APPENDIX I

1957 Report on "Method for Preparing Artificial Hemoglobin Corpuscles"

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(Figures scanned from original report with new fonts added for clarity — text reformatted but with no change from original, except errors in spelling, grammar and typing.)

The red corpuscle, once it has passed the reticulocyte stage, is almost without metabolic activity and is little more than a hemoglobin containing shell[1]. This pointed towards the possibility that perhaps an artificially produced hemoglobin corpuscle may be used as a physiologically functioning red blood cell. With this possibility in mind, first, a survey of the literature concerning erythrocytes, hemoglobin and possible artificial membranes was made; then possible methods of getting hemoglobin into corpuscles of microscopic sizes were tried.

ERYTHROCYTES

1. Structure

The normal human erythrocyte is a biconcave disc, with an average diameter in dried films of 7.2 μ. The thickness of cell is l μ at the centre and 2.4 μ near the edge. Although the cell membrane is not elastic, the cell can become distorted in order to pass through narrow capillaries,

also, the concave faces of the corpuscle can be distended to a convex shape when the cell content increases as in the case of hypotonic environment. The membrane is of lipoprotein, and when examined with electromicroscope appears to be finely granular without folds. The mean corpuscular volume of the cell has an average of 86 cubic μ.

2. Function

Hemoglobin of the erythrocyte carries out the functions of transportation of O_2 to the tissues and that of transportation of CO_2 from the tissues to the lung to be excreted. This respiratory function of the hemoglobin of the erythrocyte is the most important function. Hemoglobin and electrolytes inside cell also act as a buffering system to maintain the normal pH of 7.3–7.4. 60% of buffering action of whole blood is due to red cell, the other 40% to plasma. Carbonic anhydrase is highly concentrated in erythrocytes. This is important since the reaction $CO_2 + H_2O = H_2CO_3$ is speeded up 2500 times, resulting in the transportation of CO_2 to the lung to be excreted.

3. Enzymes and inclusion bodies

Besides carbonic anhydrase, metabolic enzymes, dehydrogenases and pyridine nucleotides; there are various other enzymes whose functions are at the presence obscure. Also there are inclusion bodies in circulating erythrocytes: Punctate basophilia, Cabot rings, Howell-Jolly bodies, siderocyte, Heinz bodies. Quite a number of these are associated with diseases, especially the Howell-Jolly bodies which are associated with severe anemia of both the iron deficiency and the pernicious type, and with leukemia, and are particularly common after splenectomy. This is mentioned, because in an artificially produced hemoglobin corpuscle it is possible to include in the cell content all sorts of combinations of enzyme systems and inclusion bodies. This way, the function and activity of the various enzymes and inclusion bodies in the erythrocyte may be studied.

HEMOGLOBIN

1. Molecular structure

Hemoglobin is the red coloring matter of the red blood cell. It is a conjugated protein, consisting of a protein part called globin and an iron-pyrrol compound known as hematin or heme[5]. Work by Svodberg, Adair and others indicates that hemoglobin has a molecular weight of 66,800 and that the molecule of hemoglobin contains four molecules of hematin. The hemoglobin molecule is 64 Å in length, 48 Å in width and 36 Å in thickness.

2. Hemoglobin content of the blood

Hemoglobin is held within the stroma of the erythrocyte. The normal hemoglobin content of the blood is usually measured colorimetrically depending upon the oxygen combining capacity or the blood. The present assessment of normality is between 15 and l6 gm per l00 ml for males and with a lower value for females. Knowing the hemoglobin content of the blood the mean corpuscular hemoglobin (M.C.H.) and the mean corpuscular hemoglobin concentration (M.C.H.C.) can be calculated from the following formulae:

M.C.H. = (Hb in gms/1000 ml blood)/Red cells in millions per mm^3.

This is expressed in μu and the normal value for male being 29 μu (with a range or 27 to 32 μu) M.C.H.C. = (Hb in gms per 100ml blood × 100)/(VOL in ml packed cells per l00ml blood) This normal value is about 33% with a range of 32–38% and shows no variation with sex.

3. Methemoglobin

The ferrous iron in hemoglobin is readily oxidized by oxidizing agents to the ferric state, which is methemoglobin. Methemoglobin is brown in solution and has an absorption band with a maximum at 634 mu. In normal human blood it is approximately 2% of the total Hb. Now, since methemoglobin does not have the power to combine with O_2, it is important in the preparation of hemoglobin corpuscles to preserve

the hemoglobin in the ferrous state. There are various enzyme systems in the red blood cell for the purpose of reduction of methemoglobin to hemoglobin. These are: triosephosphate and lactate dehydrogenating system[3], dehydrogenases and pyridine nucleotides. Other ways of reducing methemoglobin to hemoglobin are by the action of sodium sulphide, amyl nitrite in phosphate-saline solution, $Na_2S_20_4$, ascorbic acid, or alkaline solution prepared from $TiCl_3$. All these will readily convert methemoglobin to hemoglobin which does combine with O_2 and CO_2.

