THE MECHANISM OF CHANGE IN RESISTANCE OF ERYTHROCYTES TO HYPOTONIC SALT SOLUTIONS.*

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A series of interesting papers has been published recently by Ashby[1] in which she presents data tending to show that the alkali metal cations may be grouped into two classes, according to their tendency to increase or to decrease the resistance of erythrocytes to hypotonic hemolysis in NaCl solutions. $K^{\cdot}$ and $Rb^{\cdot}$ decrease the resistance of human red blood cells, which contain $K^{\cdot}$ in large amounts, while Na^{-} and $Li^{\cdot}$ increase it; but when the studies were extended to cells which contain practically no $K^{\cdot}$ these effects were reversed (dog, ox, cat).

These experiments have been repeated, in part, with the addition of parallel series in which the fragility of the cells was determined in dilute KCl solution, in order to test the possibility that a reciprocal relation existed between the ions used to produce and to test the changes in fragility. The results fail to substantiate Ashby's results in certain important respects and are given here in the hope that others will be induced to repeat the experiments to confirm or disprove the points thus brought into question.

PROCEDURE.

Ashby added 5 to 10 cc. of citrated or defibrinated blood to 100 cc. of a 0.154 N NaCl solution or to isotonic KCl, LiCl, or RbCl solutions, and incubated the mixture at 36°C. for from 1 to 3 days, shaking every 24 hours. Asepsis was maintained. The cells were then collected by centrifugation and their fragility determined by observations on the

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[1] Ashby, W., *Am. J. Physiol.*, 1924, lxviii, 239, 250, 585, 611.

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degree of hemolysis in sodium chloride solutions of various concentrations to which were added in each case sodium citrate in quantity sufficient to be isotonic with 0.06 per cent NaCl; 0.02 cc. of cell sediment was added to each 1.0 cc. portion of the test solution.

The use of citrate in these experiments seems objectionable because of the introduction of an additional ion whose effects ought to be determined separately, and since it is not indispensable our experiments were conducted with defibrinated blood only; the sodium citrate was left out of the hypotonic test solutions. In some of the experiments these test solutions were made up with KCl as well as with NaCl. The effects of Li˙ and Rb˙ were not investigated.

Extreme care was used in the choice of materials. The distilled water was distilled once from block tin, and again from a well used Pyrex glass still in which the water and its vapor touched nothing else but well steamed out glass wool and absorbent cotton; its specific conductivity when fresh distilled was of the order 5×10^{-6} ohms^{-1}. Only Pyrex glass was used, except momentarily in the case of volumetric flasks and pipettes. Five samples of salts were used: pre-war Kahlbaum's NaCl and KCl; Squibb's reagent NaCl purporting to contain no potassium, and less than 0.0039 per cent of bi- and trivalent bases; a sample of KCl several times recrystallized for the preparation of calomel half-cells; and NaCl repurified by precipitation by HCl and subsequent heating in a stream of nitrogen.[2] No differences in results were traceable to differences in the samples used. Asepsis was maintained until the moment of testing for hypotonic hemolysis. Just prior to this the suspensions of red blood cells were tested for the presence or absence of intercurrent bacterial growth by plating out measured samples on nutrient agar.

The solutions used for incubation were sterilized in Pyrex glass and their subsequent pH found to be 6.6 to 6.9, showing the presence of only minimal traces of basic materials dissolved from the glass. The solutions in which the cells were incubated were therefore of a very high degree of purity and possessed so little buffering power as to have no influence on the pH during incubation; the addition of sodium citrate for buffering purposes was therefore unnecessary. The stock

[2] The last two were furnished by the Division of Chemistry, Hygienic Laboratory.

solutions were made up to be accurate within ± 0.1 per cent of their concentration, proper correction being made for the temperature of the water at the time of diluting to the final volume. These stock solutions, in which the cells were incubated, and by dilution of which the hypotonic test solutions were prepared, were 0.900 per cent NaCl and 1.150 per cent KCl (1.140 per cent in the last two experiments— this is more exactly isotonic with 0.9 per cent NaCl). The hypotonic solutions were prepared in 20 or 40 cc. lots with an error of ± 0.002 per cent in the case of NaCl and the same proportional error in the case of KCl.

