditions of our experiments, the chemical reaction is completed within a millisecond. Essentially, therefore, the over-all rate at which nitric oxide is taken up by erythrocytes depends only upon *two* processes: the rate at which nitric oxide passes across the cellular membrane, and diffusion of the gas within the cell.

TABLE I

Combinations of Saline Solutions and Cell Suspensions Used to Obtain the Unknown and the Control Mixtures

1:80 mixture	Saline solution contains	Cell suspension (1:40) contains	Chemical composition
"Reduced" Hb control	No reacting gas	Deoxygenated Hb	0 per cent COHb or NOHb
Reaction mixture	CO or NO gas	Deoxygenated Hb	x per cent COHb or NOHb
100 per cent COHb or NOHb control	CO or NO gas	100 per cent COHb or NOHb	100 per cent COHb or NOHb

Preparation of Reactants.—It was necessary to prepare two saline solutions and two erythrocyte suspensions (or two hemoglobin solutions) for each experiment; Table I shows the composition of these solutions and the manner in which they were combined to obtain both control and experimental data at each point. We required large volumes of solutions and suspensions because of the need for measurements before and after specific alterations in the red blood cells. We withdrew 85 ml. of human venous blood of which 75 ml. was immediately mixed with saline solution

to a total volume of 3000 ml. We used the remaining 10 ml. of blood for measurements of the total hemoglobin concentration and oxygen capacity. The blood was either heparinized (0.1 mg. heparin sodium/ml. blood) or defibrinated. We used two saline solutions: in some experiments the medium was phosphate-buffered sodium chloride solution (pH 7.40); in others it was one which more closely resembles plasma in its electrolyte composition (pH 7.40, P_{CO_2} 40 mm. Hg) (5). When the latter was used, the rinsing and pressurizing gas was not pure helium, but rather helium with sufficient carbon dioxide added so as to result in a final P_{CO_2} of 40 mm. Hg in both the saline solutions and suspensions when the usual driving pressure of 250 mm. Hg pressure was reached.

When nitric oxide was the reactant gas, the system was meticulously freed of oxygen since, in the presence of oxygen, nitric oxide is instantaneously converted into brown fumes of nitrogen dioxide and tetroxide; these gases are not only highly toxic, but lead to the formation of methemoglobin. Therefore even the "pure" helium gas used in rinsing and pressurizing the system had to be purified further by bubbling through a hydrosulfite-anthraquinone mixture to absorb any traces of oxygen. Since this solution also absorbs carbon dioxide, the Gey and Gey solution with a P_{CO_2} 40 mm. Hg could not be used and we always used phosphate-buffered saline solutions in the nitric oxide experiments.

We also wished to determine, in some experiments, the rate of gas uptake by extracellular hemoglobin, free in solution, to obtain a measure of the velocity of the chemical reaction alone, uncomplicated by the diffusion of gases through the membrane or within the cell. We hemolyzed the cells by adding saponin, water, or triton in such a manner that the final concentration of hemoglobin in the solution was the same as that present in the total cell suspension.

Mixing of Reactants.—A critical measurement is the precise volume of saline solution and of cell suspension that combine to form the final reacting mixture. To determine this we measured the volume flow for 5 seconds for each of the four reagents before and after obtaining a series of experimental data; when necessary, we adjusted inflow resistances so that the volume flows were equal. As an additional check, we measured the total concentration of hemoglobin as cyanmethemoglobin for the cell suspensions flowing alone and then flowing in combination with the saline solutions; we required that the latter be equal to half the former.

Filter-Photometer System.—In the latter part of the work, we replaced the dual prism system described by Foster by a half-silvered mirror; this system is superior because light which has passed through precisely the same unit of cell suspension in the glass observation tube falls on both filter-phototube systems.

Initially, to differentiate hemoglobin from carboxyhemoglobin in a mixture of the two we utilized a red (Wratten 29) filter and a green (Wratten 61) filter (11). Later, to improve the reproducibility of the data, we substituted narrow band interference filters.[1] We selected filters with peak wave lengths of 555 and 536 mμ to distinguish hemoglobin and carboxyhemoglobin and filters peaking at 453 and 442 mμ to differentiate hemoglobin and nitric oxide hemoglobin.

Calibration of the Optical System.—(*a*) We calibrated the glass observation tube at seventeen points each 1 cm. apart, beginning at 1.0 cm. from the mixing chamber.

[1] Farrand Optical Co., New York City.

For this we prepared three 1:80 suspensions of red blood cells:—one contained 0 per cent COHb, a second 100 per cent COHb, and a third 28 per cent COHb; the concentrations were verified by chemical analysis (18). The suspensions were driven, in succession, through the observation tube and at each of the seventeen positions we measured the degree of saturation of the hemoglobin with carbon monoxide in the test mixture in relation to the 0 and 100 per cent COHb cell suspensions. From three such sets of measurements, consisting of seventeen determinations each, we found the mean concentration of COHb to be 27.85 per cent ± S.D. 1.61; the concentration of COHb obtained by chemical analysis was 28.15 per cent. Thus, although the observation tube is not optically perfect, measurements at any point are valid if bracketed with cell suspensions known to contain 0 and 100 per cent COHb.

(*b*) We prepared calibration curves for COHb and NOHb, using the filters employed in the experiments. The linear relationship reported by Forster and associates (4) to hold for the reaction between hemoglobin and carbon monoxide was confirmed. The relationship between light absorption and concentration of NOHb is, however, not a linear one when filters of 453 and 442 mμ are used, and reference to a calibration curve (Fig. 1) is necessary.

Noise Level.—Our electrical controls for the photocell system were housed in an aluminum box and it was found necessary to surround the magnetic stirrers, used for the cell suspensions, with iron shielding to prevent noise caused by the magnetic fields. The noise level then noted with flowing reagents was low enough that records could be read with an accuracy of 0.2 mm. (maximum excursion of the Brush pen motor was 40 mm.). This noise disappeared completely when the biconcave erythrocytes were sphered; indeed this change was so consistent that it could be used to predict the precise time at which discs were converted into spheres. We assume, therefore, that the noise is caused by light scattering associated with the biconcave discoidal cells and turbulent flow.

Calculation of l'_c and j'_c. The present investigation deals only with the measurement of the initial over-all rate of gas uptake with no attempt to distinguish exact values for the true initial velocity constants of association. In the notation used by Roughton, k'_c, l'_c, and j'_c represent the initial over-all rates of reaction of hemoglobin with oxygen, carbon monoxide, and nitric oxide, respectively; the subscript c refers to cells in contrast to hemoglobin solution.

During a typical experiment, we measured the COHb (or NOHb) concentration of the flowing mixture at a minimum of five positions along the observation tube, generally corresponding in time to 5, 10, 17, 24, and 34 milliseconds. Immediately thereafter, we made a duplicate set of measurements, and often, as many as four complete series were run in succession. The initial over-all velocity constant for NOHb (j'_c) was obtained by the same method employed by Forster *et al.* (4) to arrive at the velocity constant l'_c for COHb.

