A NOTE ON THE DISTRIBUTION OF THE SALTS IN HAEMOLYSIS

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In his extensive studies¹ of the comparative electrical conductivities of normal blood laked by various means, Stewart has shown that complete liberation of the haemoglobin can be produced in blood and in artificial suspensions of erythrocytes without any change or with only a slight increase in conductivity. This is the case when what he calls the less violent haemolytic agents (freezing and thawing, heat, foreign serum, strictly minimal doses of saponin, etc.) are employed. When the laked blood is subsequently exposed to one of the more energetic haemolytic agencies (water, supraminimal doses of saponin, etc.) a marked increase in the conductivity is produced. The total increase is sensibly the same whether the haemolysis has been effected in two stages, first by a gentle and then by a more violent agent, or in one stage by the original and more energetic action of a body of the second group. The action of the more violent haemolytics when consecutive to that of the less violent, is an action on the shadows or ghosts of the corpuscles already deprived of haemoglobin which reduces them to the same condition as that produced directly by the more energetic haemolytics acting alone. It is not due to the

1. *Jour. of Phys.*, Vol. XXIV, p. 211, 1899; *Ibid.*, Vol. XXVI, p. 470, 1901; *Jour. of Exp. Med.*, Vol. VI, p. 257, 1902; *Jour. of Med. Research*, Vol. III, p. 268, 1902; *Amer. Jour. of Phys.*, Vol. IX, p. 72, 1903.

breaking up of (hypothetical) compounds of colloids and electrolytes in the extracorporeal liquid¹ of the laked blood.

This phenomenon is of interest not only in relation to the mechanism of haemolysis but in relation to the physical chemistry of cells in general, since, as Stewart² has pointed out, and as has been more recently insisted on by Overton and others, it is a common property of living cell 'envelopes'³ to be relatively impermeable to the ions with which they are normally in contact.

In the interpretation of the alterations of conductivity in laking the following points must, according to Stewart, be taken account of :

1. The exit of haemoglobin from the corpuscles (supposing it to take place without interchange, on the whole, of water or electrolytes between the serum and the stromata, and without change in the permeability of the envelopes or stromata to the ions) would depress the conductivity of the laked blood by diminishing the conductivity of the intercorpuscular liquid to a greater extent than would be compensated by the diminution in volume of the badly conducting corpuscles.

2. Apart from the depressing influence of liberated haemoglobin, the exit of electrolytes from the corpuscles would increase the conductivity of the intercorpuscular liquid and, therefore, of the laked blood, provided that the volume of the corpuscles is not increased by the passage of water into them.

3. The exit of water from the corpuscles unaccompanied, on the whole, by electrolytes would cause an increase in the conductivity

1. The extra- or intercorpuscular liquid is a convenient term for the liquid in which corpuscles or ghosts are suspended. In unaltered blood it is, of course, the serum.

2. *Journal of Physiology*, Vol. XXIV, *loc. cit.*, 1899.

3. Note by G. N. S.—In this paper the question of the precise physico-chemical action produced by the various haemolytics or their precise point of attack in the corpuscle has not been entered into. Moore and Roaf (this *Journal*, Vol. III, p. 55, 1907) have recently published an interesting paper in which they argue against the view that the difference in the inorganic constituents of corpuscles and plasma can be accounted for by the existence of a 'membrane' variously permeable to different salts. I have avoided the use of the term 'membrane' in this connection, and have preferred to speak of the corpuscles as being bounded by an 'envelope,' a word which does not seem to carry with it the same preconceived histological and physico-chemical suggestions as the word 'membrane.' I have, however, demonstrated histologically (*Amer. Jour. of Phys.*, *loc. cit.*) the existence of an envelope in the large nucleated corpuscles of *Necturus*. I hope soon to have an opportunity of returning to the subject.

of the laked blood by increasing the volume of the intercorpuscular liquid relatively to that of the corpuscles, and perhaps by increasing somewhat the degree of dissociation of the serum electrolytes, although it would diminish the specific conductivity of the intercorpuscular liquid.

4. An increase in the permeability of the corpuscles or ghosts to the ions, without, on balance, any increase in the electrolytes of the intercorpuscular liquid or in its amount, would increase the conductivity of the laked blood (apart from the depressing influence of the liberated haemoglobin).

According to the same writer, the difference in the action of the two groups of haemolytic agents may be explained in two ways:—

(a) By the assumption that in the first case a relatively small, and in the second case a relatively large amount of intracorpuseular electrolytes escapes from the corpuscles; the relation of a considerable part of the electrolytes of the corpuscles to the stroma and envelope being such that an energetic action of the haemolytic on these structures is required for their liberation.

(b) By the assumption that the permeability of the envelope (and stroma) to the ions of the intercorpuscular liquid, and perhaps also to the intracorpuseular ions, is decidedly increased by the second group and slightly, if at all, by the first. The consequence of this would be that after the action of, say, a sufficient dose of saponin the laked blood would approximate more closely to the condition of a homogeneous conductor than after a less violent haemolysis, which left the shadows their 'semi-permeable' character.

