RELATION OF HEMOGLOBIN TO THE RED CELL MEMBRANE * †

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(Submitted for publication June 29, 1959; accepted July 31, 1959)

Despite the accumulation of biochemical and physiological knowledge of hemoglobin, little is certain about its physical state in the native red blood cell. Perutz and Mitchison (1) indicate that the pigment is almost surely not in the crystalline state except for sickle-hemoglobin in the reduced form. A separate aspect of the problem is the relation of hemoglobin to the cell membrane. The prevailing view is that of the respiratory physiologists who hold that the exchange of blood gases involves no chemical event in the membrane (2). Accordingly, hemoglobin is excluded from it. Drabkin has estimated that not more than about 2 per cent of the total pigment could be "anchored at the surface of the red cell" (3), while Hoffman has recently concluded emphatically that the notion that there may be structurally fixed hemoglobin must be abandoned (4). Yet, many investigators have been struck by the difficulty encountered in trying to free red cell stromata entirely from hemoglobin. For example, Zittle, Della Monica and Custer, in preparing red cell acetylcholinesterase, noted the stromata contained "considerable hemoglobin in spite of the long period of washing" (5). Ponder, in a recent review (6), favors the idea that some of the pigment is associated with the membrane. His view is based in part on microscopic observations that could bear other interpretations and afford no quantitative data, and in part on the finding that "ghosts" may contain a greater concentration of hemoglobin than the surrounding lytic solution, "provided that the amount of water used for hemolyzing the cells is relatively small." Unfortunately, with only small amounts of water lysis is likely to be incomplete, and it is accordingly improbable that there is free diffusion of pigment between cells and medium. Unless the condition of free diffusion is satisfied, differences of concentration of intracellular and extracellular hemoglobin cannot be regarded as evidence of binding between hemoglobin and stroma rather than as a reflection of membrane phenomena such as permeability.

Two main difficulties have stood in the way of approaching the issue more directly. First, there has not been available a satisfactory method of measuring amounts of hemoglobin in membranes directly, on account of their insolubility. A few investigators have measured iron, but the amounts found have been variable and inconstant and, furthermore, not certainly associated with hemoglobin itself. Secondly, although various ways of preparing ghosts have been devised, yet it is rarely clear to what extent the processing may have resulted in loss of stromal substance. Thus, it has not been common practice to measure any characteristic membrane constituent, *e.g.,* phospholipid, to see whether the quantity found approximates that of the native cell. The production of "hemoglobin-free ghosts" affords no warranty as to the degree of preservation of any of the natural components of the intact membrane and, indeed, microscopists have come to speak of "attenuated" or "thin" ghosts (6). Experiments of Lovelock illustrate the delicate constitution and fragile structure of red cells, for even repeated washings with saline at 37° C. may result in appreciable losses of lipid and protein although hemolysis is negligible (7).

For estimating residual hemoglobin attached to membranes, a simple direct method has recently been devised which involves dissolving weighed amounts of lyophilized membranes in 40 per cent urea and measuring photometrically the concentration of cyanmethemoglobin (8). It could then be shown for one way of processing cells that substantial and rather constant amounts of hemo-

* This work has been supported by a grant from the United States Public Health Service (RG-5763-C), and a grant from the Avalon Foundation.

† Presented in part at the meeting of the Association of American Physicians, Atlantic City, May 6, 1959.

globin are demonstrable in membranes even after thoroughgoing lysis and repeated washing with water. At the same time reproducible and regular quantitites of nonhemoglobin nitrogen and of lipid phosphorus were found and it was possible to describe the preparation of red cell membranes in terms of quantitative chemical analyses (8). The present work represents an amplification and extension of the observations. The applicability of the methods to cells of animals has been examined and various methods of preparing membranes compared. It will be shown that it is extremely difficult to reduce the amount of hemoglobin attached to membranes beyond a certain minimum without at the same time removing either lipid phosphorus or nonhemoglobin nitrogen or both. The data support the idea that in the native cell about 3 per cent of the total blood pigment is bound to the membrane. Since the term "ghost" has come to be applied to almost any sort of preparation of red cells which have been somehow partially hemolyzed, it seems best for present purposes to avoid its use and to speak definitively of "membrane," meaning the insoluble phase of the cell as separated *in vitro*.

