The Nature and Significance of the Bohr Effect in Mammalian Hemoglobins

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ABSTRACT The oxygenation of hemoglobins is accompanied by the dissociation of protons. The number of protons discharged is inversely related to the size of the mammal from which the hemoglobin comes. The number of mercuric ions which are immediately bound by hemoglobins is approximately equal to the number of protons dissociated during oxygenation. Pretreatment of human hemoglobin by *N*-ethylmaleimide, which appears to bind only sulfhydryl groups prevents the binding of any mercuric ions under conditions when mercuric ions would otherwise be bound. These facts suggest that those mammals with higher metabolic rates will generally possess hemoglobins with a larger number of appropriately placed cysteine residues.

Bohr (1904) discovered that carbon dioxide greatly decreases the affinity of blood for oxygen. Barcroft (1928) later found that any acid acts similarly, and stressed the physiological significance of this effect in facilitating gas exchange. Thus not only would carbon dioxide in capillaries aid in unloading the oxygen bound to the hemoglobin, but also the high oxygen pressure in the lungs would promote the discharge of carbon dioxide. Pure hemoglobin solutions exhibit both the effects of CO_2 and of changes in pH. Although Barcroft attributed the entire effect of CO_2 to its properties as an acid, Ferguson and Roughton (1934), Ferguson (1936), and Stadie and O'Brien (1937) showed that CO_2 can be directly bound to hemoglobin. In this paper, however, we shall be concerned primarily with the pH effect.

Henderson (1920) first showed that the pH effect could be explained by assuming that the hemoglobin molecule possesses acid groups which become more acidic when hemoglobin is oxygenated. German and Wyman (1937) titrated both oxyhemoglobin and reduced hemoglobin and confirmed directly that protons are discharged from hemoglobin when the molecule is oxygenated. The oxygenation-linked acid groups have been identified as im-

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idazole groups (Wyman (1939, 1948); Coryell and Pauling (1940)) or as ammonium groups (Roughton (1943–44, 1949)). In this paper we shall describe experiments which indicate that the primary oxygenation-linked acid groups are neither imidazole groups nor ammonium groups but the sulfhydryl groups of cysteine residues. Furthermore, among terrestrial mammals, the smaller the animal the greater is the number of cysteine residues per hemoglobin molecule, and the larger is the number of protons discharged during oxygenation. These experiments offer a biochemical explanation for the observations of Schmidt-Nielsen and Gjönnes (1952) and Schmidt-Nielsen and Larimer (1958) that the oxygen affinity of whole blood from terrestrial mammals is inversely related to the body weight of the animal: the smaller the animal the lower is the oxygen affinity. The experiments also offer an explanation of the observations of Foreman (1954) on small mammal hemoglobins.

Method

THE OXYGEN EQUILIBRIA The spectrophotometric procedure for the determination of the oxygen equilibria has been described (Riggs and Wolbach (1956)). Samples of blood were collected by syringe from the ear of an Indian elephant,[1] from the hearts of an English setter,[2] a Chihuahua,[2] rabbit, guinea pig, bullfrog tadpole, and bullfrog adult. Mouse blood was obtained by making a razor-nick in the tails of laboratory animals. Cattle and hog bloods were obtained from the local abbatoir. The red cells were washed three times in 0.9 per cent sodium chloride and were hemolyzed by the addition of 1 volume of glass-distilled water to 1 volume of packed cells. One volume of this hemolysate was dialyzed for 15 hours against at least 20 volumes of glass-distilled water with continuous rotation of the sac at 4°C. One volume of the dialyzed solution was mixed with 1 volume of 0.2 M K_2HPO_4–KH_2PO_4 buffer. The solutions were centrifuged for 15 minutes at 19,000 *g* at 4°C. to precipitate the ghosts. The oxygen equilibria were carried out at 35°C. The oxygen capacity of the solution was determined with Warburg manometric apparatus, using potassium ferricyanide to liberate the oxygen. The pH of each solution was determined immediately after removal from the tonometers. The smaller animals were weighed; cattle, hog, and elephant weights were estimated. Experiments on the effect of CO_2 were carried out by injecting an appropriate amount of the gas into the tonometer.