Blood from six species of animals was used: sheep and dog, whose red blood cells contain little or no K·; and horse, guinea pig, rabbit, and man, whose erythrocytes are rich in K·. The blood was obtained from the jugular vein (sheep and horse), heart, (dog, guinea pig, and rabbit, the last under light ether anesthesia), and median basilic vein (human). No effect of the ether anesthesia was observable in the case of rabbit blood.

Portions of blood were poured into ten times their volume of either NaCl 0.9 per cent, or KCl 1.15 or 1.14 per cent (both sterile) and incubated at 37.5°C. for 1 or 2 days. Longer incubation was impossible because of excessive stromatolysis.

The normal resistance to hypotonic hemolysis was determined by tests on unwashed cells or cells washed once in ten volumes of a balanced salt solution isotonic with 0.9 per cent NaCl. Except as noted in one experiment this contained NaCl, KCl, $CaCl_2$, and $MgCl_2$ in the proportions customary for Ringer-Tyrode solution. In all cases the normal cells were gathered into a small volume by centrifugation and decantation of the supernatant fluid (serum or salt solution) and tested for fragility by adding 0.05 cc. to 2.0 or 2.5 cc. of each of the hypotonic solutions.[3] The blood incubated in the sodium and potassium chloride solutions was treated in exactly the same way after incubation. The cells were resuspended once by shaking after the first

[3] The error introduced by the higher osmotic pressure of the suspending fluid added with the cells to the hypotonic test solutions is nowhere greater than one-half of the interval between adjacent solutions, and in each experiment it is always the same in any given test solution; it may therefore be neglected by comparison with other errors.

24 hours of incubation. Further details of technique are given under the individual experiments.

RESULTS.

The experimental results are given in two different forms. The first is that used by Ashby, in which the degree of hemolysis in each

TABLE I.

Hemolysis in Hypotonic Solutions of Cells Incubated in Isotonic KCl and NaCl Solutions.

Red blood cells from	Incubated in	Tested in	Degree of hemolysis in NaCl solutions of the concentrations given in per cent, and in KCl solutions isotonic with them.													
			0.270	0.315	0.360	0.405	0.450	0.495	0.540	0.585	0.630	0.675	0.720	0.765	0.810	0.855
Rabbit (Experiment 1).	Control....	KCl	4	4–	2	1	0	0	–	–	–	–	–	–	–	–
		NaCl	4	4	3	1	0	0	–	–	–	–	–	–	–	–
	KCl........	KCl	4	4	4	4	4	4	3+	–	–	–	–	–	–	–
		NaCl	4	4	4	4	4	4	4	–	–	–	–	–	–	–
	NaCl.... ..	KCl	4	4	4	4	3	1	0	–	–	–	–	–	–	–
		NaCl	4	4	3+	2+	2	1	0	–	–	–	–	–	–	–
Sheep (Experiment 3).	Control....	KCl	–	–	–	–	4	4	4–	3–	0	0	0	0	–	–
		NaCl	–	–	–	–	4	4	4–	2	1+	0	0	0	–	–
	KCl........	KCl	4	4	4	4	4	4	4	4	4	4	4	4	3+	2
		NaCl	4	4	4	4	4	4	4	4	4	4	3+	3–	2	0
	NaCl.......	KCl	4	4	4	4	4	4	4	4	3+	2	1	0	0	0
		NaCl	4	4	4	4	4	4	4	4–	2	1	0	0	0	0

Control cells washed in a solution containing NaCl, KCl, $CaCl_2$, and $MgCl_2$ in the proportions usual for Ringer-Tyrode solution, and tested immediately, without incubation.

The degree of hemolysis is indicated by the figures 0, 1, 2, 3, 4, ranging from *no hemolysis* to *complete hemolysis* as determined by macroscopic observation of the turbidity.

test solution is given, the figures 0–4 representing increasing degrees of hemolysis from that too small to detect up to complete hemolysis. In my experiments, and presumably in Ashby's also, the degree of hemolysis was estimated by macroscopic observation of the turbidity

of the test suspensions (Tables I and II). In the last two experiments (Table III and Figs. 1 to 4) the degree of hemolysis was accurately determined: the intact cells were separated by centrifugation and decantation, hemolyzed in a volume of distilled water equal to that of the decanted supernatant fluid, and the comparative color of decantate and hemolyzed sediment used as a basis for calculating the proportion of the cells hemolyzed by the hypotonic test solution. These figures,

TABLE II.