METHODS

In an extension of studies of species differences in erythrocyte phosphatides (9), variations in yields of phosphorus were traced to variations in the anticoagulant used, and a method of preparing membranes was therefore developed which overcame this difficulty. In this method, called here for convenience "standard," blood is taken into acid citrate dextrose (ACD), known from blood bank experience to be an excellent preservative. (ACD = 0.4 per cent citric acid, 1.32 per cent sodium citrate, 1.47 per cent dextrose; one part to four parts whole blood.) This and all other solutions were brought to 4° C. before use. Centrifugations were carried out in the high capacity-angle head of the International Refrigerated Centrifuge, Model PR2, at 4° C. and 10,000 rpm (11,000 × G). Samples of human adult blood were fresh or banked as noted. Human fetal blood and the specimens of animal blood were not more than a few hours old when processed. After separation of plasma, the cells were washed three times in cold saline to remove the buffy coat of white cells as well as the remaining plasma and also to bring them to 4° C. before further processing. Lysis was then carried out by repeated exposure to 10 volumes of cold water, the insoluble residue each time being deposited by high speed centrifugation. Other methods of preparation have been examined and the materials obtained have been compared in terms of certain chemical and morphological data. Measurements of lipid phosphorus in the intact red cells were made by extraction into chloroform-methanol and washing with large volumes of water, according to the procedure devised by Folch and co-workers for wet tissues (10).

The harvests of wet membranes, suspended in a minimum of fluid, were lyophilized, and portions then analyzed for nitrogen and lipid phosphorus as previously described (9). That part of dry weight representing hemoglobin was estimated by dissolving 10.0 mg. of lyophilized membranes in 10.0 ml. 40 per cent urea containing standard cyanide-ferricyanide [0.1 per cent $NaHCO_3$, 0.005 per cent KCN, 0.02 per cent K_3 Fe $(CN)_6$] and measuring the concentration of cyanmethemoglobin in a photometer. With occasional shaking a clear solution was usually obtained in an hour or two. Persistent turbidity was encountered rarely, and then only when the method of preparation under trial had involved exposure of the membranes to materials of a kind that might well have produced considerable denaturation of protein. In 20 per cent urea, solution took place more slowly; the results were otherwise no different. It has been shown that strong solutions of urea will not interfere with the absorption spectra of hemoglobin and its derivatives (11). However, it became necessary to look for a spectral disturbance resulting from prior lyophilization. A fresh solution of human hemoglobin was prepared from the supernatant after lysis of cells with water and an aliquot was lyophilized. Using the Beckman DU spectrophotometer, absorption spectra were then plotted for the following: *a*) cyanmethemoglobin standard, *b*) lyophilized hemoglobin dissolved in 40 per cent urea containing cyanide-ferricyanide, *c*) original watery predecessor of *b* after conversion to cyanmethemoglobin, *d*) lyophilized membranes dissolved in urea-cyanide-ferricyanide. The curves obtained were all superimposable and characteristic of cyanmethemoglobin. It appeared, therefore, that lyophilization did not interfere with conversion to cyanmethemoglobin, and further, that the method devised for measuring hemoglobin in membranes was a valid one. Nonhemoglobin nitrogen was estimated as the difference between total nitrogen and hemoglobin nitrogen, calculated as 16 per cent of the pigment.