MERCURIC CHLORIDE TITRATIONS Dialyzed hemoglobin solutions were prepared as indicated for the oxygen equilibrium measurements. One volume of the dialyzed hemoglobin solution was mixed with 1 volume of 0.2 M pH 7.32 K_2HPO_4 buffer with 0.2 M KCl and centrifuged 15 minutes. One ml. of the supernatant buffered hemoglobin solution was introduced into a titration vessel containing 40 ml. of pH

[1] I am indebted to Dr. Frederick Stark, Director of the San Antonio Zoo, for making an elephant available and to Dr. Robert A. Sturtevant for obtaining the sample.
[2] I am indebted to Dr. G. D. Stallworth for supplying the dog bloods.

7.32 buffer (composed of 0.1 M phosphate and 0.1 M KCl) and 0.02 ml. of Dow-Corning Anti-Foam compound B. The vessel is similar to that described by Laitinen and Burdett (1950). High purity helium was bubbled for at least 5 minutes prior to the introduction of the hemoglobin and continuously thereafter. Within 5 minutes the color of the added hemoglobin changed from orange-red to the purple characteristic of reduced hemoglobin. The sample was then titrated with 10^{-3} M $HgCl_2$, using the rotating platinum electrode procedure of Kolthoff, Stricks, and Morren (1954). All titrations were performed at 25°C. A syringe microburet was used to introduce the $HgCl_2$, and the rotating electrode was driven by a Sargent cone-drive motor. The potential of the electrode was maintained at −0.20 volt by means of a calomel cell in conjunction with a battery supply adjustable with a potentiometer. The titration itself was performed rapidly and was completed within 1 minute. Additional mercury is taken up over a longer period, but this is a non-reproducible quantity and is always associated with the precipitation of a brownish denatured hemoglobin. Titrations carried out in this manner give very sharp end-points and are reproducible with a precision better than 5 per cent. These titrations have also been performed on human carbon monoxide–hemoglobin solutions pretreated with *N*-ethylmaleimide (obtained from Schwarz Laboratories, Inc.). In the latter experiments carbon monoxide was bubbled through the titration vessel rather than helium.

We have also utilized the optical method of Alexander (1958) to measure the binding of *N*-ethylmaleimide by hemoglobin and by amino acids. The reaction between hemoglobin and *N*-ethylmaleimide can be followed at 300 mμ provided hemoglobin itself is used as a blank in the spectrophotometer.

The iron content of each sample of hemoglobin was determined according to the procedure of Thorp (1941).

RESULTS

The data for the oxygen equilibria are summarized in Table I. The relation between the logarithm of the oxygen pressure required for half-saturation of the hemoglobin (log p_{50}) and the pH is shown in Fig. 1. It is clear that the smaller the mammal the more sensitive is the oxygenation of its hemoglobin to pH. It is curious that all the oxygen equilibria except that of the mouse appear to be identical at pH 7.4. The magnitude of the Bohr effect, r, defined as $\frac{\Delta \log p_{50}}{\Delta \text{pH}}$ has been calculated numerically and plotted as a function of the body weight of the various mammals in Fig. 2. The figure shows that the size of the Bohr effect is inversely related to the body weight of the animal. Only horse and Chihuahua hemoglobins appear exceptional. The Bohr effect reflects the fact that protons are discharged from the hemoglobin when it is oxygenated. Wyman (1948) showed that the function, r, is equal to the number of protons dissociated for each oxygen molecule bound; $4r$ will be the total number of protons discharged since 4 oxygen molecules are bound. We can therefore conclude that the number of protons discharged per molecule of hemoglobin upon oxygenation is inversely related to the size of the animal.

TABLE I

OXYGEN EQUILIBRIUM DATA FOR VARIOUS MAMMALS

All measurements made at 38° C. in 0.1 M K_2HPO_4–KH_2PO_4.

n is the slope $\frac{\log \frac{y}{1-y}}{\log pO_2}$ at 50 per cent saturation; y is the fraction of hemoglobin combined with oxygen. The oxygen capacity is given in terms of microliters O_2 per ml. of the saturated solution.