4. Derivatives of hemoglobin

In the handling of hemoglobin during the preparation of the artificial hemoglobin corpuscles, it is important to be able to tell whether the hemoglobin inside the membrane is still hemoglobin, or if not, it is helpful to be able to know what substance it is changed into. The derivatives of hemoglobin may be OHb, methemoglobin, CO-hemoglobin, nitric oxide hemoglobin, hemochromogen, hematin, alkali-hematin, and hematoporphyrin. Each of these possesses a specific absorption spectrum which serves as an aid in their detection[2].

MEMBRANE

1. Reason for using collodion membrane

After much searching around, collodion is chosen for the membrane, since it has been studied quite extensively by a number of physiologists.[6,7,9,l0,11,12] It is also being widely used by bacteriologists as membranes for ultrafiltration[8]. It is used despite the fact that being a plastic material, it cannot be used as the membrane of cells to be perfused into the circulation since once injected, it will remain as such in the circulation. However, since the problem of finding a suitable membrane would already be a project in itself and since the amount of time available for this project is limited, collodion was used as the membrane. Of course, a lipoprotein membrane would be most suitable.

2. Studies made on collodion membrane

Much can be made used of the information obtained by the physiologists and bacteriologists concerning the permeability of collodion to various molecules and methods of preparing collodion membrane with any desired pore size. In 1907, Bechhold discovered that porous membranes of collodion could be precipitated by water from non-aqeous solution. His first paper (Bechhold H. Z. Physik Chem 60,257,1907; 64,328,1908) is still among the best on this subject, here he sets forth many principles and applications. Elford[8] classified colloidal membranes into two main types:

1. Microgel: It is an acetic collodion membrane which has relatively course structure with pores of microscopic order.
2. Ultragel: Which is ether alcohol collodion membranes. The structure or this type or membrane is not resolved by microscopic apparatus. Ultaviolet light microphotography show that it is built up of particulate matter.

He also found that dilution of the ether alcohol Collodion with ether gives a thinner and less permeable membrane, whereas thickness and permeability increases with addition of amyl alcohol. Bauer[6] found that the addition or 1 ml of glacial acetic acid to 200 ml of diluted collodion solution would reduce average pore size to 200 mμ. The addition of 2 ml reduce the pore size to l00 mμ, and the addition of 3 ml reduces pore size further to 15 mμ. Field made collodion membranes which held back potassium chloride and sucrose. Kistler[9] found that collodion membrane can be made as dense as desired by filtering through them solution of cellulose or of collodion.

3. Final constituents of collodion solution used in this experiment

The collodion solution purchased is the commercially prepared Collodion U.S.P. XI. It is a solution of 4 gms of pyroxylin (chiefly tetranitropcellulose) in 100 ml of a mixture of 1 vol alcohol and 3 vol ether. Its specific gravity is 0.765–0.775. When exposed in thin layers it

evaporates and leaves a tough somewhat opaque and permeable film. During the experiment it was found that the hemoglobin in solution was precipitated by the addition of this collodion solution. To decide the substance causing this precipitation, hemoglobin solution was added to each of pure ether, alcohol and it was found that whereas Hb is precipitated by alcohol, it is not precipitated when added to ether. The collodion solution was then evaporated to dryness, and then redissolved in ether, when hemoglobin is added to this ether collodion solution, hemoglobin remains in solution without precipitating. Thus it is concluded that it is the alcohol that precipitated the hemoglobin, and from here on, only ether collodion solution was used.

METHOD

The basic problem is how to make a hemoglobin corpuscle of about 7 μ diameter with a thin and permeable collodion membrane. Various methods were tried.

Method I

The arrangement for this method is as shown in the diagram (Fig.1).

A jet of air is sent into the funnel leading to a very narrow nozzle. Hemoglobin crystals, which are finely ground to microscopic sizes, are put in at X, so that the powder is sent through a stream of collodion. It was hoped that, each crystal in passing through the stream of Collodion would be coated with a coating of collodion which on drying would form a membrane.

Three problems arise immediately in attempting this method. First, using this method, the amount of Hb crystals required is quite enormous, and attempts to crystallize a large amount of hemoglobin from the solution was not too successful.

Secondly, a stream of collodion exposed to the air would be evaporated very rapidly, and in order to maintain such a stream approximately 1000 ml/3 mins is required, and the amount available is only sufficient for very small scale experimentation. However, the most severe problem is that for the hemoglobin crystals to be sent through