RESULTS

With the standard method of preparing membranes by repeated exposure to 10 volumes of water at 4° C., the supernatant was usually virtually free of pigment after four times, and the membranes deposited were distinctly reddish. The number of applications of water was increased in many experiments, but further loss of hemoglobin from the membranes was negligible. For instance, in one experiment, a portion of the membranes was washed 12 times in water and during both the eleventh and twelfth washings allowed to stand in the water overnight. The amount of hemo-

TABLE I

Average composition of lyophilized standard membranes prepared after repeated lysis in water at 4° C.

Sources of cells	Hemoglobin	Total nitrogen	Phosphatide*
	mg./100 mg. dry weight		
Human adult (24)†	54	13	10
Human fetal (4)	58	14	11
Rabbit (1)	58	13	15
Guinea pig (1)	56	14	14
Ox (2)	48	11	16

* Calculated as 25 × lipid phosphorus.

† Figures in parentheses represent numbers of individuals. The total yield of dry material was generally just under 2 per cent of the wet weight. One ml. packed cells yielded about 18 mg. dry weight. Standard deviation for human adult = 3 per cent.

globin in all of the washings and in the membranes was measured and it was found that the further loss of "attached" hemoglobin after the fourth wash was only about 5 per cent. The routine was therefore adopted of washing four times with water or, if then the supernatant was still pink, once or twice more. Membranes prepared in this way occupied a packed wet volume about 50 to 60 per cent of that of the intact cells. When examined with the ordinary light microscope, they appeared as disks. This impression was confirmed by phase contrast microscopy (Figure 1). Furthermore, the degree of completion of lysis could be appreciated microscopically, since intact red cells are much more densely pigmented than those that have been lysed. Only very few or no intact red cells were observed after the standard method of lysis. The membrane composition is given in Table I. It can be seen that the amount of hemoglobin found is in the neighborhood of 50 per cent of the total dry weight. The range of values extended from 48 to 58 per cent. This corresponds to about 3 per cent of the total hemoglobin of the intact cell. With human blood banked for one to four weeks, the figure was somewhat lower, usually just below 50 per cent. Included among the human adults were cases of acute and chronic leukemia, idiopathic thrombocytopenic purpura, acquired hemolytic anemia, as well as nonhematological disorders such as cardiac failure. The amounts of hemoglobin found did not vary significantly with anemia or reticulocytosis.

Since the amount of phospholipid nitrogen is so small that it can be neglected, the total nitrogen

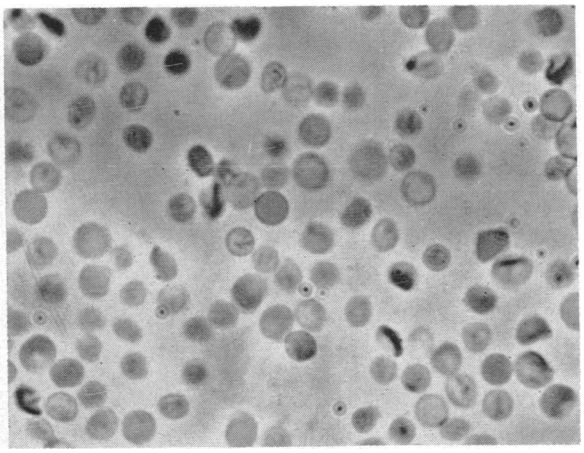

FIG. 1. PHASE CONTRAST PHOTOMICROGRAPH OF "STANDARD" MEMBRANES SUSPENDED IN WATER

Varying degrees of lightness and darkness are determined by optical properties such as depth, and do not signify variations in content of hemoglobin.

may be assumed to represent in effect the contributions of hemoglobin and nonhemoglobin peptide or protein ("stromatin"). The latter, then appears to make up about half as much of the dry weight as does hemoglobin.

