THE INFLUENCE OF TEMPERATURE ON HEMOLYSIS IN HYPOTONIC SOLUTIONS.[1]

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During the year 1905–1906 it was my privilege to assist Dr. Theobald Smith in determinations of the resistance of the erythrocytes of horses to hemolysis by water. The method in use was a modification of that originated by Hamburger in which the resistance of the cells is measured by the amount of neutral salt required to protect them against the laking effect of distilled water. Struck by the accuracy of the results obtained and familiar in some degree with the conditions under which the hemolytic effect of the complex hemolysins is determined, I was led to try the influence of temperature on the destruction of erythrocytes in dilute solutions, a phenomenon supposed to depend on purely physical conditions.

The experiments made have shown conclusively that hemolysis becomes progressively more marked as the temperature is decreased from 37° C. to 5° C. Aside from any importance which may be ascribed to this fact, the experiments deserve to be recorded in some detail because they illustrate the superiority in point of delicacy and accuracy of the technique employed as contrasted with the original method on which it is based.

Hamburger[2] is the only experimenter who until very recently had attempted to determine the influence of temperature on hemolysis in hypotonic salt solution. He tested beef corpuscles in solutions of KNO_3, NaCl, and cane sugar in varying concentrations, and at temperatures 0° C., 14° C. and 34° C. He found no difference in the amount of laking at these temperatures. He estimated the

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[2] The original article (*Arch. f. Anat. u. Physiol., Physiol. Abt.*, 1886, 476) is transcribed without essential changes in this author's Osmotischer Druck u. Ionenlehre, Wiesbaden, 1902, i, 172.

degree of hemolysis by comparison of the point of beginning hemolysis in the separate series. It will be developed later that it was this use of the point of beginning hemolysis which accounts for his failure to detect differences dependent on temperature.

Kiss[3] in the present year publishes observations in accord with those here presented. He used beef erythrocytes only, and his method was such that he was forced to base his judgment on relatively small percentages of corpuscles hemolyzed. This author corrects this mistake of Hamburger's as to the influence of temperature on hemolysis, and undertakes on the basis of his results in this respect and others even more important to combat the whole idea that the erythrocyte is essentially a semipermeable membrane enclosing fluid contents, and that physical and chemical hemolytic agents act in accordance with what would be expected on such an analogy. As will be seen, the present paper was constructed on the older conception as a foundation. As I am not well prepared by experience to discuss most phases of the work of Kiss and as I have been chiefly concerned with method and fact rather than theory, it seems best to allow my paper to stand as written, omitting an extended discussion of the important monograph of this author.

As already stated I have used in my work the modification of Hamburger's method introduced by Theobald Smith.[4, 5] For a detailed description, the interested reader is referred to the original papers. Briefly stated the method is as follows: sodium chloride solutions of graded strength from about .40 per cent. to about .70 per cent. varying by a constant difference of .02 per cent. are carefully prepared and preserved. At the time of use a given quantity of each solution is measured into a small test tube. The tubes used are selected to approach closely a uniform diameter. Each conveniently takes 3 c.c. of fluid. The concentrated red blood corpuscles are added to the fluid from a pipette, the measure being by drop.

[3] Kiss, J., Das periodische System der Elemente und die Giftwirkung, Wien u. Leipzig, 1909.

[4] Smith, T., The Pathological Effects of Periodic Losses of Blood, *Jour. of Med. Research*, 1904, xii, 385.

[5] Smith, T. and Brown, H. R., The Resistance of the Red Blood Corpuscles of the Horse to Salt Solutions of Different Tonicities, before and after Repeated Withdrawals of Blood, *Jour. of Med. Research*, 1906, xv, 425.

The tubes are thoroughly shaken at once. After a given time, preferably several hours, the amount of hemolysis in each tube is ascertained by comparing the color of the supernatant fluid with that in a series of tubes of the same diameter containing varying percentages of hemoglobin. The hemoglobin solution is freshly prepared each time by laking a quantity of erythrocytes equal to that used in each tube of the test series in a quantity of distilled water also equal to that of the salt solution used in each tube. From a multiple of these amounts used as a 100 per cent. hemoglobin solution, suitable dilutions are made. Where experiments are conducted with a single suspension of blood corpuscles no great precaution need be taken to secure comparative results. If the corpuscles of different individuals are to be used, then one must refer each test series to a hemoglobin solution obtained with the particular blood suspension used in that series, or at least assure one's self that the suspensions are of equal value. If normal horse blood is used it suffices to allow the corpuscles to sediment over night in order to attain suspensions of equal hemoglobin content. If the blood of cattle, rabbits or guinea-pigs is used one must centrifugate at high speed to a constant corpuscle volume to attain this result. If the corpuscle suspension is well concentrated the factor of error due to the adherence of a small amount of blood serum is a constant and is too small to embarrass the result.