Hemolysis in Hypotonic Solutions of Cells Incubated in Isotonic KCl and NaCl Solutions. Experiment 4.

Red blood cells from	Incubated in	Tested in	Degree of hemolysis in NaCl solutions of the concentrations given in per cent, and in KCl solutions isotonic with them.																
			0.18	0.225	0.27	0.315	0.36	0.405	0.45	0.495	0.54	0.585	0.63	0.675	0.72	0.765	0.81	0.855	0.90
Man.	Control...	KCl	4	3—	2—	1	0	0	0	0	0	—	—	—	—	—	—	—	—
		NaCl	4	3	2—	Tr.	0	0	0	0	0	—	—	—	—	—	—	—	—
	KCl......	KCl	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	2
		NaCl	4	4	4	4	4	4	4	4	4	4	4	4	3+	2+	0	0	0
	NaCl.....	KCl	—	—	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0
		NaCl	—	—	1+	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sheep.	Control...	KCl	—	—	—	4	4	4	3	1+	0	0	0	—	—	—	—	—	—
		NaCl	—	—	—	4	4	4	3+	1+	0	0	0	—	—	—	—	—	—
	KCl.....	KCl	4	4	4	4	4	4	4	4	4	4	4—	3	1	0	0	0	0
		NaCl	4	4	4	4	4	4	4	4	4	4	4—	3	1—	0	0	0	0
	NaCl....	KCl	—	—	4	4	4	4	4	4	3	2	1	0	0	0	0	0	0
		NaCl	—	—	4	4	4	4	4	4	3+	2+	1—	0	0	0	0	0	0

Controls and legend as in Table I.

in per cent, are properly corrected and plotted against the concentration of the test solutions as abscissæ (Figs. 1 to 4). The resistance of the cells is considered to be inversely proportional to the concentration of NaCl in which a given degree of hemolysis occurs; the mean of the values for 20, 40, 60, and 80 per cent hemolysis is here used.[4] The

[4] The details and advantages of this method of titration are given in Brooks, S. C., *J. Med. Research*, 1919–20, xli, 399.

theoretical significance of resistance as determined in this way is perhaps dubious, but it is still at least a concrete measure of the phenomena in which we are here interested, and as such it will be used.

Experiments 1, 3, and 4 (Tables I and II) show clearly that perfectly pure KCl solutions acting on cells not at any time exposed to such extraneous substances as sodium citrate, is uniformly injurious[5] regardless of whether the cells contain much or little potassium. NaCl, under similar conditions has little effect, either injurious or otherwise; an increase in the resistance of human cells might possibly be deduced from Table II. This will be referred to below.

These first experiments fail almost completely to show the reversal of the effects of Na˙ and K˙ which Ashby's experiments would lead us to expect when they act upon one of the two types of cells (K˙-rich and K˙-poor) instead of the other. But in these experiments the normal cells were washed once in a balanced salt solution. The effects of washing the cells are inadequately considered in Ashby's work and hence direct comparison would be unfair, especially as she does not tell how her control cells were treated. If washing in the modified Ringer-Tyrode solution increased the resistance, as might be expected from the work of Brinkman and van Dam,[6] then any increase in resistance caused by NaCl would be compensated for by a similar increase in the case of the washed control cells, and would thereby be concealed.

For this reason further experiments were done, in which the effects of washing in 0.9 per cent NaCl and in balanced salt solutions isotonic with 0.9 per cent NaCl were compared with the effects of incubation in the NaCl and KCl solutions. Ashby's method of evaluating resistance to hemolysis was so unsatisfactory for quantitative purposes that the precise method of hemolytic titration devised by the writer[4] was adapted to the present purpose as already outlined. Unwashed cells were used as controls, and all cells were suspended in just enough additional supernatant fluid to make them easy to handle, the final volume of the stock suspension of cells being only 15 to 25 per cent greater than that of the cell sediment obtained by centrifugation. In the first four experiments, each lot of cells had been suspended in a

[5] The term "injurious" is here to be understood as meaning only that the resistance of the red blood cells to hypotonic hemolysis is decreased.