Animal	Weight	pH	log p_{50}	n	Oxygen capacity	$\frac{\Delta \log p_{50}}{\Delta \text{ pH}}$ $(= r)$	$4r$
	kg.						
Mouse	0.03	7.03	1.540	2.86	66.8	0.96	3.84
		7.16	1.415	2.80	54.7		
Guinea pig	0.57	7.18	1.295	2.77	43.2	0.79	3.16
		7.44	1.090	2.69	43 (est.)		
Chihuahua	1.36	7.04	1.413	3.30	40.6	0.64	2.56
		7.36	1.190	2.90	36.8		
		7.80	0.935	2.71	30.2		
Rabbit	1.50	7.12	1.330	2.87	42.5	0.75	3.00
		7.32	1.165	2.79	53.5		
		7.68	0.920	2.60	39.3		
English setter	27.2	7.07	1.370	2.94	51.4	0.65	2.60
		7.34	1.175	2.85	47.5		
		7.73	0.950	2.53	52.7		
Man	62	7.26	1.225	3.04	35.0	0.62	2.48
		7.38	1.135	3.05	33.9		
		7.81	0.930	2.70	33.9		
Hog	102	7.06	1.335	2.94	45.5	0.57	2.28
		7.38	1.125	2.59	44.8		
		7.82	0.915	2.52	57.0		
Horse	544	7.12	1.348	3.10	76.0	0.68	2.72
		7.29	1.215	3.08	80.5		
		7.62	1.024	2.95	76.5		
Cattle	454	7.16	1.455	3.15	31.6	0.52	2.10
		7.50	1.315	2.75	15.2		
		7.82	1.110	2.85	30.3		
Elephant	3140	7.08	1.255	2.75	33.5	0.38	1.52
		7.38	1.150	2.65	34.0 (est.)		
		7.77	0.995	2.59	33.5 (est.)		

The mercuric chloride titrations, summarized in Table II, indicate that the smaller the mammal, the greater is the number of mercuric ions which can be *immediately* bound by its hemoglobin. Furthermore, a linear relation, shown in Fig. 3, exists between the size of the Bohr effect and the number of mercuric ions bound. We can extrapolate the line in Fig. 3 to the point at

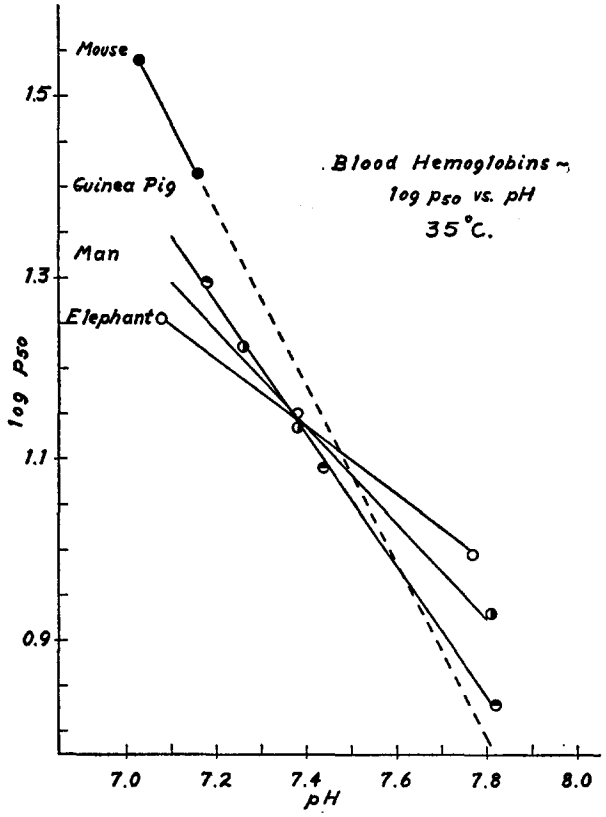

FIGURE 1. The variation of the log p_{50} value for various mammalian hemoglobins as a function of pH. The pressure units are millimeters of Hg.

which zero mercuric ions are bound. The value of r at this point is 0.10. This is the value of r for horse muscle hemoglobin (Theorell (1934)). It is known that horse muscle hemoglobin does not possess any —SH groups (Tristram, 1949). If the mercuric ions are binding —SH groups, then it appears that there is a direct relation between the number of —SH groups and the magnitude of the Bohr effect. A comparison of the total number of protons liberated during oxygenation ($4r$ in Table I) with the number of mercuric ions bound (Table II) indicates a quantitative correspondence which adds strength to the identification of the oxygenation-linked acid groups as —SH groups.