Because of the numerous technical difficulties involved in the many phases of this study and of the gradual evolution of solutions to these problems, we do not consider it valid to compare data for l'_c or j'_c obtained during experiments in the early part of this investigation with those obtained in the later phases. We do consider valid, however, all data from experiments in which control measurements could be compared directly with measurements made on the same cells, using the same con-

trol and reacting fluids, the only difference being an induced alteration in the erythrocytes. This is because, to compare initial over-all rates of uptake *within* the same experiment, it is not necessary to know the exact concentration of reactant gas in

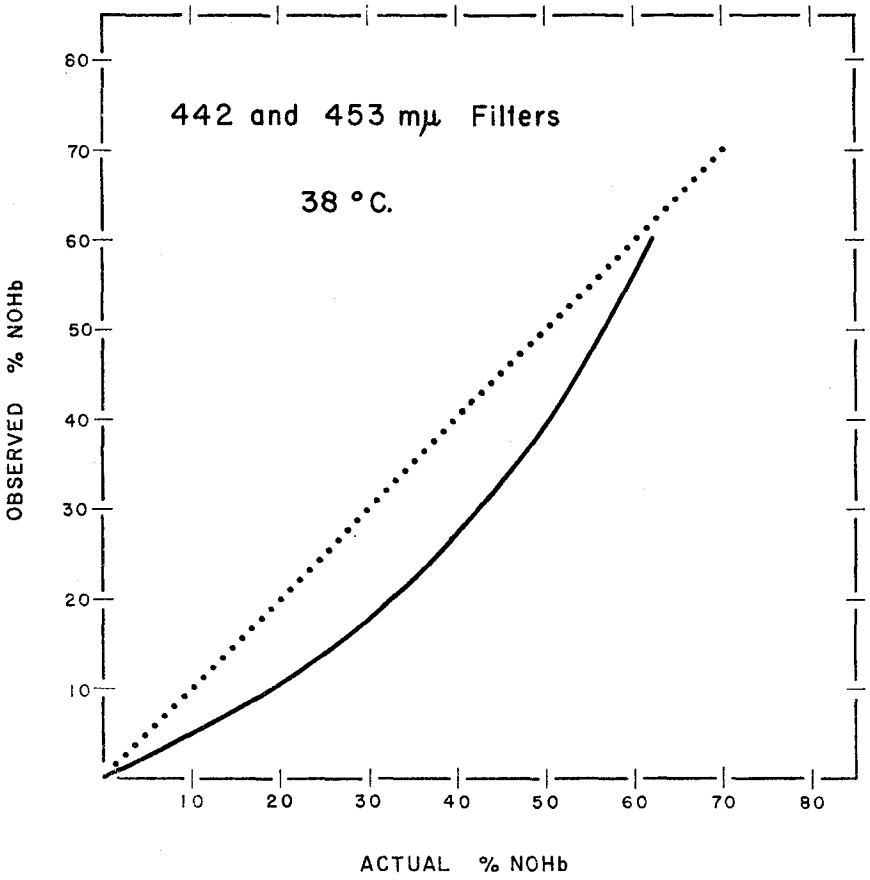

FIG. 1. Calibration curve for nitric oxide hemoglobin performed on suspensions of normal human erythrocytes at 38°C., using Farrand narrow band interference filters with maximal peaking at 442 and 453 mμ. Dotted line represents the concentration of nitric oxide hemoglobin that would be obtained if the relationship between the actual and observed concentrations were linear. Solid line is the relationship found experimentally. All data on the rate of uptake of nitric oxide by cells were corrected according to this calibration curve.

the saline solution as long as the same gas concentration is used throughout the experiment.[2]

[2] For comparisons *between* experiments in which saline solutions with different gas concentrations were used, it is necessary to know precisely the gas concentra-

Methods Used for Inducing Changes in Cellular Shape and/or Volume:

1. *Shape Changes without Appreciable Changes in Volume.*—To transform the red blood cell from a biconcave disc to a sphere without accompanying changes in cellular volume we tried, without success, some of the well known chemical sphering agents, particularly sodium taurocholate and sodium cetyl phosphate (courtesy of Dr. Merkel H. Jacobs). We were unable to obtain a homogeneous suspension of perfectly sphered erythrocytes which would persist for a reasonable period of time without undergoing reversal of effect, further shape change, or hemolysis. Upon standing, the deoxygenated cells often assumed various spheroidal shapes, whereas the COHb cells became discoidal.

We then proceeded to take advantage of the observation made by Ham, Shen, Fleming, and Castle (6) that human red blood cells, heated to some critical temperature between 48.6 and 50.0°C., depending upon the duration of heating, change irreversibly into spheres with minimal hemolysis and no significant increase in cellular volume (hematocrit value). Our data upon temperature and duration of heating show remarkably good correspondence with those of Ham *et al.* and reaffirm their conclusion that cells sphere within a narrow range of temperature. This technique permitted us to compare rates of reaction in discs with those of the same cells transformed into spheres.

We obtained first several complete sets of measurements of gas uptake by discoidal cells at 38° or at 46–48°C. Our own experience and that of Ham *et al.* have shown that human erythrocytes may be kept at a temperature of 46° for an hour or longer with no demonstrable changes; we have been able to expose the cells to 48–49°C. for short periods of time without alterations in shape. The temperature of the water bath then was raised cautiously and continuously to 50°C. By repeated sampling and microscopic observation it was noted that at least 90 per cent of the cells changed suddenly into perfect spheres when the temperature reached 49.6–50.0°C.

We then followed one of two procedures:—(*a*) we repeated immediately measurements of the reaction rates at the sphering temperature or (*b*) without making any measurements of reaction rates, we rapidly cooled the blood to the temperature at which the control data had been obtained (38, 46, or 48°C.); since erythrocytes once sphered by heat do not revert to discs on cooling, we could repeat the measurements on spherocytes at the control temperature. (In the latter experiments, the bottle containing the reactant saline was maintained at control temperatures in a separate bath to avoid changes in gas concentration.)

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tion. Unfortunately, we were unable to obtain close agreement between the values for gas concentration arrived at by chemical analyses and those obtained from calculation using the solubility coefficient and partial pressure. Normal values are presented in Tables V and VI and Fig. 8 for l'_c and for j'_c using (*a*) the *calculated* concentration of the gas, and (*b*) the *measured* concentration of the gas in the saline solution. The actual values probably lie somewhere between; they are given to show the approximate range of l'_c and j'_c. We are attempting to improve our techniques to obtain better agreement between the two values.

In regard to procedure (*a*), since we found the temperature coefficient of the reaction for intraerythrocytic combination of carbon monoxide with hemoglobin to be small (Q_{10} = 1.25), we considered it valid to compare reaction rates at 48° (discs) with those at 49.6° (spheres), corrections being made for the slight decrease in gas concentration in the reactant saline owing to the increase in temperature.

2. *Shape Changes with Appreciable Changes in Volume.*—Control measurements were made upon normal discoidal cells suspended in 0.9 per cent phosphate buffered saline solution at 38°C. Deaerated 30 per cent sodium chloride solution was then injected into each bottle in sufficient quantity to increase the concentration of salt in the medium to 2.0, 2.5, or 3.0 per cent depending upon the degree of hypertonicity desired. The cells became crenated spheres with a decrease of 20 to 55 per cent in mean corpuscular volume, and an increase in mean corpuscular hemoglobin concentration, in some experiments, approaching the probable point of maximum concentration of 59 per cent (8).

To measure gas uptake in cells with a volume *larger* than normal, the cells were suspended in 0.6 per cent phosphate buffered saline solution at the outset of the experiment and the kinetic studies made. A quantity of deaerated 30 per cent sodium chloride solution was injected sufficient to raise the tonicity of the medium to an equivalent concentration of sodium chloride of 0.9 per cent; the measurements were repeated on the cells which thus had reassumed their original discoidal shape and volume.

Methods of Observing and Measuring Changes in the Erythrocytes.—Throughout the course of this work the appearance of the red blood cells was followed microscopically, using an oil immersion lens (970-fold magnification), and vinylite slides and coverslips. Care was taken to use well washed slides and to observe the cells before drying could take effect. Because identification of the cells was critical, plastic slides were used in place of glass slides since the latter induce a disc-to-sphere change, apparently the result of an elevated intracellular pH (2).