Stewart arrived at the conclusion that an increase in the conductivity of blood after laking is not due altogether to a surrender of electrolytes by the corpuscles to the fluid, but rather that some alteration in the corpuscles which allows ions to pass through them more freely is caused by the laking process.

Foa, whose views as to the structure of corpuscles are quite at

variance with those of Rollett and of Stewart, observed¹ that repeated freezing and thawing of blood with nucleated corpuscles lowered its freezing-point by stages. He, believing that in the laking of non-nucleated corpuscles electrolytes do pass into the surrounding fluid, holds that any indications to the contrary furnished by measurements of the electrical conductivity of the fluid of laked blood are not admissible, in that we do not know what the relations between the albuminous components of the blood and the electrolytes may be, and what effect their relation may have on electrical conductivity. Moreover, by a method which he used, of separating the fluid of laked blood from the ghosts, he found the ash in the former in excess of that found in the serum of an equal quantity of unlaked blood.

Since it seems doubtful if a complete separation of serum and corpuscles, or of ghosts and fluid in laked blood, can be effected by simple centrifugalisation, I made, some time ago (at Dr. Stewart's suggestion), to test the constancy of the results, determinations of the ash in as much serum of measured quantities of blood and in as much intercorpuscular fluid of measured quantities of laked blood as could, after centrifuging, be separated as cleanly as possible from the corpuscular elements with a pipette. It was found impracticable—especially with laked blood, always darkened—to discern the exact line of separation; and there were always, even with the best available centrifugalisation, loose, flocculent particles, barely visible, above the seeming line of separation. This, I think, ought to preclude the possibility of an exact reading of the comparative volume of ghosts and of fluid in laked blood. It is less easy to imagine how a perfect separation of serum and corpuscles or fluid and ghosts can be effected by the method which Foa used of centrifuging in tubes with stop-cocks at the bottom, and then allowing the sediment to flow off through a small opening in the cock. If blood is centrifuged very long and rapidly, the corpuscles or ghosts will be packed together and pressed against the wall of the containing tube so tightly that they will adhere

1. *Archivio di Fisiologia*, Vol. I, Fasc. 2, 1904.

to the wall of the tube, and will continue to do so after the contents of the tube are poured out.

The numerous comparative determinations of the ash from serum and from the fluid of laked blood—made by trying to draw off all of the fluid parts carefully with a pipette—were made with measured amounts of chicken blood because it was expected that the corpuscles of such blood, still ballasted, so to speak, by the nuclei, would sediment well. The results are so inconstant that it is held to be wholly impossible to ascertain the ash from the fluid part of blood—especially when laked—by separating all of the fluid from the corpuscular elements.

With horse blood, also, determinations of the ash from the serum and from the fluid of the blood, laked by freezing and thawing, were made, the fluids being separated as completely as possible from the corpuscular elements after centrifuging a long while. The results of determinations in the fluid of the laked blood were far apart. The proportion between the amount of ash (L) found in the fluid of the laked blood and the ash (S) in the serum were even much greater in each case than Foa found with horse blood by his method of separation. Thus in two experiments I obtained :—

$$L : S :: 1.196 : 1.00$$

$$L : S :: 1.3558 : 1.00$$

which compares with Foa's result —

$$L : S :: 1.1388 : 1.00$$

The gross irregularities in the increases in the ash of the fluid of the same blood laked by the same procedure but separated from the ghosts by such doubtful means, make it quite a certainty that some of these increases are due to salts which get into the ash determination in ghosts or in solid particles which do not sediment perfectly.

It seemed to me that it might be ascertained if salts come out of the corpuscles into the fluid on laking, by simply comparing the ash of a measured volume of serum of unlaked blood with a measured volume of fluid of laked blood, both being taken from the upper layers

only of portions well centrifuged in closed tubes, while at the same time the relative proportion of serum and corpuscles in the unlaked blood and of liquid and ghosts in the laked blood was determined by the haematocrite. But the same difficulty of effecting a complete separation of the ghosts presented itself here. I was, therefore, forced to leave out of account the possibility that the relative volumes of fluid and corpuscular elements of blood may change on laking without alteration in the proportion of salts. This would be the case if a quantity of water holding the same amount of salt as the same volume of serum, were transferred one way or the other. As, however, I found positive differences in the amount of ash, this objection does not render my observations valueless.

In these experiments the blood to be tested was handled in centrifuge tubes fitted with rubber caps, such as bacteriologists use. With these caps put on the tubes as soon as filled, there can be no evaporation and hence no alteration of volume of the contents. Some of the tubes were centrifuged, immediately after filling, to provide the normal serum; others were subjected to the laking process, well stirred up and then centrifuged. Carefully measured volumes of serum and of fluid of laked blood were pipetted off and brought into crucibles in which their ash-content was weighed after incinerating *lege artis*. In this way the relation of ash (S) in the serum of blood to the ash (L) in the fluid of the same blood laked was found in the following cases to be :—

L : S :: 0.917 : 1.00 (chicken blood laked by heat).