Horse and rabbit hemoglobins, however, apparently liberate more protons than the number of mercuric ions bound. The data for the other hemoglobins indicate a closer correspondence: the number of protons liberated is usually only slightly greater than the number of mercuric ions bound. Perhaps this small difference reflects the existence of weakly linked non-sulfhydryl groups such as that which exists in horse muscle hemoglobin. The number of protons liberated during oxygenation is compared with the number of mercuric ions

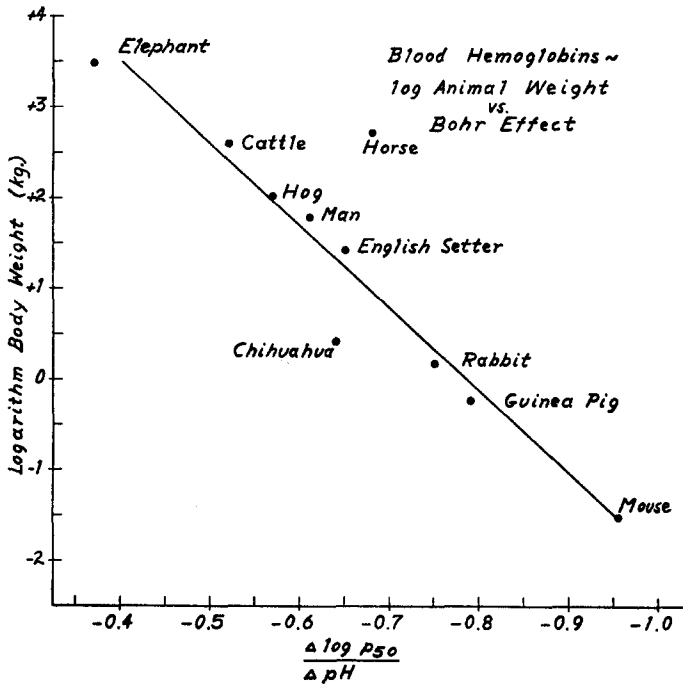

FIGURE 2. The relation between the logarithm of the body weight of various mammals and the size of the Bohr effect. The smaller the animal the larger is the sensitivity of its hemoglobin to pH.

which may be independently bound in Fig. 4 from the data of Tables I and II.

These experiments identify the primary oxygenation-linked groups in hemoglobin with those groups which bind mercuric ions. The assumption that we are dealing with —SH groups rests upon the specificity of the mercury titration. I have carried out the following experiments to test this assumption. Human hemoglobin binds about 2.35 Hg^{2+} ions per mole (Table II). The previous addition of just 2.4 moles of *N*-ethylmaleimide abolishes the mercury titration completely. The mercury titration is not affected by histidine. The presence of 0.1 M histidine in a solution of 10^{-3} M glutathione has no effect whatever on the mercury titration of the glutathione. I find that

0.71 Hg^{2+} ion is bound per mole of glutathione in the absence of histidine and 0.73 Hg^{2+} ion per mole of glutathione in the simultaneous presence of 10^{-3} M glutathione and 0.1 M histidine.

The method of Alexander (1958) has been used to examine the possibility that *N*-ethylmaleimide (NEM) might react with histidine. The absorption at 300 mμ of 10^{-3} M NEM is unaffected by 2 hours' incubation in the presence of 10^{-2} M histidine at pH 7 and 25°C. Under these conditions 10^{-3} M glu-

TABLE II

MERCURIC CHLORIDE TITRATIONS OF VARIOUS HEMOGLOBINS

1 ml. of hemoglobin solution is titrated in each measurement. The hemoglobin concentration is one-fourth the measured iron concentration.

Animal	10^{-3} M $HgCl_2$	Hemoglobin concentration	Hg^{2+}/hemoglobin
	ml.	*moles* × 10^3 *per ml.*	
Elephant	1.23	0.955	1.29
Cattle	2.05	1.065	1.93
Horse	1.95	1.125	1.73
Rabbit			
Animal I	1.60	0.815	1.96
Animal II	1.33	0.730	1.82
Man	1.20	0.510	2.35
English setter	3.20	1.230	2.60
Guinea pig	1.46	0.450	3.24
Mouse	2.67	0.730	3.66
Bullfrog adult			
Animal I	1.91	0.545	3.50
Animal II	1.93	0.400	4.83
Bullfrog tadpole	0.465	0.360	1.29

tathione reacts completely in less than a minute. We have determined the effect of NEM on the magnitude of the Bohr effect in human hemoglobin. Five moles of NEM lower the Bohr effect value from 0.65 to 0.28 at 35°C.