[6] Brinkman, R., and van Dam, E., *Biochem. Z.*, 1920, cviii, 35.

volume equal to that of the blood from which it had been derived; when considerable hemolysis had occurred during incubation, correspondingly fewer cells were added to each test solution. This error
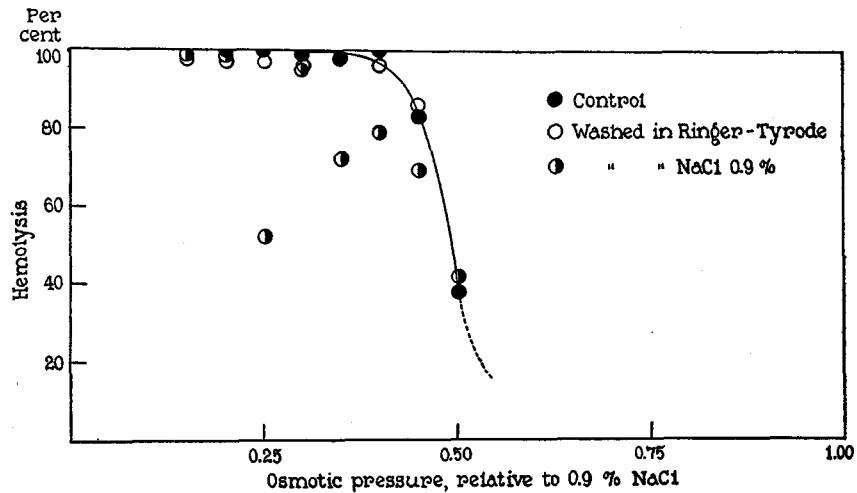

FIG. 1. The effect of different preceding treatments of dog erythrocytes on the degree of hemolysis (ordinates) produced by various dilutions of a 0.9 per cent NaCl solution (abscissæ). (Experiment 5.)
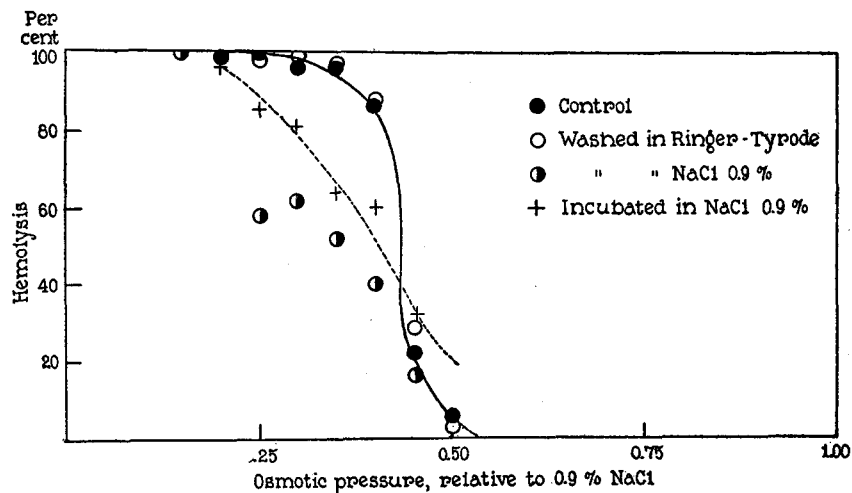

FIG. 2. The effect of different preceding treatments of guinea pig erythrocytes on the degree of hemolysis (ordinates) produced by various dilutions of a 0.9 per cent NaCl solution (abscissæ). (Experiment 5.)

was therefore eliminated by the change in procedure, and in addition
the protective effects of the salts or serum constituents carried in with
the supernatant fluid of the cell suspension were considerably reduced,
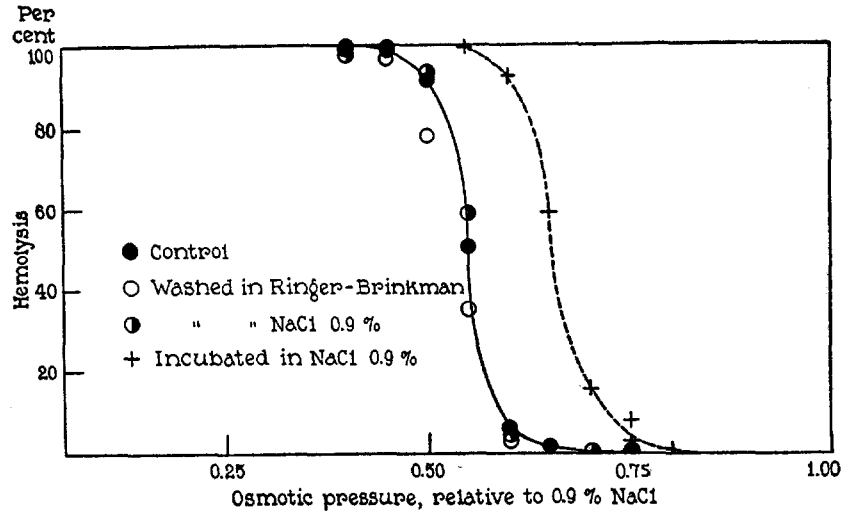