The amounts of lipid phosphorus recovered varied somewhat with the species of animal. For human cells, several experiments were carried out to compare the amounts of lipid phosphorus in standard membranes to the amounts in their native wet precursors. Fresh wet red cells subjected to extraction with chloroform-methanol [Folch and associates (10)] yielded 65 to 70 μg. lipid phosphorus per ml. Equivalent amounts of the same samples were processed in the standard way and lyophilized, and the yield of lipid phosphorus found to be 58 to 66 μg. per ml. wet. The loss of phospholipid was thus not more than 10 per cent. With blood that had been banked, losses of phosphorus during processing were occasionally greater, once as much as 30 per cent. It appeared, therefore, that the method employed conserved most of the phospholipid and at the same time yielded regular amounts of packed, wet membranes which had a fairly uniform microscopic appearance as intact disks with little fragmentation, and which gave reproducible analytical values for nitrogen, phosphorus and hemoglobin. It now became of interest to see how these results might be influenced by alteration of the experimental conditions.

TABLE II

Average yield and composition of membranes: Anticoagulants ACD and oxalate compared*

	Lipid phosphorus		
	Native cells	Membranes	Total solids
	μg./ml. wet native cells		*mg./ml. wet native cells*
ACD	65	66	18
Oxalate	70	45	6

* Acid citrate dextrose.

In the first step of the processing, *i.e.*, prevention of coagulation, the use of 1.4 per cent potassium oxalate instead of ACD resulted in a considerable diminution of membrane substance during the subsequent lysis. This is illustrated in Table II, where it can be seen that much of the membrane solids, including phospholipids, was lost when oxalated blood was processed. Also, on microscopic examination, fragmentation of the membranes into fine particles was seen after the third application of water. A systematic study of other anticoagulants was not made, nor were efforts directed toward finding substitutes for ACD. A few trials of heparin and of defibrination revealed membrane injury like that observed with oxalate, since early fragmentation was observed microscopically when lysis was undertaken.

The use of ACD appeared to be a much more important factor in obtaining reproducible results than did the control of temperature, at least for the range 4° to 18° C. However, it was observed that bovine cells treated at 18° C. rapidly became brownish in color, probably from methemoglobin formation, and on this account as well as on the general desirability of maintaining low temperature in handling proteins, all processing was carried out routinely in the cold.

Attention was next directed to the effect of varying the composition of the fluid used for lysis of blood drawn into ACD. Instead of water, hypotonic solutions at acid, alkaline and neutral pH were employed. At pH > 8, lysis was associated with micrscopic fragmentation or thinning of membranes and grossly apparent loss of substance, as for instance with 0.015 M K_2HPO_4, pH 8.5, and the hemoglobin content was reduced to the neighborhood of 25 per cent of dry weight. On the other hand, with 0.015 M KH_2PO_4 or NaH_2PO_4, pH 4.5 to 4.6, membranes were obtained that closely resembled the "standard" ones in appearance and chemical composition. Because of Parpart's report of having obtained hemoglobin-free membranes with carbonic acid (12), particular attention was given to the effect of this substance. When lysis was carried out with water saturated with CO_2, pH 3.8 to 4.0, floating particles (lipid?) were encountered after the third or fourth application. At this time also most of the sedimented residue appeared amorphous and fibrous microscopically, though it still contained some 25 per cent hemoglobin as dry weight. Entirely similar results were obtained with water half-saturated and quarter-saturated with CO_2 and with 0.01 M citric acid, pH 2.7, and citrate-phosphate, pH 4.2. It was concluded that the optimum pH range for processing membranes was somewhere in the range 4.5 to 8.5 and that the use of carbonic acid had best be avoided.