These data suggest that hemoglobins with small Bohr effects should have a small number of —SH groups. Since I had previously determined (Riggs, 1951) that bullfrog tadpole hemoglobin has almost no Bohr effect whereas the hemoglobin of the adult is very sensitive to pH, an opportunity existed to test the generality of this suggestion. I therefore performed mercury titrations and iron determinations on both bullfrog adult and tadpole hemo-

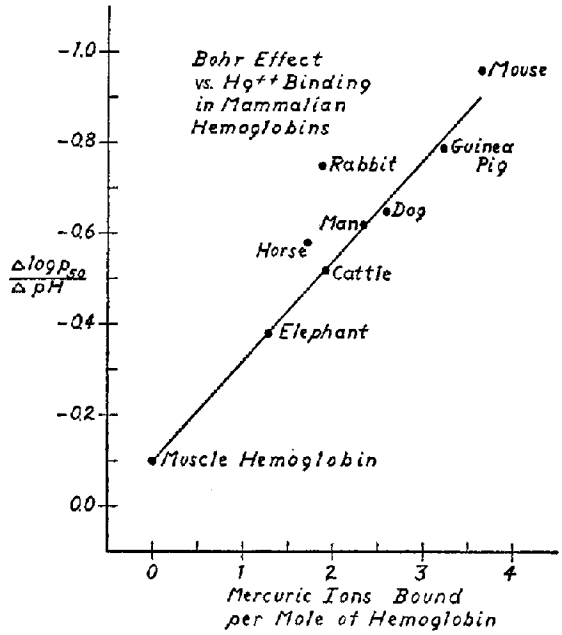

FIGURE 3. The size of the Bohr effect compared with the number of mercuric ions bound by reduced hemoglobin. The value of the Bohr effect of muscle hemoglobin is that of the horse from Theorell (1934).

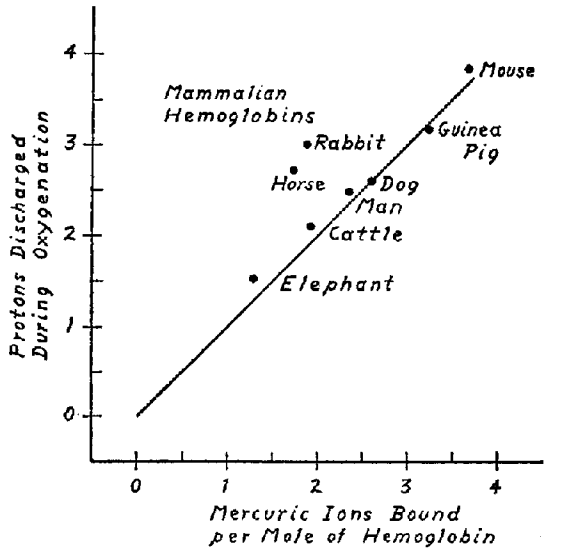

FIGURE 4. The relation between the number of protons discharged during the oxygenation of various hemoglobins and the number of mercuric ions which are bound by the reduced hemoglobin.

globins. These data, given in Table II, indicate that the metamorphosis of hemoglobin in the bullfrog involves a large increase in the number of —SH groups per molecule.

TABLE III

THE EFFECT OF CARBON DIOXIDE ON MAMMALIAN HEMOGLOBINS

0.1 M K_2HPO_4-KH_2PO_4 buffers, 35°C.; CO_2, when present, is at 40 mm. Hg.

Animal	pH	log p_{50} (+CO_2)	log p_{50} (−CO_2)	Δlog p_{50}
Mouse	7.22	1.490	1.405	+0.085
Guinea pig	7.26	1.270	1.236	+0.034
Rabbit	7.31	1.310	1.178	+0.132
Chihuahua	7.14	1.405	1.340	+0.065
English setter	7.08	1.400	1.353	+0.047
Human	7.20	1.290	1.247	+0.043
Hog	7.20	1.290	1.240	+0.050
Elephant	7.18	1.255	1.222	+0.033

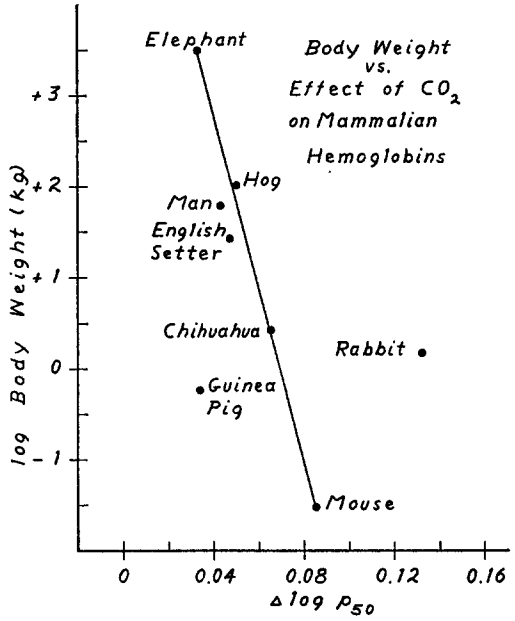

FIGURE 5. The effect of carbon dioxide on the oxygen equilibria of mammalian hemoglobins. With two exceptions, the smaller the animal the greater is the influence of CO_2, *at constant pH.*

Physiologically, the effect of CO_2 on the transport of oxygen by hemoglobin might be of greater significance than the effect of pH changes alone. We therefore examined the effect of CO_2 on the oxygen equilibria of the hemoglobins of eight species of mammal, being careful to keep the pH constant.