Aliquots of whole blood were saved for the determination of oxygen capacity (method of Van Slyke and Neill) and of total hemoglobin concentration, measured as cyanmethemoglobin. Samples of 1:40 and 1:80 cell suspensions were analyzed also for total hemoglobin concentration to verify the degree of dilution, adequacy of stirring, and equality of the rates of flow.

Since the over-all reaction rate of free hemoglobin with carbon monoxide and particularly with nitric oxide is much more rapid than that of intracellular hemoglobin it is essential to know of the presence and extent of hemolysis resulting from the experimental techniques.[3] Samples of the 1:40 and 1:80 cell suspensions were centrifuged and the supernatant measured for hemoglobin as cyanmethemoglobin.

The number of red blood cells/mm.^{3} was determined in the usual routine clinical manner. Hematocrit determinations were performed on the same 1/200 dilution

[3] Small amounts of hemolysis could be corrected for, in the case of the NO and Hb reaction, since the almost instantaneous combination of NO with free Hb resulted in an upward displacement of the baseline of the NOHb *vs.* time curve and the reaction of NO with intracellular Hb could be measured as beginning from this new "zero" line.

used for red cell counts in the following manner: Van Allen hematocrit tubes were individually calibrated to find the exact ratio of the volume of the stem to the total volume of the pipette. After centrifugation, the length of the packed cell column was converted by the calibration factor into the hematocrit value. (The error in the method was 0.8 per cent and the accuracy of the method was 0.61 per cent.) From the hemoglobin determinations, the red blood cell counts, and the hematocrit reading,

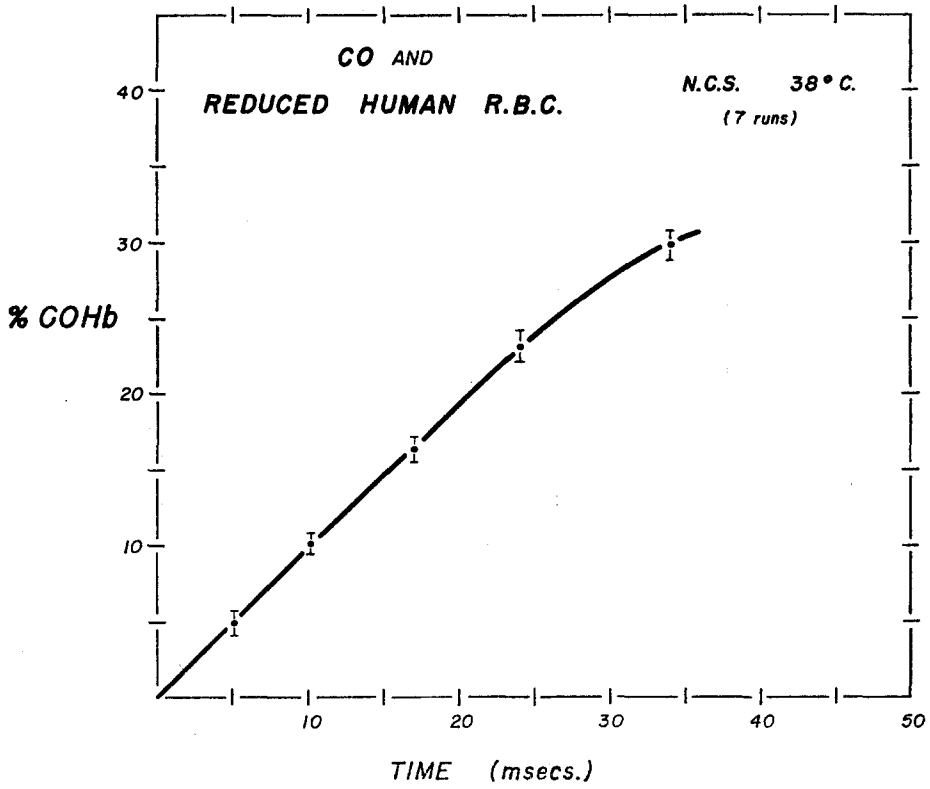

FIG. 2. Initial over-all rate of uptake of carbon monoxide by normal human cells at 38°C. Each point is the mean of seven measurements, the bars representing one standard deviation from the mean. This illustrates the reproducibility of the method within a single experiment.

we calculated the mean corpuscular volume, the mean corpuscular hemoglobin, and the mean corpuscular hemoglobin concentration for each stage of an experiment.

RESULTS

Reproducibility within an Experiment.—Fig. 2 illustrates the reproducibility of our measurements of reaction rates. The curve represents the mean of seven sets of measurements of the initial rate of carbon monoxide uptake

by normal discs at 38°C. (Experiment 53, N.C.S.); the bars signify plus and minus one standard deviation from the mean. This reproducibility within

TABLE II

Over-all Initial Rate of Carbon Monoxide or Nitric Oxide Uptake by Biconcave Discs Compared with Spheres of Similar Volume

Experiment No.	Sub ect	Gas	Filters	Biconcave discs				Spheres			
				Temperature	Hemolysis	l'_c(mM^{-1}·sec.$^{-1}$)		Temperature	Hemolysis	l'_c(mM^{-1}·sec.$^{-1}$)	
						Calculated*	Measured*			Calculated*	Measured*
			mμ	°C.	*per cent*			°C.	*per cent*		
16	J. H. C.	CO	W 29,61	49.0	0.0	101	—	49.5	0.5	101‡	—
22	J. H. C.	CO	W 29,61	49.0	2.0	139	145	50.0	4.1	139‡	145‡
23	J. H. C.	CO	W 29,61	49.0	1.6	121	—	49.6	3.0	121‡	—
28	J. H. C.	CO	W 29,61	48.0	1.3	178	196	49.6	2.0	178‡	196‡
25	L. C.	CO	W 29,61	47.8	2.4	88	—	49.8	5.6	88‡	—
33	C. O.	CO	W 29,61	47.0	1.1	141	173	50.5	8.4	145	176
37§	J. H. C.	CO	555,536	46.0	0.4	80	104	50.0	2.2	79	102
38	D. J.	CO	555,536	47.5	0.5	78	114	50.0	10.0	76	110
39	D. J.	CO	555,536	47.5	2.1	78	102	50.0	8.4	80	105
41	D. J.	CO	555,536	47.5	2.5	77	90	47.5	8.8	74	85
42	D. J.	CO	555,536	47.0	0.0	83	83	47.0	10.3	83‡	83‡
43	D. J.	CO	555,536	47.0	1.7	76	85	47.0	6.0	66	73
50	R. B.	CO	555,536	47.0	0.8	83	106	47.0	7.2	83‡	106‡
51	P. K.	CO	555,536	38.0	0.3	63	74	38.0	2.6	63‡	74‡
						j'_c				j'_c	
52	R. L. J.	NO	555,536	38.0	0.8	114	—	38.0	5.9	114‡	—
55	J. H. C.	NO	453,442	38.0	1.3	118	—	38.0	6.0	118‡	—
56	R. B.	NO	453,442	38.0	1.2	113	174	38.0	6.5	110	168

* Based on the calculated [CO] or [NO] in the saline solution, or on the measured [CO] or [NO].

‡ In these experiments the graphed points of milliseconds *versus* per cent COHb or per cent NOHb were so close to those for the control data on biconcave discs that no distinction could be made between them.

§ In Experiments 37 and on, the new electrical system, narrow band interference filters, and half-silvered mirror were employed.

a single experiment is sufficiently good to detect small changes in initial reaction rates that might result from alterations in the red blood cell.