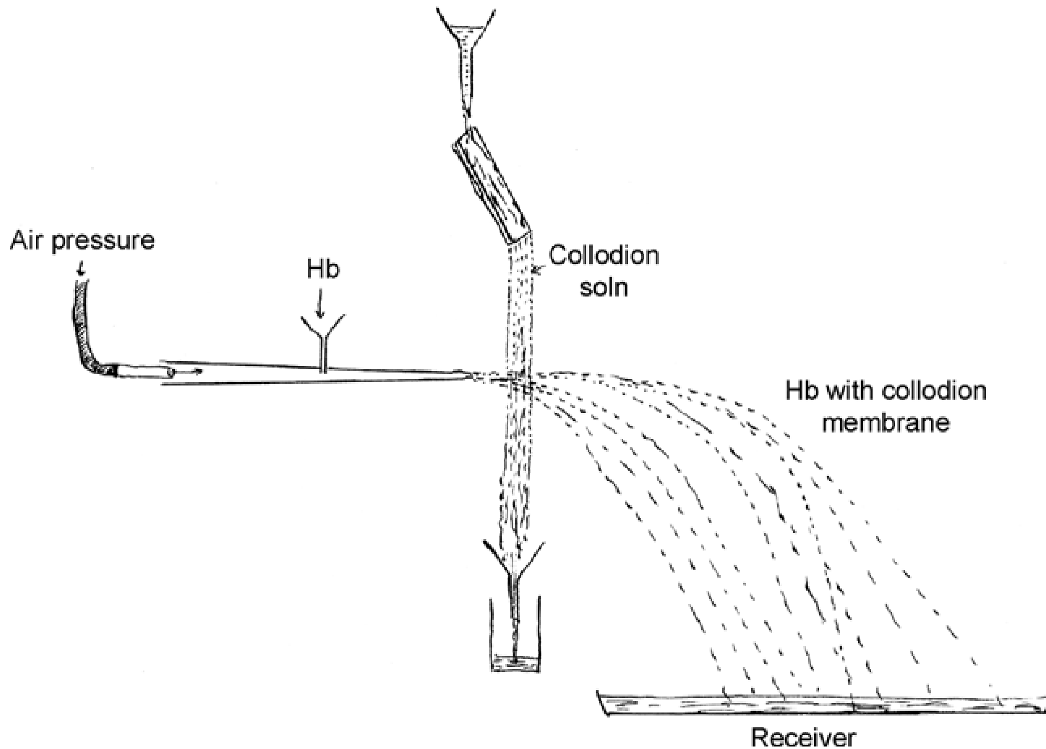

Fig. 1. Arrangement of Apparatus.

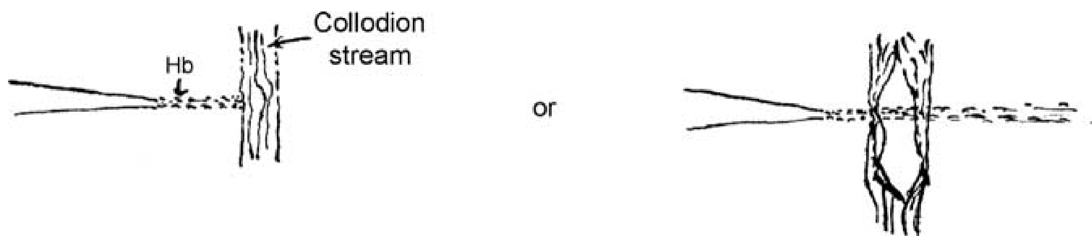

Fig. 2.

the stream of collodion, it would be necessary for the jet of air to be blown through the collodion at the same time. It was found that if the force of the air jet was not strong enough, then it would not be able to pass through the stream of collodion solution. However, when the force is increased, nothing can penetrate the collodion stream until a hole is made by the jet of air in the collodion stream, and the crystals would then be sent through by the jet of air without coming into contact with the stream of collodion. A sort of an all-or-none effect.

Fig. 3.

The solution to this problem is to send the crystals through the stream of collodion by means other than air pressure. One possible way is to eject the crystals through the use of a very powerful spring, and one was made as shown.

This way, the crystals are not carried by a jet of air, and it is hoped that therefore each crystal might be able to pass through the stream of collodion individually due to their force of momentum. However, further experiments shows that this method will turn out to be rather elaborate, since in order for the fine crystals to attain the necessary speed to penetrate the collodion stream, they will have to be ejected in a vacuum to avoid the resistance of air to such small particles. However, instead of increasing the speed of the crystals, it would be just as good to decrease the viscosity of the collodion stream. Thus another method was attempted.

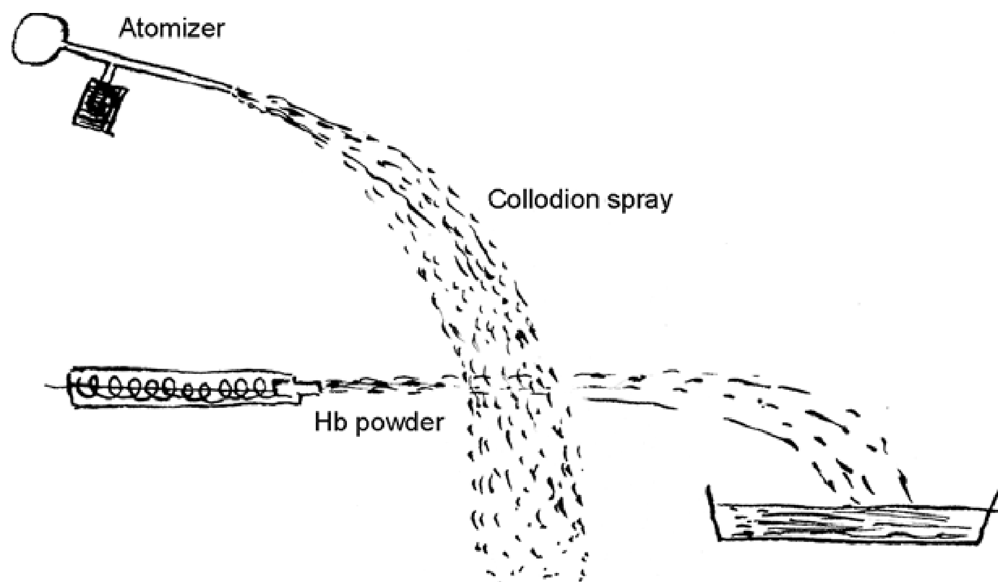

Fig. 4.