These experiments are summarized in Table III and in Fig. 5. These data suggest a relationship between body weight and the sensitivity of the hemoglobin to CO_2, although both guinea pig and rabbit hemoglobins appear to be exceptions. If this effect of CO_2 is determined by the actual binding of CO_2 to hemoglobin, then it would appear that, with two exceptions, the smaller the animal, the greater is the quantity of CO_2 which its hemoglobin can bind.

DISCUSSION

These experiments with hemoglobins identify a specific molecular adaptation. They show that the smaller the mammal the greater is the sensitivity of the oxygen equilibrium of its hemoglobin to pH, and the greater will be the promotion of gas exchange in the tissues and lungs. This graded adaptation

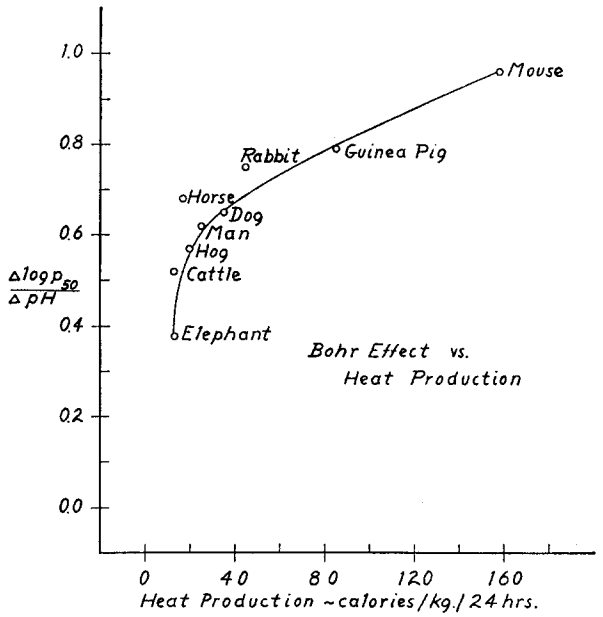

FIGURE 6. The relation between the size of the Bohr effect in various hemoglobins and the rate of heat production by the animals from which the molecules come. The heat production data are those of Benedict (1938).

is directly related to the metabolic rates of the animals from which the molecules come. This relationship is illustrated in Fig. 6 in which the heat production by various mammals is compared with the size of the Bohr effect. The heat production data are those of Benedict (1938).

In Fig. 7 the rates of oxygen consumption by various mammals (compiled by Spector *et al.*, 1956) are compared with the oxygen pressure (p_{50}) required to saturate 50 per cent of the hemoglobin or blood. In this figure our data on

hemoglobin solutions at pH 7.0 are compared with the recent data obtained by Schmidt-Nielsen and Larimer (1958) for the whole blood of various mammals. Our conditions differ from theirs in at least three ways: (*a*) the concentration of hemoglobin in our experiments is about 10 per cent of that existing within the intact red cells; (*b*) our hemoglobin solutions contained no carbon dioxide, while the whole blood preparations of Schmidt-Nielsen and Larimer