A comparison was made of lysis of cells by water, 0.1 per cent NaCl, 0.3 per cent NaCl, and by 1, 2.5 and 5 per cent glycerol in water. All of these agents produced microscopically intact membranes giving a similar appearance and yielding comparable quantities of total dry weight solids; the amounts of hemoglobin as dry weight found were: for water 58 per cent; for 0.1 per cent NaCl, 45 per cent; for 0.3 per cent NaCl, 45 per cent; all glycerol solutions, 40 per cent. It seemed probable, therefore, that a number of solutes might be found which in hypotonic solution at neutral pH could serve much like water for lysis of cells and preparation of membranes. However, when in the course of lysis a change was made in the lytic agent, then loss of membrane substance was often seen. This is illustrated in Table III. Here a method of preparing membranes with progressively decreasing concentrations of NaCl, sometimes referred to as "osmotic shock" (13), is examined. It may be noted that the treatment resulted in a very considerable loss of total substance, representing hemoglobin, nonhemoglobin protein, as well as phospholipid. It was concluded that although certain hypotonic solutions, if used alone, might perhaps be substituted for water as lytic agents, yet a shift from one of these to another during the processing introduces the possibility of denaturation and fragmentation. In any case, the phenomenon of lysis by water or by hypotonic solutions probably involves forces which

TABLE III

Yield and composition of membranes: Water and decreasing NaCl concentrations compared as lytic agents

Lytic agents	Recovered in membranes/ml. wet native cells			
	Total solids	Hemoglobin	Non-hemoglobin peptide	Phosphorus
	mg.	*mg.*	*mg.*	*μg.*
A. Water, 4 washes	18	9	5	70
B. NaCl, 3 washes *1.* 0.45%; *2.* 0.23% *3.* 0.11%	7	3	3	50
C. B, then water	5	2	1	35

are obscured in descriptive terms such as "osmotic shock" or "osmotic fragility." It is by no means clear, for instance, to what extent the explosive hemolysis effected by water is determined simply by diffusion.

From the observations detailed above it seemed likely that a small amount of blood pigment is so constantly found in association with the red cell membrane that it might properly be regarded as a membrane constituent. However, among other objections to this idea, it could be said that the hemoglobin is merely trapped within a membrane that has become impermeable to it by, say, denaturation. To meet this argument, fresh preparations of "standard" wet membranes in water were homogenized for 15 minutes in a Virtis homogenizer at 45,000 rpm. This resulted in a very fine fragmentation; few intact membranes could be seen microscopically. The residues were now washed further with 10 volumes of water, in the usual way, three times more. After lyophilization the hemoglobin content was found to be 43 per cent. This figure is, to be sure, lower than the mean (54 per cent), yet considerable enough to refute the idea that trapping is the principal factor accounting for the association of hemoglobin with the membrane.

Standard wet membranes suspended in water exhibited the absorption bands of oxyhemoglobin when examined with the Hartridge reversion spectroscope. It was of interest to see whether conversion of the pigment, at an early stage of the processing, to the derivatives, reduced hemoglobin, carboxyhemoglobin and methemoglobin, might affect the amounts found attached to the membranes. Membranes were prepared *a*) under N_2 with equilibration by bubbling, *b*) after equilibration with CO, *c*) after treatment with nitrite. The amounts of hemoglobin recovered from the membranes as dry weight were: 52 per cent for *a*; 47 per cent for *b*; 43 per cent for *c*; and 49 per cent for control (oxyhemoglobin). It appeared, therefore, that the state of oxidation or oxygenation of hemoglobin did not materially affect its attachment to the membrane.

DISCUSSION

Among the factors that may influence or alter the structure of membranes obtained by any method of hemolysis are: species of animal, kind of anticoagulant, ionic composition of lytic agent, pH, and temperature. There is perhaps nothing astonishing here, since the membrane contains proteins as well as complexes about which little is known, and any of them could be easily denatured. Nonetheless, for those concerned with the chemistry or metabolic behavior or ultrastructure of membranes, it is of importance to delineate as specifically as possible the variables which may condition the composition of the object of study. While it is impossible to define the native membrane properly, yet some knowledge, however imperfect, of its chemistry and architecture can be obtained. But if the results of electron microscopy are to be compared with, say, those of chemical analysis, the methods of preparation had best be uniform. At the same time it is entirely probable that any given method of obtaining membranes, including the one called here, for convenience, "standard," may denature or remove certain constituents. For some purposes, therefore, lysis with hypotonic salt or carbohydrate could well prove superior. The method described above was developed for the principal end of analyzing phosphatides and its further utility remains to be determined. It would be of interest, for example, to know what the enzymatic and metabolic activities of "standard" membranes may be.