Comparison between Discoidal and Spherical Erythrocytes in Rate of Gas Uptake.—

(*a*) *Shape Change without Volume Change.*—Table II contains data from seventeen experiments in which biconcave discoidal erythrocytes were trans-

formed into spherical cells by heating the suspension to a temperature between 49 and 50°C. The average increase in mean corpuscular volume, as calculated from the red cell count and hematocrit value, was 5.4μ^3, or a 6.6 per cent increase over the original mean corpuscular volume. Data from experiments in which the initial hemolysis exceeded 2.5 per cent have been discarded. Fig. 3 portrays the data obtained in a typical experiment (No. 51). In the first nine of the experiments included in the table (Nos. 16 to 39), the control measurements on discoidal cells were made at temperatures between 46.0 and 49.0°C.,

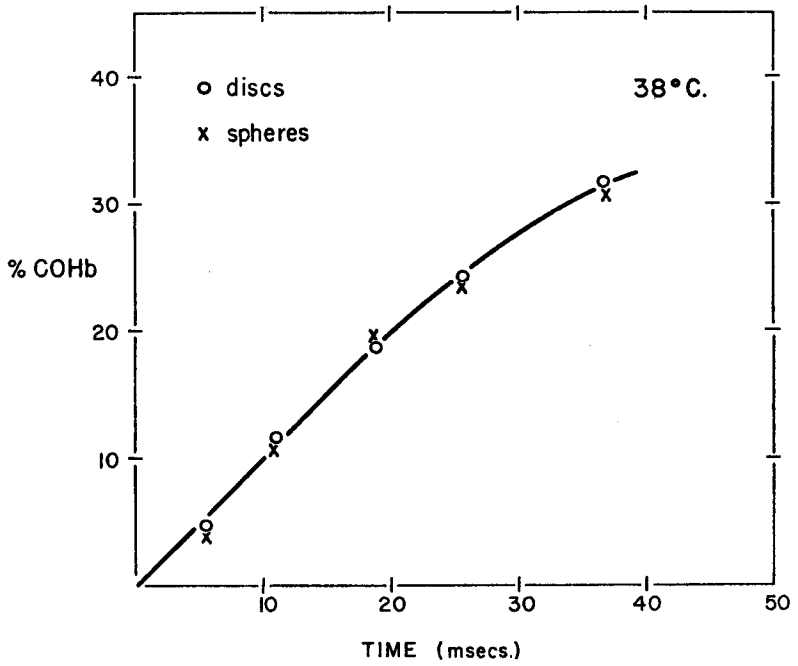

FIG. 3. Comparison of the initial over-all rate of uptake of carbon monoxide by normal biconcave discs with that of cells of the same volume sphered by heat (Experiment 51, P. K.). Identical results were obtained with nitric oxide.

and the experimental measurements on spherical cells were performed at 49.5 to 50.0°C. Every set of measurements was performed in duplicate or in triplicate. In the subsequent four experiments (Nos. 41, 42, 43, 50), after sphering the temperature was returned to 47°C. for experimental measurements; in Experiments 51, 52, 55, 56, the temperature was returned to 38°C. This was to eliminate the need for any correction of the carbon monoxide concentration and to quench further changes in the erythrocytes resulting from heat. To rule out the possibility that the chemical reaction between hemoglobin and carbon monoxide was the limiting factor, the rate of uptake of nitric oxide was measured also (Nos. 52, 55, 56); the latter rate is presumed to be diffusion-

limited. We found no significant difference between the rate of CO or NO uptake by biconcave discs and that by the same cells of similar volume in the form of spheres.

Objections may be raised to the use of heat as a means of sphering cells on the grounds that heat may produce structural changes in the cells themselves, alterations in the cellular membrane, or even changes in the chemical nature of the hemoglobin. Consequently, a comparison of the reaction rate of normal cells with that of heated cells may involve more than a comparison of discoidal and spherical cells. With respect to the first of these arguments, since irreversible shape change results from heating, it is probable that some structural alteration in the red blood cell architecture does occur. The results of Ham *et al.* (6), however, indicate that notable increases in the osmotic and mechanical fragilities of the cells do not occur until considerable fragmentation has taken place; in our experiments, spherocytosis preceded fragmentation. The very slight increase in cellular volume suggests that the permeability of the heated membrane had not been altered appreciably with respect to the electrolytes in the environment. Ham *et al.* (6) showed that normal and heated cells display parallel reductions in volume when suspended in hypertonic solutions of sodium chloride, demonstrating that the membrane permeability for sodium chloride and water is not altered materially.

The third possibility concerns the state of the hemoglobin. Gasometric determinations of the oxygen capacity of heated blood compared to that of the same blood, unheated, showed no consistent decrease. Furthermore, when aliquots of heated and unheated cell suspensions and of hemoglobin solutions were compared on a recording spectrophotometer (courtesy of Dr. David L. Drabkin), no significant differences could be found, thus indicating the absence of appreciable quantities of pigments other than those originally present. Also, the identical kinetic curves which we obtained using heated and unheated cells, would suggest, when included with the above information, that the hemoglobin was not detectably altered.

(*b*) *Change in Shape with Increase in Volume.*—In Table III are presented data from four experiments in which normal erythrocytes were converted, with minimal hemolysis, into swollen spheres by suspension in phosphate buffered saline solution equivalent osmotically to 0.6 per cent NaCl. Despite an approximate increase in cellular volume of 30 per cent, the initial rate of gas uptake by the enlarged spherical cells was so similar to that of discs of the normal volume that no difference could be noted between the experimental and control measurements (see Fig. 4).

(*c*) *Change in Shape with Decrease in Volume.*—Table IV includes data from eight experiments in which the rates of uptake of nitric oxide by discs of normal cellular volume are compared with those of the same cells in the form of crenated shrunken spheres produced by suspension in a hypertonic medium. In an

additional experiment (No. 63, J.R.) the rate of uptake of carbon monoxide was measured so as to be certain that the results were not peculiar to the use of nitric oxide. The calculations were corrected for the small amount of free hemoglobin resulting from the addition of concentrated salt solution.

To discard the possibility that the hemoglobin was denatured by the increase in salt concentration, the oxygen capacity of cells suspended in 3.0 per cent saline solution was compared gasometrically with that of cells from the same blood suspended in 0.9 per cent saline solution. No significant decrease in the

TABLE III

Initial Rate of Nitric Oxide Uptake by Biconcave Discs Compared with Spheres of Greater Volume (38°C.)

442 and 453 mμ filters.

Experiment No.	Subject	Equivalent per cent NaCl	Mean corpuscular volume		Mean corpuscular hemoglobin			Hemolysis	j'_c(mM^{-1}·sec.$^{-1}$)	
			μ^3	Increase	μg.	[Hb]	Decrease in [Hb]		Calculated [NO]*	Measured [NO]*
				per cent		*per cent*	*per cent*	*per cent*		
60	J. H. C.	0.6	114.3	32.3	28.0	24.5	24.8	0.5	130	177
		0.9	86.4	—	28.2	32.6	—	0.7	130‡	177‡
61	J. H. C.	0.6	113.8	35.3	28.3	24.8	25.8	0.8	113	144
		0.9	84.2	—	28.2	33.5	—	1.2	113‡	144‡
62	C. G.	0.6	114.9	23.3	29.5	25.6	19.5	0.9	126	184
		0.9	93.2	—	29.7	31.8	—	1.4	126‡	184‡
64	C. G.	0.6	119.5	25.4	30.5	24.5	23.2	0.4	124	220
		0.9	95.3	—	30.4	31.9	—	0.8	124‡	220‡

* Based on the calculated [NO] in the saline solution, or on the measured [NO].