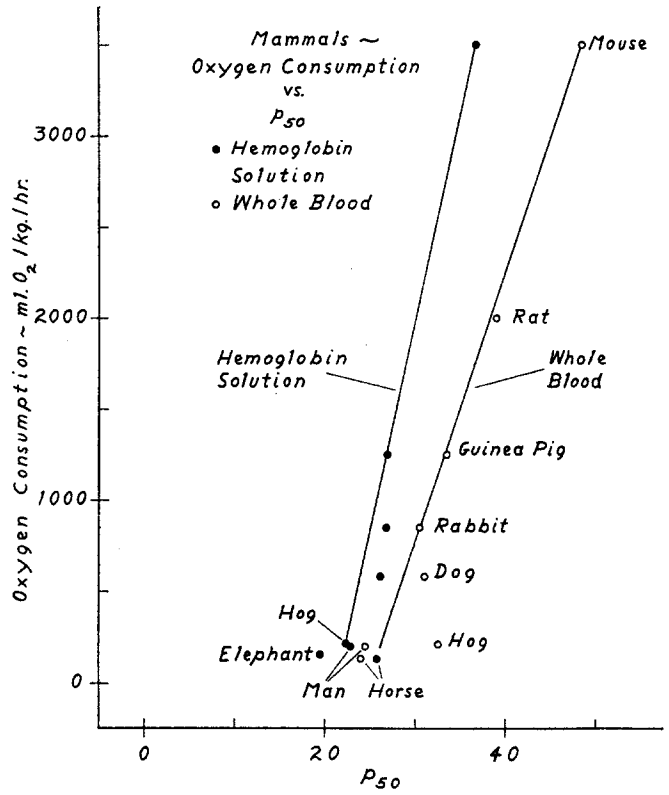

FIGURE 7. The relation between the rate of oxygen consumption by various animals as compiled by Spector *et al.* (1956), and the pressure of oxygen required to saturate 50 per cent of the hemoglobin or blood. The data for whole blood are those of Schmidt-Nielsen and Larimer (1958). The hemoglobin solution measurements were made at pH 7.0, 35°C.; those for whole blood were made at 37° under 40 mm. Hg p CO_2.

were equilibrated under 40 mm. Hg p CO_2; (*c*) our solutions were buffered and maintained at constant pH.

We have found that the presence of CO_2 depresses the oxygen affinity *even when measured at constant pH* (Table III, Fig. 5). Furthermore, this CO_2 effect—the original Bohr effect—is apparently also related to some extent to the size of the animal. Thus mouse hemoglobin is greatly affected by CO_2 whereas elephant hemoglobin is affected relatively slightly. This effect by

itself largely explains the differences between our data on hemoglobin solutions and those of Schmidt-Nielsen and Larimer on whole blood provided that we assume that the hemoglobins in red cells are behaving as if they were in a solution whose pH is about 7.0. The CO_2 effect suggests that the smaller the mammal the greater will be the amount of CO_2 actually bound to the hemoglobin. It is significant that Larimer (1959) finds that the smaller the mammal the greater is the content of carbonic anhydrase in the red cells.

The quantitative relation between the number of protons discharged during oxygenation and the number of mercuric ions which may be immediately bound during amperometric titration suggests strongly that the oxygenation-linked acid groups are sulfhydryl groups. These groups have been previously identified as imidazole groups by Wyman (1939, 1948) and Coryell and Pauling (1940) or as ammonium groups by Roughton (1943–44, 1949). However, these identifications were based on pK values and apparent heats of dissociation. Unfortunately, the pK values and heats of dissociation of these groups vary greatly with the electrostatic environments so that much overlap exists between their properties. A detailed discussion of these overlapping properties is given by Edsall and Wyman (1958), and by Barnard and Stein (1958). The justification for the assertion that we are here dealing with —SH groups rests upon the specificity of the amperometric mercury titration. If we were titrating imidazole groups, it would be difficult to understand why we obtain an extremely sharp end-point at 1.73 moles mercury per mole of horse hemoglobin when there are a total of about 36 imidazole and 38 lysine residues in the molecule (Tristram (1949)). Furthermore, Cole, Stein, and Moore (1958) have found that the chromatographic determination of cysteine as cysteic acid from human globin matches the determination by uptake of *N*-ethylmaleimide (NEM). Their data indicate a total of 4 to 6 —SH groups in human hemoglobin. Our data indicate that about 2.35 groups per mole of reduced hemoglobin are *immediately* available to mercuric ions. Our present experiments indicate that 2.4 moles of NEM per mole of human hemoglobin are just sufficient to abolish the mercury binding. Alexander (1958) has shown that the optical absorption of *N*-ethylmaleimide at 300 mμ decreases almost to zero when combination with —SH compounds occurs. He found that the assay of —SH compounds by this procedure is unaffected by the presence of any of 18 unspecified amino acids. We have found that the absorption at 300 mμ of NEM is completely unaffected by a large excess of histidine. Furthermore, our titrations are not affected by a 100-fold excess of histidine. If the irreversible reagent, NEM, binds the oxygenation-linked acid groups one would expect that it would also either destroy or greatly reduce the magnitude of the Bohr effect. The present experiments indicate that this is so.