Fig. 3. The effect of different preceding treatments of dog erythrocytes on
the degree of hemolysis (ordinates) produced by various dilutions of a 0.9 per
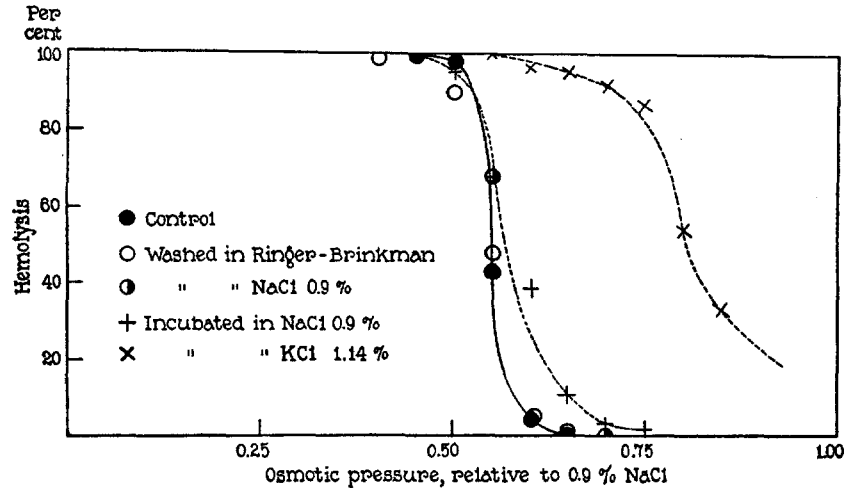cent NaCl solution (abscissæ).   (Experiment 6.)

Fig. 4. The effect of different preceding treatments of horse erythrocytes on
the degree of hemolysis (ordinates) produced by various dilutions of a 0.9 per
cent NaCl solution (abscissæ).   (Experiment 6.)

as was necessary to keep pace with the greater precision of the titration method.

Figs. 1 to 4 are the actual titration curves of these experiments, and the degrees of resistance calculated from them are given in Table III. In the first of the two experiments thus presented the incubation period of 2 days was so long that all but one of the lots of cells were nearly or quite completely hemolyzed, and no cells were left to be tested for resistance. In the other experiment the incubation period was reduced to 1 day, but even in this time the dog erythrocytes in the KCl solution were completely hemolyzed.

The error in these titrations is probably not much less than 3 per cent, and differences less than that may, for our purposes, be neglected. The error might have been greatly reduced by doing the tests in duplicate or triplicate, but this did not seem worth while since Ashby's limit of accuracy was from 5 to 15 or even 50 per cent, and the differences which she observed were even greater.

Washing the cells in a balanced solution has no material effect, whether modified Ringer-Tyrode solution or the solution employed by Brinkman and van Dam[6] be used, as in Experiment 6.[7]

Washing in NaCl 0.9 per cent had considerable effect in one case, that of guinea pig blood. The resistance was increased about 20 per cent, the irregularity of the titration curve making the uncertainty of this figure very great. In all other cases washing with NaCl solution had effects smaller than the experimental uncertainty.

The effects of incubation in pure salt solutions were as follows: potassium chloride led in all cases to a decrease in resistance, while

[7] The exact composition of this solution was as follows:

NaCl.. 7.60 gm.
NaHCO$_3$.. 1.70 "
KCl... 0.20 "
CaCl$_2$, 2H$_2$O.................................. 0.133 "
H$_2$O $q. s$..................................... 1,000 cc.

This may for convenience be called Ringer-Brinkman solution. These proportions of CaCl$_2$ and NaHCO$_3$ are supposed to determine the hydrion and calcion concentrations, as 0.45×10^{-7} N and 0.75×10^{-3} N respectively, but the solution here used had a pH of 8.35, (H· $= 0.45 \times 10^{-8}$), and (Ca··) was presumably about 0.075×10^{-4} N. The NaHCO$_3$ used was found to have contained more than the declared amount of Na$_2$CO$_3$.

sodium chloride decreased the resistance of dog blood markedly, that of horse blood by an amount hardly greater than the experimental error, and increased that of guinea pig blood by an amount considerably less than that due to washing in the same solution.