‡ In these experiments the graphed points of milliseconds *versus* per cent NOHb were so close to those obtained in the control experiments that no differentiation could be made between them.

carrying capacity of the hemoglobin for oxygen was found in those cells suspended in 3.0 per cent saline solution.

The resulting percentage decrease in mean corpuscular volume ranged from 22 to 40 per cent, depending upon the degree of hypertonicity. The percentage increase in mean corpuscular hemoglobin concentration was between 20 and 71 per cent, so that the hemoglobin concentration per cell rose to as high as 57 per cent, believed to be close to the upper limit of intracellular hemoglobin concentration.

In every instance there was a marked decrease in the initial over-all rate of gas uptake when the cells were dehydrated (Fig. 5). In Fig. 6 the j'_c values

obtained are plotted against the mean corpuscular hemoglobin concentrations of the cells suspended in solutions of varying salt concentration. The values obtained within a single experiment are connected by straight lines to show the steepness with which the initial rates of gas uptake declined following cellular shrinkage. Included also are the values found in the four experiments in which the cells were first suspended in a hypotonic environment, with an

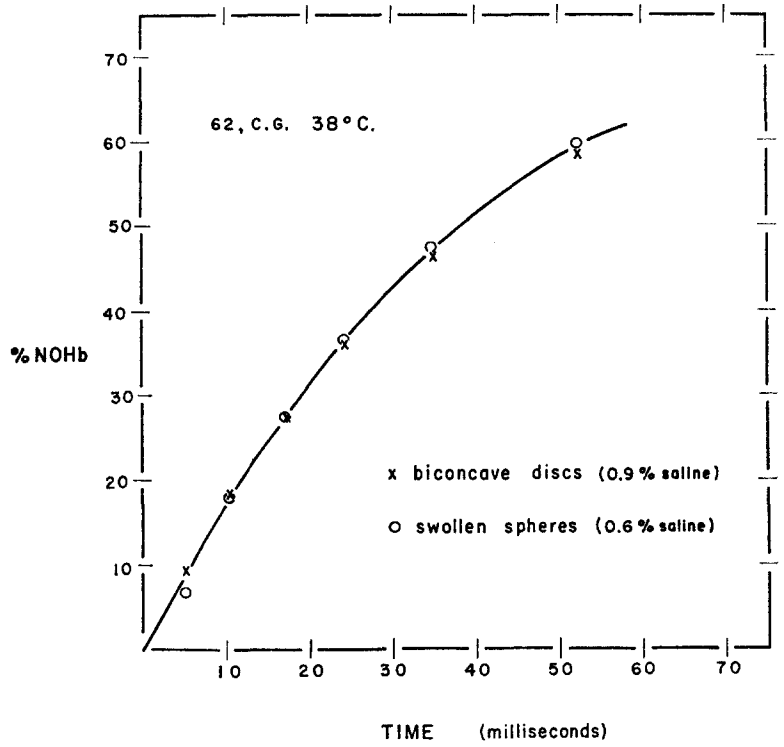

FIG. 4. Comparison of the initial over-all rate of uptake of nitric oxide by biconcave discs with that of cells of greater volume sphered by immersion in hypotonic saline solution at 38°C. Each point is the mean of three measurements.

average hemoglobin concentration per cell of 25 per cent; *i.e.*, 23 per cent less than normal. It appears from these data that gas uptake is markedly and consistently slowed when the red cell shrinks to a volume such that the hemoglobin concentration per cell has risen to only 40 per cent (No. 58, R.L.J.), and may occur even before this.

DISCUSSION

Our experiments have tested for the first time several concepts of physiological importance and have yielded two unexpected findings. The first of these

is that disc-to-sphere transformations, accomplished in two different ways (by heating and by immersion in hypotonic saline solution), are not accom-

TABLE IV

Over-All Initial Rate of Nitric Oxide Uptake by Normal Biconcave Discs Compared with Shrunken Spheres (38°C.)

Experiment No.	Subject	Equivalent per cent NaCl	Mean corpuscular volume		Mean corpuscular hemoglobin			Hemolysis	j'_c (mM^{-1}·sec.$^{-1}$)	
			μ^3	Decrease	$\mu\mu$g.	[Hb]	Increase in [Hb]		Calculated [NO]*	Measured [NO]*
				per cent		*per cent*	*per cent*	*per cent*		
57	M. S.	0.9	83.3	—	26.2	31.4	—	0.6	102	124
		2.0	61.8	25.8	26.7	43.3	37.9	1.4	57	69
58	R. L. J.	0.9	84.1	—	26.2	31.2	—	0.6	129	190
		2.0	65.2	22.5	26.3	40.3	29.2	2.6	88	130
59	P. K.	0.9	79.8	—	27.4	34.3	—	2.0	123	166
		2.0	54.4	31.8	26.4	48.6	41.7	4.9	74	100
59	J. M. B.	0.9	80.8	—	26.5	32.8	—	1.2	110	149
		2.0	51.9	35.8	26.1	50.3	53.4	3.4	61	83
60	J. H. C.	0.9	86.4	—	28.2	32.6	—	0.7	130	177
		2.0	62.2	28.0	29.6	47.6	46.0	2.8	78	106
		2.5	52.9	38.8	29.3	55.4	70.0	2.7	65	89
61	J. H. C.	0.9	84.2	—	28.2	33.5	—	1.2	113	144
		3.0	50.4	40.2	28.8	57.2	70.8	3.1	50	64
62	C. G.	0.9	93.2	—	29.7	31.8	—	1.4	126	184
		3.0	63.5	31.9	31.3	49.3	55.0	3.0	61	89
64	C. G.	0.9	95.3	—	30.4	31.9	—	0.8	124	220
		2.5	64.8	32.0	30.4	47.0	47.3	1.8	56	99
	Carbon monoxide uptake								l'_c (mM^{-1}·sec.$^{-1}$)	
63	J. R.	0.9	100.6	—	32.6	33.1	—	1.3	64	80
		2.0	74.5	35.0	32.6	43.7	32.0	1.6	45	57

* Based on the calculated [CO] or [NO] in the saline solution, or on the measured [CO] or [NO].

panied by any measurable change in the rate of gas uptake. The second is that reduction in the size of the human red blood cell (by immersion in hypertonic saline solution) does indeed lead to a change but this is a rather dramatic *reduction* in the rate of gas uptake. It would be tempting to conclude from

the first case that the erythrocytic membrane is more of a limiting factor than are intraerythrocytic diffusion distances, and from the second case, that the concentration of intraerythrocytic hemoglobin to a value greater than 34 per cent imposes mechanical restrictions on diffusion, thus confirming the hypothesis of Perutz (12). Our experimental procedures, however, undoubtedly produced many physical and chemical changes. For this reason we cannot say at