Our finding that the number of cysteine residues in the hemoglobin mole-

cule is related to the size of the animal is supported by the analytical data of Birkofer and Taurins (1940), which clearly indicate an inverse relation between cysteine-cystine content and body weight in seven species of mammal, ranging in size from jackal to cattle.

What is the relation between the number of Hg^{2+} ions bound and the number of —SH groups present? Ingram (1955) has suggested that one Hg^{2+} or one organic mercurial ion will be bound between a pair of —SH groups in each half-molecule of the native protein. The conclusion was based on his finding that horse hemoglobin would bind 4 Ag^{+} ions, but only two *p*-chloromercuribenzoate or Hg^{2+} ions. The simplest interpretation of our data would indicate a 1:1 relation between —SH and Hg^{2+} ions in the native protein. It is reasonable to assume that a 1:1 correspondence exists between the number of protons discharged during oxygenation and the number of acidic groups involved. We have obtained an approximate 1:1 relation between protons discharged during oxygenation and the number of Hg^{2+} ions bound. This correlation suggests that a 1:1 relation exists between —SH groups and Hg^{2+} ions. This conclusion agrees with that of Allison and Cecil (1958) who have suggested that Ingram's results could better be explained by assuming that silver ions may bind other groups in addition to —SH groups. Rabbit and horse hemoglobins appear exceptional: the number of protons discharged during oxygenation is greater than the number of mercuric ions which the molecules may bind. This difference might be due to some —S—Hg—S— linkages, to a steric hindrance to the binding of mercury, to the existence of some oxygenation-linked groups which are not sulfhydryl, or to a combination of these factors.

Allen, Guthe, and Wyman (1950) concluded that the hemes are functionally indistinguishable. Our data suggest that the hemes in each molecule must, in general, be functionally different from one another. If the oxygenation of all four hemes of elephant hemoglobin involves the discharge of just over one proton, it appears most improbable that the hemes are all indistinguishable.

None of our data indicates an integral number of —SH groups per molecule. Three explanations for this deviation from integral numbers may be suggested: we might assume a heterogeneous population of molecules; we might suppose that the non-integral number merely reflects an equilibrium in which not quite all the —SH groups are binding Hg^{2+}; or we could suppose some Hg^{2+} ions bind groups other than —SH to a small extent. The number of —SH groups in human hemoglobin as determined here is less than the total number determined analytically by Cole, Stein, and Moore (1958). This presumably means that there are cysteine residues in positions which are not influenced by oxygenation, and are not readily bound by mercuric ions unless the structure of hemoglobin is altered by denaturing agents.

The hemes in each molecule of mammalian hemoglobins interact with

one another in such a way that the oxygenation of one heme greatly increases the oxygen affinity of some of the other hemes. The oxygen equilibria for all the mammals studied, from elephant to mouse, appear to have identical or almost identical patterns of heme-heme interaction. That is, the value of n, an empirical measure of interaction, does not vary significantly among the mammalian hemoglobins studied. Since the number of immediately available —SH groups varies from about 1.3 in elephant hemoglobin to almost 3.7 in mouse hemoglobin, a mechanism of heme-heme interaction which *requires* —SH groups cannot be correct. Bullfrog tadpole hemoglobin has the same heme-heme interaction as does either bullfrog adult hemoglobin or mammalian hemoglobins (Riggs (1951)), yet it possesses a very small number of —SH groups.

Ingram (1957) has produced evidence which indicates that human sickle cell hemoglobin (Hb-S) differs from normal human hemoglobin (Hb-A) by the replacement of a single residue of glutamic acid by valine. This abnormal hemoglobin appears to be due to a single gene. We must assume that other amino acid positions may likewise mutate, perhaps with varying frequencies. We suggest that the primitive mammalian hemoglobin might have possessed either a small number of functional —SH groups or else none at all. It has been suggested that hemoglobins might have evolved from cytochrome oxidase (Barcroft (1928); Wald and Allen (1957)). It may be significant that Wald and Allen's measurement of the carbon monoxide equilibrium of cytochrome oxidase indicates complete lack of a Bohr effect. If an organism has such a physiology and lives in such an environment that a large Bohr effect would be advantageous, any mutation which would increase the magnitude of the Bohr effect would be selected. A mutation resulting in an increase in the number of appropriately placed cysteine residues would be selected by an animal with a relatively high metabolic rate. The fact that bullfrog tadpole hemoglobin has very few —SH groups while that of the adult has a large number, indicates that this mechanism of molecular adaptation is not confined to mammals and may be widespread.

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