Method II

Instead of using a stream of collodion, a spray of collodion is used. Here the viscosity of the collodion is extremely small.

But here, there is a serious problem, since it takes a considerable amount of time for the collodion membrane to be perfectly set. When hemoglobin covered by a membrane of collodion which has not set touches water, the hemoglobin would just simply dissolve in water. Another problem is that, the droplets are so close together that once they are coated with collodion they would stick together.

Experimentation with collodion

Knowing that one of the important problem is the 'setting' of the collodion as a membrane, a study was made of the way in which a collodion membrane is set. Two useful observations were made. First it was observed that a thin collodion layer sets slowly (10 to 15mins) after it comes in contact with water. Secondly, a film of collodion is attached to the surface of water when it comes in contact with it. With these facts in mind, it was felt that it might be more feasible to approach the problem from a different angle.

Method III

Collodion was placed on top of a liquid which is immiscible with water and which has a specific gravity greater than collodion but less than saline. (In this case paraffin oil was used.) A small drop of hemoglobin solution was dropped about 1/2 feet from the surface of the collodion solution. This height is to give the hemoglobin droplet the neccessary force to penetrate the collodion solution. As the hemoglobin droplet passes through the collodion solution a film of collodion is attached to its surface. Due to the force of gravity, this droplet falls into the paraffin oil through the collodion layer. The purpose of the paraffin oil is to allow the collodion membrane time to set on the droplet of the hemoglobin solution since if this droplet was to enter water right away the membrane would rupture since it is still immaturely set. About 30 minutes was allowed for the droplet to remain in the paraffin oil. At

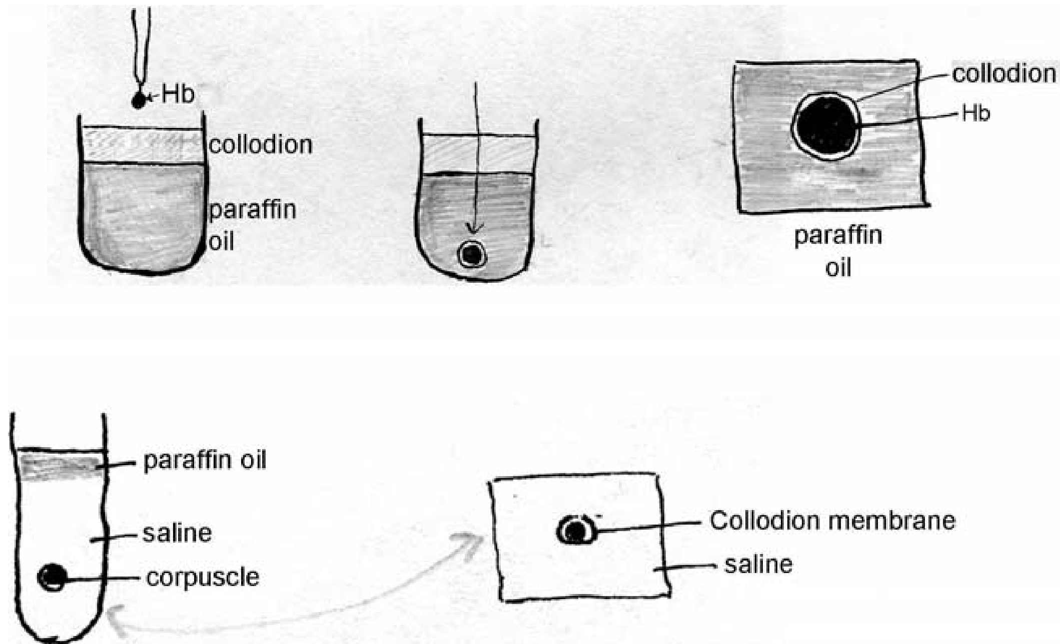

Fig. 5.

the end of this time the membrane was perfectly set, and the collodion layer together with as much as possible of the paraffin oil was removed. The remaining paraffin oil, together the hemoglobin corpuscle was then emulsified in saline, the paraffin oil having been emulsified and float to the surface leaving behind the hemoglobin corpuscle in saline.

With this method, it was successful in preparing hemoglobin corpuscles with size of about 0.2 mm in diameter. The trouble with this method being that the surface tension between collodion and paraffin oil was such that only hemoglobin droplets of appreciable size would be able to penetrate the collodion-paraffin oil interface.

Method IV — successful method

With the above principle, methods for obtaining hemoglobin corpuscles of microscopic sizes were then attempted.

Introduction: A possibility that comes into mind was the method of emulsion. Books on colloidal chemistry were read. For a while the method of emulsion appeared to be rather impractical in this case, since in the emulsions used by the chemists, it is only possible to

emulsify one liquid in another and the emulsion would only remain in the liquid in which it is emulsified. While on the verge of discarding the method for emulsion, the principle found in using collodion layer and paraffin layer comes into mind. Many attempts were then made to combine this principle with the principle of emulsion.