The observed amounts of hemolysis or hemochromolysis agreed with the measures of the volumes of intact cells and ghosts after incubation, and with the effects of incubation on resistance to hemolysis, all of them showing that injury was greatest when K·-poor cells were exposed to KCl, and less in the order: K·-poor, with NaCl; K·-rich, with KCl; K·-rich, with NaCl. Reduction of oxyhemoglobin was marked in the first of these combinations and decreased in the same order as the degree of injury; this fact suggests that autoxidation processes were accelerated when injury occurred. A cause and effect relation might conceivably be present, but it seems more likely that the phenomena are end-results of common causes.

DISCUSSION.

The results of my experiments are in conflict with Ashby's in that there is no case in which a clear-cut increase in resistance to hemolysis can be attributed to incubation of red blood cells of either type, K·-rich or K·-poor, in either NaCl or KCl solutions. The one case in which any increase of resistance is recorded, namely that of guinea pig blood in 0.9 per cent NaCl, is based on a very uncertain titration curve, and may well be meaningless. Accurately determined titration curves of other blood cells of the K·-rich group show no such effect. It must be noted, however, that merely washing another portion of these same guinea pig cells in 0.9 per cent NaCl was in itself enough to cause an apparent increase in resistance, which much exceeded that found after incubation in the same solution. Evidently the resistance decreased during incubation, and merely appeared to be increased because, due to the effect of washing, it started high; or else, possibly exposure to pure solutions of NaCl first increases and later decreases the resistance of red blood cells like those of the guinea pig.

The results of my experiments also conflict with Ashby's in that, irrespective of the type of cell, KCl was always more injurious than NaCl. The conflict of results may be due to impurity of materials

used by Ashby, to the use, by her, of such extraneous substances as sodium citrate, to mechanical injury of the cells during defibrination in the case of the present experiments, or to the fact that we have studied different phases of time processes which produce opposite effects according to the stage at which they are interrupted.

Mechanical injury due to defibrination as used in these experiments may be discounted since the serum of the blood used was found to be but slightly discolored by hemoglobin even after the blood had been standing at 3–25°C. for the duration of the experiment.

The presence of traces of citrate or of bivalent metals dissolved from glassware and carried into the incubation mixtures in Ashby's experiments may have so far antagonized the effects of really pure KCl or NaCl as to mask their real effects. If so, Ashby's conclusions do not concern $K^{\cdot}$ and $Na^{\cdot}$, but unknown mixtures of salts in partially balanced solutions.

The study of the last possibility requires further experiments with solutions of known purity in which the blood cells, free from substances foreign to blood, are incubated for various periods of time and their resistance to hypotonic hemolysis then determined by precise titration. In view of the unknown influence exerted by serum constituents it is doubtful whether more profitable lines of study might not be found. Enough has been said to demonstrate that Ashby's work requires much substantiation before we may regard it as proven that the effects of $Na^{\cdot}$ and $K^{\cdot}$ on red blood cells are opposite and that the sense of their effects is reversed according to whether the erythrocytes used are $K^{\cdot}$-rich or $K^{\cdot}$-poor. With this, her conclusion that the effects of $K^{\cdot}$ and $Na^{\cdot}$ are due wholly to the nature of the cells upon which they act falls to the ground also.

SUMMARY.

Ashby's work on the effects of KCl and NaCl on the resistance to hypotonic hemolysis of $K^{\cdot}$-rich and $K^{\cdot}$-poor erythrocytes has been repeated with great attention to purity of materials and refinement of technique. The results fail to agree with those of Ashby.

1. KCl produces greater loss in resistance to hypotonic hemolysis than does NaCl, irrespective of the species of the animal from which the cells are taken.

2. While cases of an increase in resistance have been encountered in my experiments, they are either very slight, or else the particular determination is subject to very great uncertainty. The great increases in resistance found by Ashby are not even approached in any of the present series of experiments.

3. Ashby's generalization that KCl and NaCl have opposite effects on red blood cells, and that the sense of these effects depends on whether the cell is K·-rich or K·-poor is not substantiated.