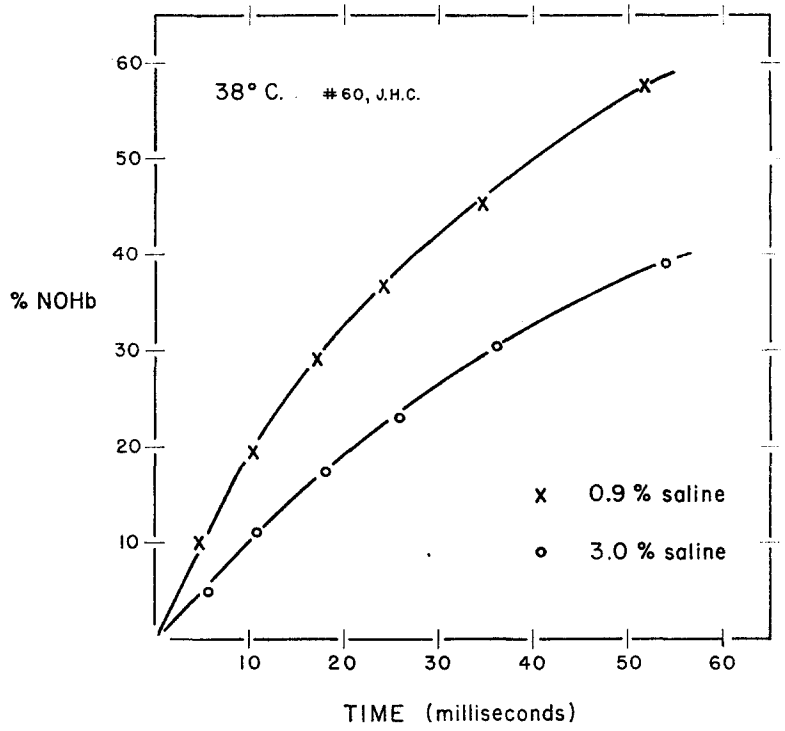

FIG. 5. Comparison of the initial over-all rate of uptake of nitric oxide by normal biconcave discs with that of cells shrunken by immersion in hypertonic saline solution at 38°C.

this time which change (s) critically limits gas uptake, but we can eliminate certain factors previously thought to be of considerable importance.

1. Shape of the Erythrocyte.—Hartridge, in 1920, was the first to suggest that the unique discoidal shape of the normally circulating erythrocyte is ideal for rapid and uniform diffusion of gas from the periphery of the cell to its interior (7). He reasoned that there are two simple forms, a sphere and an infinitely thin disc, which would permit gas molecules, diffusing from the surface, to reach the central regions at the same time. The sphere, however, would offer little surface relative to its volume, whereas the infinitely thin disc would

be fragile if not protected by a thick surface envelope to impart sufficient rigidity of form; if the disc were not infinitely thin, the combined effect of gas

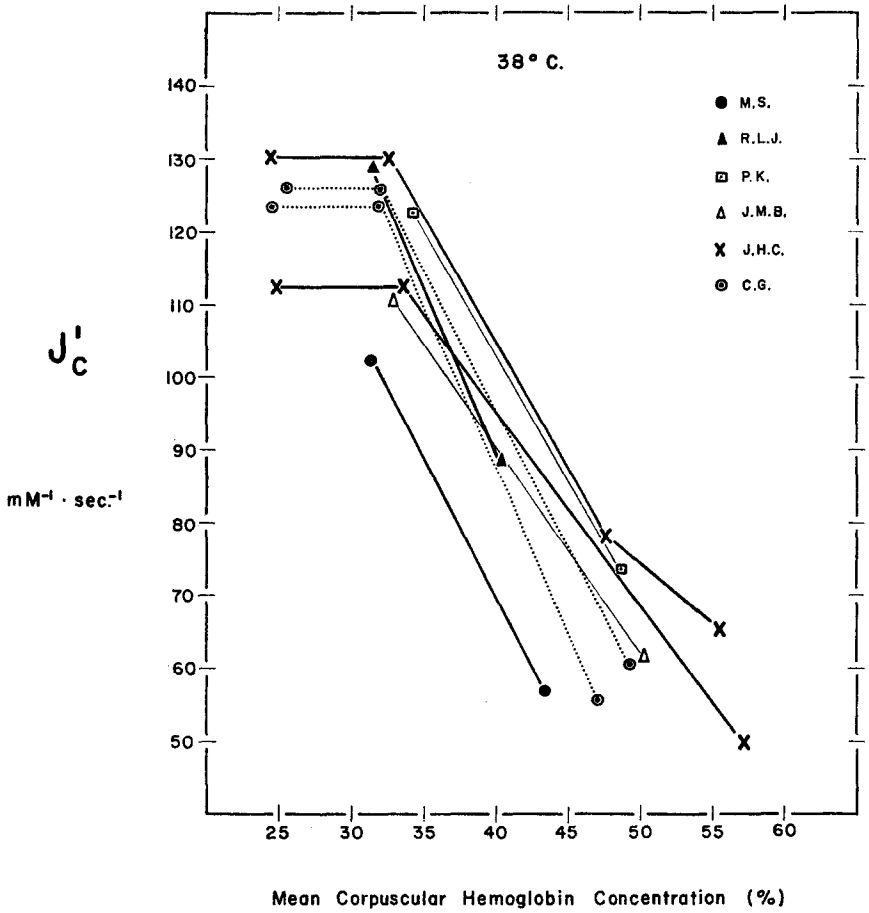

FIG. 6. A graph of the values obtained in eight experiments in which the initia over-all rate of uptake of nitric oxide (j'_c) is plotted against mean corpuscular hemoglobin concentration, to illustrate the effect of shrinkage upon the rate of gas uptake. Data are included also for cells in hypotonic media obtained during the same experiment.

molecules entering laterally and through the ends would cause saturation of the end regions before the central area. A biconcave disc is, in contour, a compromise form between the sphere and the infinitely thin disc; it provides an extensive surface relative to its volume and, since the ends are thickened, gas molecules diffusing from any part of the surface would penetrate to the

interior in a uniform manner. Following this, Ponder attempted to describe mathematically the form of the mammalian erythrocyte (13, 14); though unsuccessful, he concluded that the effectiveness of the biconcave disc for gas exchange must be very great compared to other forms of the same volume and that this special shape is clearly related to the respiratory function of the cell, it being excellently adapted to even and orderly diffusion and far more effective than a sphere.

On a purely geometrical basis, such statements are valid, and might apply to the erythrocyte if its contents were inert with respect to the gas involved. When one is considering, however, the uptake of gases which combine chemically with hemoglobin, both the rate of diffusion and that of the chemical reaction are involved, and such simple deductions no longer apply. By means of theoretical equations developed for the case of diffusion combined with a first order chemical reaction, Roughton has calculated (17) that the initial rate of entry of carbon monoxide into spherical sheep erythrocytes would not differ significantly from that into biconcave discs of the same volume. Thus, for erythrocytes with the dimensions of sheep red blood cells (volume $30\mu^3$, area 67 μ^2, and 5.2 μ) (15), disc-to-sphere transformation theoretically would have no notable influence upon gas uptake; he did not make similar calculations for human erythrocytes and did not verify this conclusion experimentally.

Roughton mentions that some of his previous kinetic measurements were probably performed unknowingly on spherical cells, rather than on discs as supposed. This, of course, makes the experimental verification of Roughton's calculations all the more important since most of the known reaction rate velocity constants have been measured in his laboratory, and to the best of our knowledge his group has not examined microscopically their red cell suspensions, nor measured mean corpuscular volume or hemoglobin concentration.

The only experimental approach of which we are aware to the effect of alterations in shape of the erythrocyte upon gas uptake has been the study by Valtis and Baikie (19) of the blood oxygen dissociation curve of guinea pigs. The oxyhemoglobin curve, however, is a static expression of the equilibrium existing between the rate of formation of O_2Hb and its rate of dissociation. Shifts in the equilibrium could result from changes in the velocity constant for either association or dissociation, or from changes in both to varying degree, thus obscuring the actual reaction affected; moreover, it is difficult to see how a chemical description of the degree of binding of oxygen to hemoglobin could yield information on the influence of the cellular shape upon the rate of diffusion of gas into or out of the cell.