L : S :: 0.891 : 1.00 („ „ saponin).

L : S :: 1.0284 : 1.00 (horse blood laked by heat).

L : S :: 1.0544 : 1.00 („ „ saponin).

L : S :: 1.0618 : 1.00 (horse blood laked by freezing and thawing).

L : S :: 1.048 : 1.00 („ „ „ „).

In all cases where saponin was used as a laking agent the small amount of ash contained in it was deducted from the total ash of the incinerated laked fluid. In all cases where the ash contained iron derived from the haemoglobin of the fluid of laked blood it was included, since it was seen that the whole excess of L over S in the mammalian blood could be accounted for by the iron, and that was all I aimed at determining.

It will be seen from the above that the increase of ash from the fluid of horse blood laked by freezing and thawing is not nearly so large as Foa found by his method, and may be accounted for by the iron of the haemoglobin. If the intercorpuscular fluid contained only ten per cent. of haemoglobin (and it would almost certainly be more, since the whole, or nearly the whole, of the pigment is in the fluid), this would correspond, say, to 0.035 per cent. of Fe or 0.05 per cent. of Fe_2O_3 . When the original serum contains 0.8 per cent. of ash this would make the ratio $\text{L} : \text{S} :: 1.06 : 1$, if no salts were exchanged between the corpuscles and the liquid. With chicken blood the quantitative relations above recorded between the ash of the serum and the ash from the fluid of laked blood are in accord with Foa's belief that there is a difference in the mode of laking of nucleated and the mode of laking of non-nucleated corpuscles. Stewart also found that in heat-laking of blood with nucleated corpuscles the conductivity might be markedly diminished.

On the other hand the relations found between the amount of the ash from the serum of blood with non-nucleated corpuscles and from the fluid of such blood laked in different ways are not easy to interpret. In blood laked by freezing and thawing we find that the increase of proportion of the ash from the fluid is almost as much in one series, and more in the other series than the increase of proportion of ash from the fluid of blood laked by saponin, whereas in blood laked by heat it is distinctly less.

Yet, as has been said, saponin increases the conductivity of the laked blood much more than heat or freezing and thawing. So far as these determinations go, then, they support the view that in none of these forms of laking is there a notable increase in the percentage of electrolytes in the intercorpuscular liquid, and that the increase in conductivity produced by saponin must be due either to an increase in the total volume of the liquid or to an increased conductivity of the corpuscles, or to both.

That a change in the conductivity of the corpuscles, not necessarily associated with the liberation of electrolytes, is caused by

saponin is indicated by determinations of ash in the fluid of suspensions of formaldehyde-hardened corpuscles treated with saponin, which bear out Stewart's view as to the behaviour of the corpuscles during the laking process. With suspensions of such corpuscles complete separation of the fluid can be made after centrifugalisation since they sediment to form a viscid mass from which the supernatant fluid can even be poured off; and the corpuscles can be washed and re-washed in M/4 cane sugar solution. Although formaldehyde-hardened corpuscles will not give up haemoglobin when subjected to the influence of laking agents, a suspension of them in isotonic solutions of salt will undergo an increase in its electrical conductivity when treated with saponin as great as if the corpuscles were fresh.

It is very easy to determine that the increase in conductivity of the suspension of formaldehyde-hardened corpuscles after treatment with saponin is not due to a passage of electrolytes from the corpuscles into the fluid of the suspension. Such suspensions, having been treated with weighed amounts of dried saponin containing a known amount of ash and allowed to stand from five to forty-eight hours, were centrifuged, and measured volumes of the supernatant fluid then taken for determinations of the ash obtainable from them, and these were compared with determinations of the ash from the supernatant fluid of a like centrifuged suspension, which had not been treated with saponin. It was found that the excess of ash from the supernatant fluid of the saponin-treated suspension could be accounted for by that added with the saponin when the suspension was in salt solution.

Or in other series, the suspensions of formaldehyde-hardened corpuscles in salt solution were first washed with M/4 solution of cane sugar and suspended in the latter when treated with saponin. After centrifuging such sugar solution suspensions, the ash from the supernatant fluid of the saponin-treated suspension was found to exceed that from the supernatant fluid of untreated suspensions in the exact proportions in which it was added with the saponin; or in a case where the whole supernatant fluid was taken for ash determinations, the excess of ash from the fluid of the saponin-treated

suspension was found to coincide almost exactly with that added with the saponin. The supposition that formaldehyde-hardened corpuscles on being treated with saponin, which raises the conductivity of a suspension containing them, do not give up any of their electrolytes to the surrounding fluid is therefore, by this method, well substantiated. The increase of conductivity must, then, be due to a change in the envelope (or stroma) which permits an easier passage to the ions of the liquid in which the corpuscles are suspended.