After many fruitless and unsuccessful attempts, a method was found. This method is based entirely on the principle of the previous method.

Procedure

1. Place one part of concentrated hemoglobin solution in 10 parts of ether. Shaking this gives a dispersion of hemoglobin in ether, but this dispersion is unstable, since as soon as shaking is stopped ether and hemoglobin separate into two layers almost immediately
2. Collodion solution (ether-collodion) is put in drop by drop while shaking continuously. Collodion on entering the solution and coming into contact with the aqueous hemoglobin solution dispersed in ether, would form a layer of collodion around the hemoglobin droplets.(As shown by the observations obtained in method III). When enough collodion is adhered to the surface of the hemoglobin droplets, the dispersion becomes stable, i.e. the dispersion remain as such after shaking is discontinued, since now around each droplet is a thin layer of collodion acting as a sort of emulsifier. When about 1 part of collodion to 10 parts of solution has been added it was found that a collodion membrane with sufficient strength would be formed.
3. The problem now is to remove the hemoglobin droplets with the collodion membrane from the ether. Here, two problems were encountered. Collodion is soluble in ether, thus there is collodion in the ether containing the hemoglobin droplets. Now, collodion sets when comes in contact with water, thus water cannot be used to separate the droplets of hemoglobin from the ether since on the addition of water, the whole solution would "coagulate". Another problem is that since collodion is soluble in ether, the collodion membrane of the hemoglobin droplets would be incompletely set

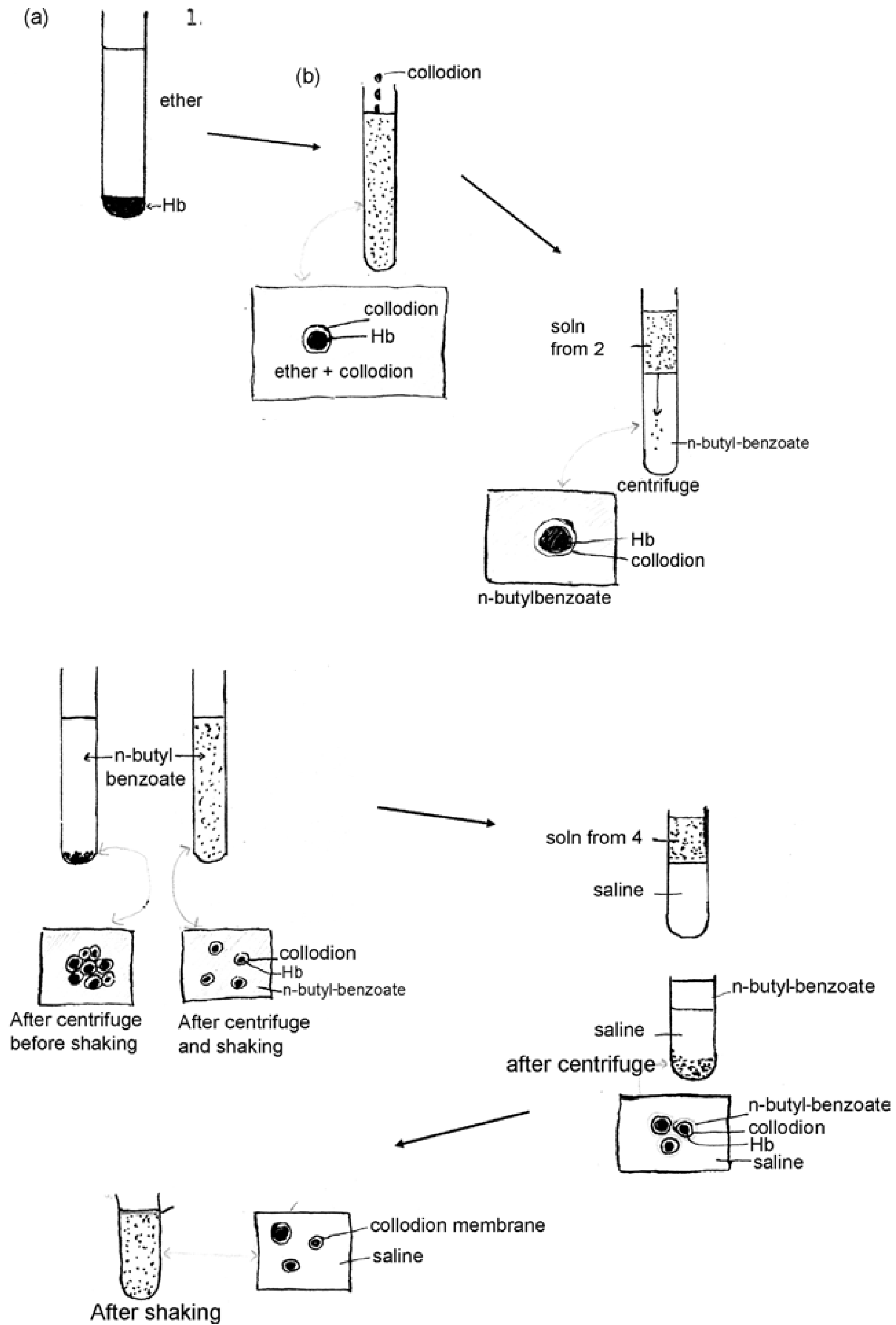

Fig. 6.