Our experimental results are at variance with the suggestions made by Hartridge and by Ponder, but provide support for the theoretical calculations of Roughton. By reference to Fig. 7, it may be seen, from geometrical considerations, that conversion of a biconcave disc into a sphere of equal volume

decreases the surface area available for diffusion by 40 per cent and increases more than twofold the distance gas molecules must travel from the surface to penetrate the innermost regions of the cell. If diffusion distance were an important limiting factor, such a transformation should retard the rate of uptake of dissolved gas by the cells. We found no difference between the over-all initial rate of uptake of carbon monoxide by normal human biconcave discs and that of cells of the same volume sphered by heat. One could object that the rate of combination of carbon monoxide with hemoglobin within the cell may

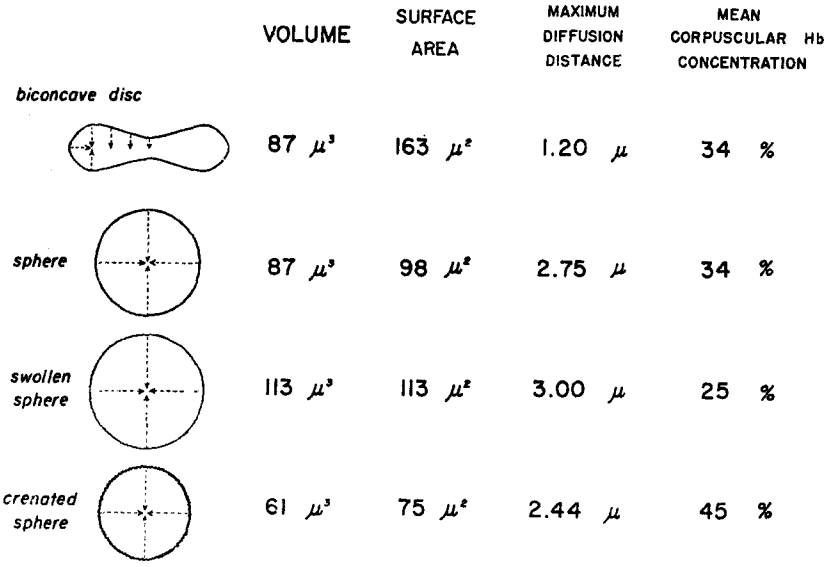

FIG. 7. Comparisons of physical measurements. The values for volume, surface area, and diffusion distance for the biconcave disc were obtained from Ponder (15). Values for the swollen sphere are calculated on the basis of a 30 per cent increase in volume over that of the disc, and the values for the shrunken sphere are calculated on the basis of a 30 per cent decrease in volume.

have been slow enough to be the limiting factor in the over-all rate of gas uptake, but the results were identical with nitric oxide which reacts almost instantaneously with free hemoglobin.

Another objection to our experimental procedure concerns the use of heat which may have produced a number of changes, both physical and chemical; those changes which tended to decrease the rate of gas uptake might exactly balance those which tended to increase the rate of gas uptake. The cells made spherocytic by suspension in hypotonic saline solution, however, were never heated beyond 38°C., yet the initial over-all uptake of nitric oxide by these cells was the same as that of normal biconcave discs. Moreover, these swollen

spherocytes had a smaller surface area and greater maximal distance for diffusion than the heated cells and these changes would exaggerate any differences if intracellular diffusion distances were critical.

The decrease in over-all initial rate of uptake of nitric oxide by shrunken, crenated erythrocytes (produced by suspension in hypertonic saline solution) also supports the concept that the distance for diffusion is not an all important

TABLE V

Initial Over-All Rate of Nitric Oxide Uptake By Normal Human Biconcave Discs at 38°C. 442 and 453 mμ interference filters.

Experiment No.	Subject	J_c' (mM^{-1} sec.$^{-1}$)	
		Calculated [NO]*	Measured [NO]*
56	R. S. B.	113	174
57	M. S.	102	124
58	R. L. J.	129	190
59a	P. K.	123	166
59b	J. M. B.	110	149
55	J. H. C.	118	—
60	J. H. C.	130	177
61	J. H. C.	113	144
62	C. G.	126	184
64	C. G.	124	220
63	J. R.	120	139
Average		119	167
±1 S.D.		8.7	28.2
555 and 536 mμ interference filters			
47	R. S. B.	121	—
52	R. L. J.	114	—

* Values based on the calculated gas concentration in the saline solution, or on the measured concentration. Satisfactory agreement between the calculated and analyzed values for gas concentration was never achieved; thus we present the data both ways.

factor for cells of this size; the maximal distance in the shrunken cells, though greater than that of the biconcave discs, is less than that of spheres produced by heat or by immersion in hypotonic solutions and thus, should have increased rather than decreased the rate of gas uptake if intracellular distance were important.

The experiments on shrunken crenated cells support to a certain extent the concept that the surface area for diffusion may be a limiting factor inasmuch as the surface area of the cells is reduced to one-half that of the disc. The surface

area, however, is reduced for all cells converted to spherocytes, yet no change in over-all initial rate of gas uptake occurred in cells sphered by heat or by immersion in 0.6 per cent saline solution. It is true that our calculations of

TABLE VI

Initial Over-All Rate of Carbon Monoxide Uptake by Normal Human Biconcave Discs

555 and 536 mμ interference filters.

Experiment No.	Subject	$1'_c$ (mM^{-1} sec.$^{-1}$)	
		Calculated [CO]*	Measured [CO]*
38°C.			
36	R. B.	71	95
43	D. J.	62	69
50	R. S. B.	65	84
51	P. K.	63	74
53	N. C. S.	66	85
63	J. R.	64	80
Average		65	81‡
± 1 S.D.		3.2	9.1
48°C.			
36	R. B.	84	110
37	J. H. C.	80	104
38	D. J.	78	114
39	D. J.	78	102
40	D. J.	82	124
41	D. J.	77	90
42	D. J.	83	83
43	D. J.	76	85
50	R. S. B.	83	106
53	N. C. S.	85	108
Average		80	102
± 1 S.D.		3.3	13

* Values based on the calculated gas concentration in the saline solution, or on the measured concentration. Satisfactory agreement between the calculated and analyzed values for gas concentration was never achieved; thus, we present the data both ways.

‡ Forster *et al.* (4) obtained a value of 79 as a mean for three subjects.

surface area are based only on mean corpuscular volume and shape (observed microscopically) and ignore such considerations as infolding of the membrane or fine crenations. We feel, however, that our experiments indicate that an increase in maximum distance for diffusion within the erythrocyte and a decrease in surface area of the membrane, of the magnitude produced by us, are