The mere association of blood pigment with membrane does not, of course, constitute convincing evidence that the two are somehow bound, and indeed most investigators have regarded the phenomenon as an artifact. On the other hand, the amounts of hemoglobin that have been found during this investigation have been so considerable

(about 50 per cent of the total dry weight) and so regular (range, 48 to 58 per cent) as to cast doubt on the popular idea that the hemoglobin is nothing more than a contaminant. Admittedly, the view that hemoglobin is indeed a constituent of the membrane must be tested further, particularly with attempts to find out more about the nature of the binding, perhaps by isolation and definition of a hemoglobin complex such as a lipoprotein. It may be pointed out that firm binding of a well-characterized soluble protein to the erythrocyte membrane is already known. Acetylcholinesterase travels with the stroma and can be extracted therefrom by non-ionic detergents. This has led to the suggestion that the link between enzyme and membrane may be lipid (5). Moreover, Chargaff, Ziff and Hogg have shown that some phosphatides may react with oxyhemoglobin at neutral pH and even destroy its characteristic spectrum (14). However, since little is known about the forces uniting peptide to lipid in lipoprotein complexes and since some may be dissipated even by removal of water (15), the problem posed by the relation of hemoglobin to the membrane is not likely to be settled easily. The instability of certain lipoprotein complexes offers a possible explanation of so-called "hemoglobin-free ghosts," if hemoglobin is a constituent of the membrane. In any case, however, "hemoglobin-free ghosts" have not usually been analyzed chemically. A well know method of obtaining membranes, by lysis with carbonic acid, is that of Parpart (12), whose preparations contained < 0.01 per cent iron. The species of animal used in the studies is not given, nor are the data that are otherwise supplied recorded in enough detail to permit a judgment as to the degree of resemblance of the final product to the native membrane. With the various methods employed in the present investigation, including that of lysis with carbonic acid, hemoglobin was never lost alone.

It is worth noting that the regular amount of hemoglobin found to be attached to the membrane (3 per cent of the total in the cell) is in the neighborhood of the amount (2 per cent) calculated by Drabkin as unlikely, yet permissible, on the basis of his assumptions as to cell surface and molecular size of hemoglobin (3). But if indeed hemoglobin is a constituent of the membrane, it is at present a matter of speculation as to how it may be laid down and whether it is in equilibrium with the bulk of the cellular pigment.

The problem of the fixation of hemoglobin is only a part of the more general one of membrane constitution. Despite the obvious difficulties, recent development of methods for the analysis of all types of lipids offers promise of progress. For instance, it has been shown that lecithin may be lacking from the red cell membranes of the true ruminants (9) and that the lipid complexes, presumably lipoprotein in nature, of the curious oval red cells of the camel are peculiarly resistant to the disruptive action of alcohol (16). The data at hand are still meager, yet it can scarcely be doubted that any information about the complex structure of a given membrane would be of advantage for physiological studies such as those of ion distribution and permeability.

SUMMARY

The problem of the relation of hemoglobin to the red cell membrane is reviewed. A method is described for preparing membranes and it is shown that they contain reproducible amounts of hemoglobin, nitrogen, phosphorus and total solids. Denaturation and fragmentation of membranes occur readily with variation of the experimental conditions in any stage of the processing and the anticoagulant used is of particular importance. A simple direct method for measuring hemoglobin attached to the insoluble membranes is described. The amount of hemoglobin found with the membrane is so regular and so large ($\pm$ 50 per cent of dry weight) as to indicate that the two are somehow bound.

ACKNOWLEDGMENT

The authors appreciate the technical assistance of Mrs. Judy Chang, and are indebted to Mrs. Helena H. Benitez of the Laboratory for Cell Physiology, Department of Surgery, for expert help with phase contrast microscopy.

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