i.e. the part that is in contact with the hemoglobin solution is set, but the part in contact with ether is not set. To solve this problem, the ether containing the hemoglobin droplets with their membrane, is placed on top of n-butyl-benzoate which has been recommended as a good emulsifier. This is put in for centrifuging, so that the microscopic hemoglobin droplets could pass through the interface into the n-butyl-benzoate.
(After centrifuge, the corpuscles are in the n-butyl-benzoate. Since the collodion outside being in contact with ether previous to centrifuging is not completely set and thus is still adhesive. As a result, they tend to stick together. Here is where n-butyl-benzoate comes into use. Being a good emulsifying liquid, on shaking this, the corpuscles would be dispersed in the n-butyl-benzoate. Quite a bit of shaking is required to disperse the corpuscles which have adhered together. This dispersion allows the corpuscles to be separated from one another and thus when the collodion membrane sets, they would not be stuck together. After leaving it in this condition for 15 mins the membrane would be set completely.)

5. The dispersion of corpuscles in n-butyl-benzoate is placed over saline and centrifuge. The hemoglobin corpuscles would then be centrifuged into the saline. Now, some n-butyl-benzoate would be stuck to the corpuscles resulting in the corpuscles having the appearance of being in an aggregation in the saline. Here, another advantage is made use of n-butyl-benzoate, since on shaking the corpuscles inside the saline, the n-butyl-benzoate which may have adhered to the surface of the corpuscle would be emulsified in saline leaving behind the corpuscles with their collodion membrane.

Proof that collodion membrane is formed

To ensure that the membrane of the corpuscle is collodion and not n-butyl-benzoatel the following experiment was done:

1. Disperse hemoglobin in n-butyl-benzoate
2. Centrifuge over saline as before.

3. Hemoglobin inside n-butyl-benzoate would be centrifuged into the saline, but when this is shaken as before, the n-butyl-benzoate is emulsified and hemoglobin is left behind and dissolves in saline. From this experiment it is concluded that the membrane in the previous method was not n-butyl-benzoate. Since, collodion is the only other alternative, it may be concluded that a membrane of collodion has been formed around each hemoglobin droplet.

RESULTS

i. Size of corpuscles

Microscopic studies show that the size of the corpuscles approximates that of the red blood cells. However, there are some which are either too big or too small. It is suggested that a solution with an uniform size of corpuscles could be obtained as follows: Using the ultrafiltration membrane of the bacteriologists, first the solution could be filtered through filter which allows all particles smaller than 8 μ to pass through — this gets rid of the large corpuscles. Following this the solution is filtered through filter which allows anything smaller than 5 μ to pass through. Thus what is left behind in the filter would be corpuscles with diameters of between 5 μ and 9 μ.

ii. Content of corpuscles

Microscopic studies show that the corpuscles contain a colored material. Spectrometric studies show that these pigments are oxyhemoglobin. (This is observed after the alcohol-ether solvent of the collodion solution is replaced by ether.)

iii. Oxygen content

It was found that using the principle of oximetry, a dissociation curve was obtained. However, it must be mentioned that this particular experiment is extremely unreliable, since it is found that during the experiment there has been a considerable amount of hemolysis of the corpuscles due to too much shaking.