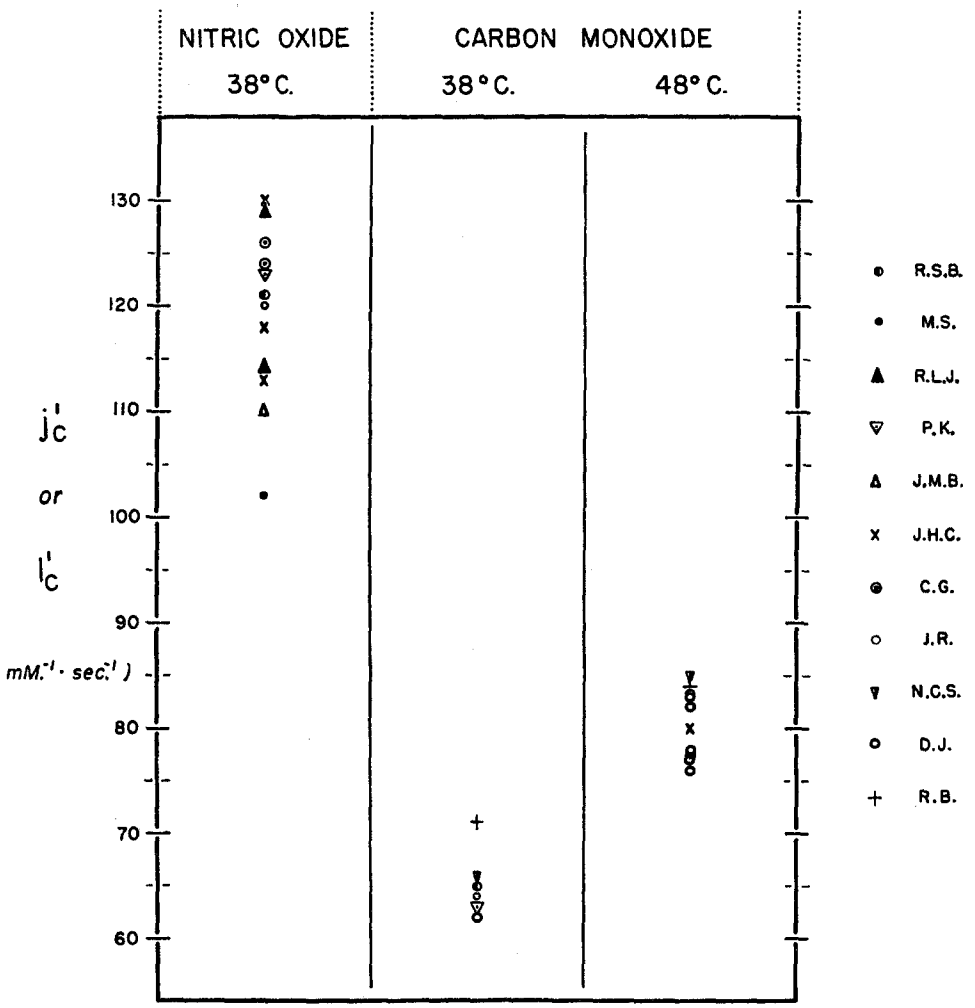

FIG. 8. Graphic portrayal of the data contained in Tables V and VI (based upon the calculated concentrations of gas in the saline solution). The Q_{10} for the combination of carbon monoxide with intraerythrocytic hemoglobin is 1.25 ± 0.053. The rate of combination of intraerythrocytic hemoglobin with nitric oxide is approximately 1.7 times faster than that with carbon monoxide.

not important factors limiting the over-all rate of uptake by the cells of gases which react with hemoglobin.

If forced to reason teleologically about the shape of biconcave discoidal red blood cells, we would on the basis of these experiments choose to discard the concept of "respiratory efficiency" and agree rather with those who believe

that red cells are so shaped to withstand better being buffeted and squeezed through narrow vascular beds, and to survive any swelling owing to slight osmotic changes in the circulating blood.

2. Changes in Erythrocytic Membrane.—When the true surface area of a red blood cell decreases, it is logical to assume that the membrane has become thicker, though capacity measurements by Curtis (3) point to a small degree of thinning of the membrane as the disc becomes a sphere. Unfortunately, we know very little concerning the actual structural changes in the membrane when discs become spheres or crenated cells. It is even possible that infoldings, wrinkling, or crenations account for the so called "change" in surface area and that the actual area and thickness of the membrane may remain unchanged. If the first possibility were fact, the delay in gas uptake by the shrunken crenated cells could be explained, at least in part, by thickening of the membrane; however, a certain degree of thickening would have existed also for the swollen sphere and the sphere without volume change and these cells showed no similar slowing of the rate of gas exchange. At present there is no direct experimental means of measuring the diffusion of gas through the erythrocytic membrane, and that alone. We, like Roughton, have measured gas uptake by red blood cells and then repeated the measurements after hemolysis. Using carbon monoxide, the uptake of gas by free hemoglobin was approximately four times faster than that of the red cells; with nitric oxide the combination with free hemoglobin is almost instantaneous whereas the uptake by Hb contained within cells is of the same order as that for CO. This suggests that the membrane barrier is the most important factor. Such a conclusion may not be valid, however, because in the solution, the molecules of hemoglobin are distributed evenly throughout the medium as a 0.25 per cent solution of hemoglobin, whereas in the suspension, the hemoglobin exists as discontinuous packets of 34 per cent hemoglobin in a medium peculiar to the interior of the red blood cell. There is no direct proof that the chemical reaction rates of hemoglobin in dilute solution and in concentrated form are identical. Unfortunately, a 34 per cent hemoglobin solution cannot be used in our constant flow apparatus because it is not possible to secure sufficient light transmission through a 2 mm. thick layer of such concentrated material. Reasoning from indirect evidence, then, it would appear that the cellular membrane normally impedes the uptake of gas considerably but slight changes in its apparent thickness are not of great consequence (comparing the normal disc, the sphere, and the swollen sphere). In the case of the shrunken sphere, the 2 to 3 per cent saline solution might act not by thickening the membrane but by evoking a reorientation and concentration of the component molecules within the membrane, thus altering its permeability to gases. The hypothetical pores in the membrane might close or narrow during immersion of the cells in hypertonic solution but this should not be an important factor if we are correct in assuming that gases diffuse over

the entire surface of the cell and not solely through special channels. There is the possibility, of course, that although gases might diffuse through the entire surface of the cell, they might diffuse much more rapidly through the watery pores. It would be interesting to know if hypertonic solutions would alter selectively the rate of penetration of substances which presumably enter the cell exclusively *via* pores without affecting rates of entry of other substances.

3. Concentration of Intracellular Hemoglobin.—The most striking feature of the interior of the red blood cell is the intimacy of the hemoglobin molecules to one another. It is conceivable that such close packing could present mechanical obstruction to substances diffusing intracellularly. Perutz has hypothesized that any increase in concentration of hemoglobin above 34 per cent would hamper the rate of intracellular diffusion by narrowing the channels between the hemoglobin molecules and thus slow the speed of reaction (12). The x-ray studies of Bateman *et al.* (1) showed that in 2.0 per cent salt solution intracellular molecules of human hemoglobin do, indeed, move closer together reaching an intermolecular distance of 65 A, corresponding to a maximum hemoglobin concentration of 59 per cent.

We found a marked decrease in the rate of gas uptake when the cells shrank to the point that the hemoglobin concentration rose to 40 per cent, and greater retardation as the concentration increased further. Thus Perutz's hypothesis appears to be an attractive one. A difficulty with this explanation is encountered, however, in the diffusion coefficients which Longmuir and Roughton determined for nitrogen in hemoglobin solutions ranging in concentration from 0 to 44 gm./100 ml. (10); they noted that rates of diffusion for 35 and 44 per cent hemoglobin concentration were not greatly different. It may be, however, that in the process of disrupting the cells and reconcentrating the hemoglobin, the molecular alignment of the hemoglobin molecules in bulk solution was no longer the same as within the erythrocyte.

We thus conclude from our observations that the surface area available for diffusion and the maximal distance for diffusion, are not factors *critically* limiting gas uptake by red cells of the dimensions with which we are dealing. It appears, therefore, that the biconcave discoidal form of the human erythrocyte is not essential for optimal gas uptake as was postulated by Hartridge and by Ponder. The most likely causes for the consistent and marked retardation in the rate of gas uptake by shrunken cells seem to be a concentration and reorientation of the intracellular hemoglobin and/or of the component molecules within the cellular membrane. This supports in part the hypothetical suggestion of Perutz that 34 per cent concentration of hemoglobin represents the maximum concentration consonant with rapid gas exchange. We believe that further experiments designed to explain this finding of marked retardation of gas uptake by cells immersed in hypertonic media will lead to better under-

standing of the functional structure of the red blood cell and of the factors facilitating and limiting gas exchange.

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