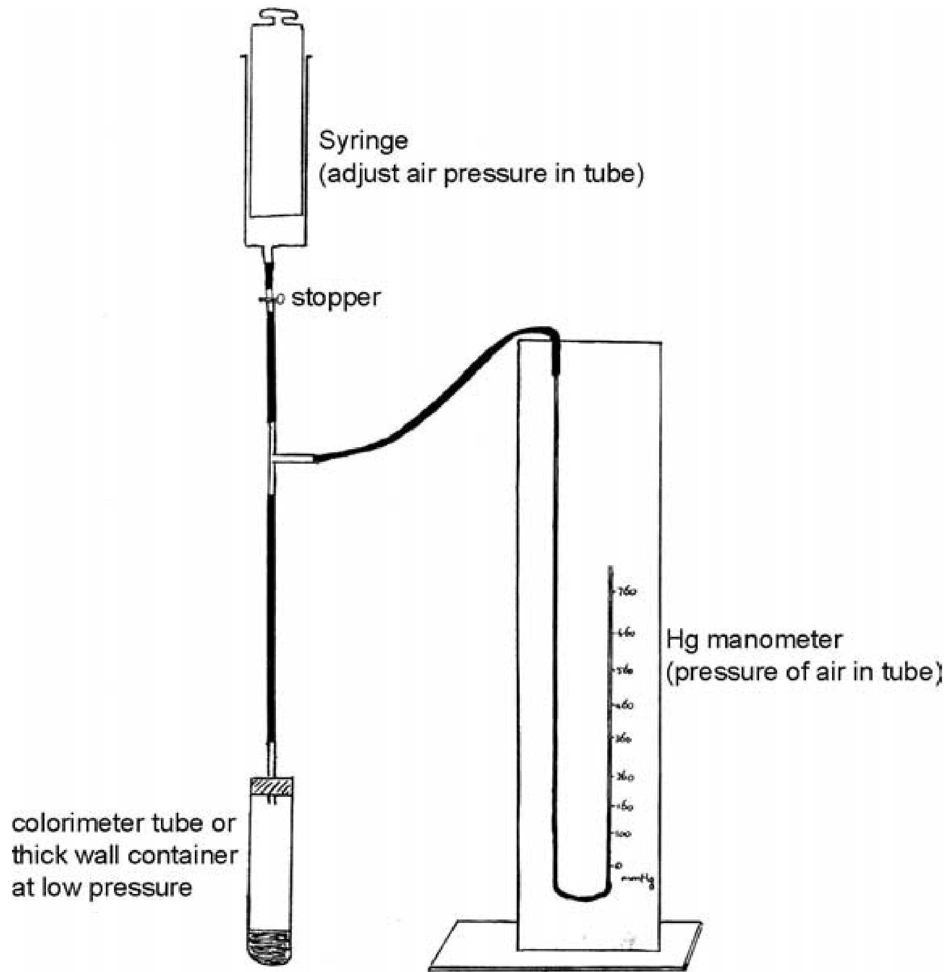

Fig. 7. Arrangement of Apparatus for Oxygen Dissociation Curve.

Method: The solution was placed in container(C) the atmospheric pressure in the container was adjusted by the syringe, and, at low pressures, by vacuum pump, to the desired pressure which is read from the manometer(M). The container was shook intermittently to allow for equilibrium to set up. After 20 minutes, the solution was read in the colorimeter with a wave length of 650 mu.

The dissociation curve obtained is then plotted as shown.

Actual amount of oxygen contained: Using a syringe with its needle inside a stop cork, a vacuum can be made in the syringe and the gas that evaporate from the solution can be measured. In one experiment the amount of gas obtained for that of a fully oxygenated solution of 0.6 ml is 0.l ml, and that for a fully reduced one is about 0.02 ml

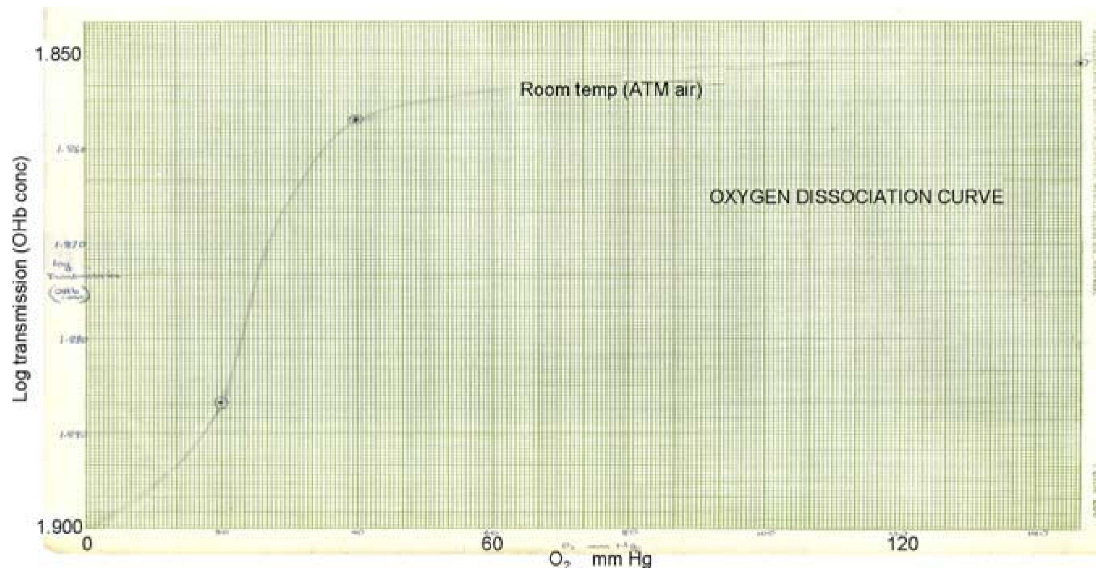

Fig. 8.

(very approximate.) It is realized that using such a small amount of solution, the results obtained is extremely unreliable. However, the solution of n-butyl-benzoate ordered has not as yet arrived and not sufficient amount was left to prepare anything more than 0.6 ml. Since it is quite inaccurate and impossible to do a dissociation curve with the possible range of gas of less than 0.08 ml, this was forced to be omitted.

(N.B. Many other possible experiments on this preparation could and should be done, but with the sudden realization that more than 120 hours have been unknowingly spent on this project at the expense of other work, and with the approaching of the final, it was necessary to put and end to this project — not without reluctance!!)

DISCUSSIONS

How far does this preparation fulfill the function of the red blood cells? Those prepared in this experiment are so crude that nothing can be said of them. However, further perfection may lead to the fulfillment of most of the main functions of the red blood cells, as discussed in the following sections.

Transportation of O_2 and CO_2

The kinetics of the formation of oxyhemoglobin from hemoglobin in the red blood cells is different from that in solution. With a pO_2 of 75 mm Hg and at 10–20°C, and with the same concentration, the half time for oxyhemoglobin saturation of the corpuscles is 0.05 seconds (i.e. for the erythrocytes), whereas that for hemoglobin solution the half time is 0.004 second. This difference in speed could be due to one or more of the following factors[5]

1. Presence of gradients of dissolved gas in the fluid surrounding the corpuscles thus leading to retardation owing to the limiting effect of diffusion.
2. Delay due to time of diffusion through membrane.
3. Dissolved gas that enter first combine with hemoglobin on the periphery of the erythrocyte and succeeding dissolving gas having to penetrate a finite distance into the corpuscle before combining with hemoglobin.

Thus in preparing artificial hemoglobin corpuscles, these factors have to be considered. Now, in the circulation the cells are well stirred and thus there is no appreciable concentration gradient of dissolved gas in the fluid surrounding the corpuscles, thus factor one can be disregarded. As far as factor 2 is concerned, that of delay due to time of diffusion through membrane, the artificial membrane used must be such that it allows easy passage of dissolved O_2 and CO_2. Also it should not be thicker than necessary, since according to Fick's equation:

$$\text{rate of diffusion} = D(C_1 - C_2)/dx$$

Where x is the thickness of the cell membrane. The studies of Weech and Michaelis[10], shows an interesting factor of permeability of collodion. He found that urea passes through the collodion membrane many times more rapidly than glycerine and about 250 times faster than for glucose. Also, he found that collodion is freely permeable to water molecules. From these observations it would appear that there shouldn't be too much trouble for O_2 or CO_2 to pass through the membrane. However, it would be interesting to

find out the permeability of the collodion membrane to dissolved O_2 and CO_2, and to prepare a membrane (by method described under the section "Membrane") which affords the best permeability to O_2 and CO_2 without allowing hemoglobin molecules to leak through.

As far as factor three, that of dissolved gas which enter first combine with hemoglobin on the periphery of corpuscle, and succeeding dissolved gas that enter having to penetrate a finite distance into the corpuscles before combination with hemoglobin occurs. The solution is to have the greatest surface area for a given volume of hemoglobin solution, and to have a shape which is such that it gives the smallest depth. Of course, the biconcave disc shape of the erythrocytes is the best solution to this problem, since with this shape all the requirements mentioned are fulfilled. Would it be possible to make a biconcave hemoglobin corpuscle artificially? It has been observed under the microscope that some of the artificial corpuscles have a sort of biconcave shape-others have surface as shown:

This may have resulted from the fact that the collodion when set, tends to shrink slightly — Since the setting of membrane does not take place simultaneously in the surface of any single corpuscle. The part that still has not set is still very elastic, thus when one part of the membrane is set the volume of the corpuscle is decreased slightly, and a pressure is exerted on the enclosed solution. This pressure in turn is transmitted from the solution onto the part of the membrane which has not yet set, thus causing them to bulge out slightly. Thus it is a matter of pure chance for the biconcave, triconcave or tetraconcave and 'polyconcave' shape of the corpuscles. These shapes may in a way compensate for the lack of the actual biconcave disk shape of the erythrocytes.

Enzyme systems in the erythrocyte

As tar as the various enzyme systems present in the erythrocyte are concerned, it wouldn't be too much of a problem since it is possible to include any enzyme system in the hemoglobin solution when preparing the corpuscles. One of the most important enzyme in the erythrocyte is carbonic anhydrase in the way of transportation of CO_2, this can easily be included in the hemoglobin solution.

Buffering action of erythrocyte

Hemoglobin and potassium ions in the erythrocyte play an important part in the buffering of the pH of the body. Now, in the prepared corpuscles only hemoglobin is present for this action, and potassium ion is absent. (This may be simply a speculation, however, from the wide variety of potassium concentration in different species of animal and from the fact that hemoglobin may be present as a potassium salt — it is suspected that perhaps part of the potassium ions are kept inside the cells despite the higher concentration because of their loose ionic association with hemoglobin; the other part being kept in by the metabolic activity of the membrane. Even before this project was started, this question has always been present. Thus it would be interesting to see whether there is any concentration gradient of potassium ions in the case of an artificial membrane. If there is a gradient in concentration in the case of an inactive collodion membrane, then it may point towards the possibility of the potassium ions being present in an ionic association with the hemoglobin.)

From what has been said before, it would appear that further perfection may make it possible for the artificially produced corpuscles to meet the requirement of the erythrocytes as far as the physiological function is concerned. However, to perfect this for perfusing is quite another question. Predominant of the numerous problems would be the problem of finding a suitable membrane — a lipoprotein membrane. Although it is not a hopeless case, a tremendous amount of work has to be put in before anything could be perfected.

SUMMARY

I. Four methods for preparing artificial hemoglobin corpuscles were attempted.
II. One of the four methods was practical and appeared to be usable.
III. From this method, corpuscles approximating the size of erythrocytes were produced. The content of these corpuscles inside the collodion membrane was found to be oxyhemoglobin under spectrometric studies.
IV. An oxygen dissociation curve was obtained and the oxygen content of these preparations was found. But it is felt that the results obtained in IV is not reliable, since only one set of result was done on these, since there was not enough time for repeating these measurements.

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