The experiments here reported were carried out by the following extension of the method as above outlined. A series of tubes was prepared for each temperature to be considered, and filled with 3 c.c. of the varying strengths of salt solution. The tubes of each series were placed in the room, ice chamber, or thermostat as the case might be and allowed to remain half an hour to attain the required temperature. The erythrocytes which had been preserved in the ice chest were in the meantime allowed to attain room temperature. The several series of tubes were brought out separately, the corpuscles added, shaken and returned at once to ice chest or thermostat. The period of temperature inaccuracy was thus but two or three minutes. After five hours, a convenient but not essential period, the tubes were brought out and the percentage of hemolysis ascertained.

The results obtained are shown in the following tables and charts. The few experiments given are but examples of a considerable number which have given the same general result without a single exception.

TABLE I.

Sodium Chloride Solution "C." Erythrocytes of Horse 95, Concentrated, 2 Drops. Time of Exposure 5 Hours.

Per cent. s. s.	Per cent. hemolysis.		
	38° C.	28° C.	5° C.
.40	100	100	100
.42	99	99	100
.44	90	90	100
.46	75	85	99
.48	25	50	90
.50	8	17	70
.52	3	5	55
.54	1.25	2	22
.56	.5	1	7
.58	0	.5	2
.60	0	0	.25
.62	0	0	0
.64	0	0	0

TABLE II.

Horse Erythrocytes. Sodium Chloride Solution.

Per cent. s. s.	Per cent. hemolysis.			
	42° C.	35° C.	20° C.	6° C.
.54	38	8	10	30
.56	38	5	6	22
.85	2	0	0	0

Erythrocytes—Horse 96—2 drops.

Salt solution 3 c.c. After five hours at the temperature given the tubes were shaken and put in the ice box over night. Readings at 20 hours.

That as the temperature is increased from 5° C. to 37° C. the amount of hemolysis is decreased is clearly shown in each instance. This is irrespective of the kind of blood used. It occurs when either sodium chloride or cane sugar is used to give tonicity to the solution. It may also be stated that the difference is clearly marked in the first few minutes of contact of corpuscle and solution, being discernible as soon as the laking has gone far enough to permit

TABLE III.

Calf Blood Corpuscles.

Per cent. s. s.	Per cent. hemolysis.			
	42° C.	38° C.	28° C.	5° C.
.40	—	—	100	100
.42	—	—	100	100
.44	—	—	100	100
.46	100	—	99	100
.48	99	—	98	100
.50	90	90	95	99.5
.52	62	80	82	98
.54	45	60	65	95
.56	25	45	48	85
.58	17	25	32	65
.60	10	12	18	38
.62	5	7	7	20
.64	3	3	4	12
.66	2	—	1	8

Calf blood centrifugalized ½ hr. to constant corpuscle volume, 2 drops. Salt solution "D"—3 c.c. Time 5 hours.

TABLE IV.

Guinea-Pig Erythrocytes. Sodium Chloride Solution.

Per cent. s. s.	Per cent. hemolysis.			
	42° C.	35° C.	20° C.	6° C.
.46	18	20	25	35
.48	8	11	15	23

Guinea-pig cells, 2 days old, centrifugalized to constant volume, 2 drops. Time 5 hours.

Salt solution 3 c.c. Control corpuscles in .85 per cent. s. s. heated to 42° C. Shown hemolysis + 2 per cent.

TABLE V.

Rabbit Erythrocytes. Sodium Chloride Solution.

Per cent. s. s.	Per cent. hemolysis.			
	43° C.	37° C.	26° C.	5° C.
.48	60	85	99	100
.50	45	60	95	98
.52	18	25	35	65
.56	4	5	10	15

TABLE VI.

Horse Erythrocytes. Cane Sugar Solution.

Per cent. sugar.	Per cent. hemolysis.		
	38° C.	27° C.	5° C.
3	100	100	100
3.25	97	98	100
3.50	80	90	95
3.75	65	80	85
4.00	45	55	65
4.25	25	28	45
4.50	15	18	32

Corpuscles—Horse 95—2 drops. Sugar solution 3 c.c. Exposure 5 hours. Ice box. Readings 24 hours.

TABLE VII.

Rabbit Erythrocytes. Cane Sugar Solution.

Per cent. sugar.	Per cent hemolysis.			
	43° C.	37° C.	26° C.	5° C.
4.50	28	25	35	75
5.00	8[a]	7[a]	7[a]	35

Erythrocytes. 2 drops of concentrated suspension.
Sugar solution 3 c.c.
a = pronounced agglutination.

comparison. Furthermore the difference once developed by exposure to the different temperatures is maintained if the tubes are then brought to an equal temperature. Thus, if, after the five-hour reading, the tubes are all shaken and put in the ice box and the readings taken again at the end of twenty-four hours, it will be found that there has been a slight and equal increase in the hemolysis in all tubes but that the difference developed by the first exposure to varying temperatures has been maintained.

The facts shown by the tables may be somewhat further analyzed with advantage. It was pointed out in the work of Smith and Brown above cited that if the hemolysis resulting in different percentages of salt solution be graphically illustrated by plotting the salt solution percentages on the abscissæ, and the percentages of corpuscles hemolysed in each per cent. salt solution as ordinates, the resulting curve in normal horses approaches the form of a simple, unimodal, nearly symmetrical curve with range limited in

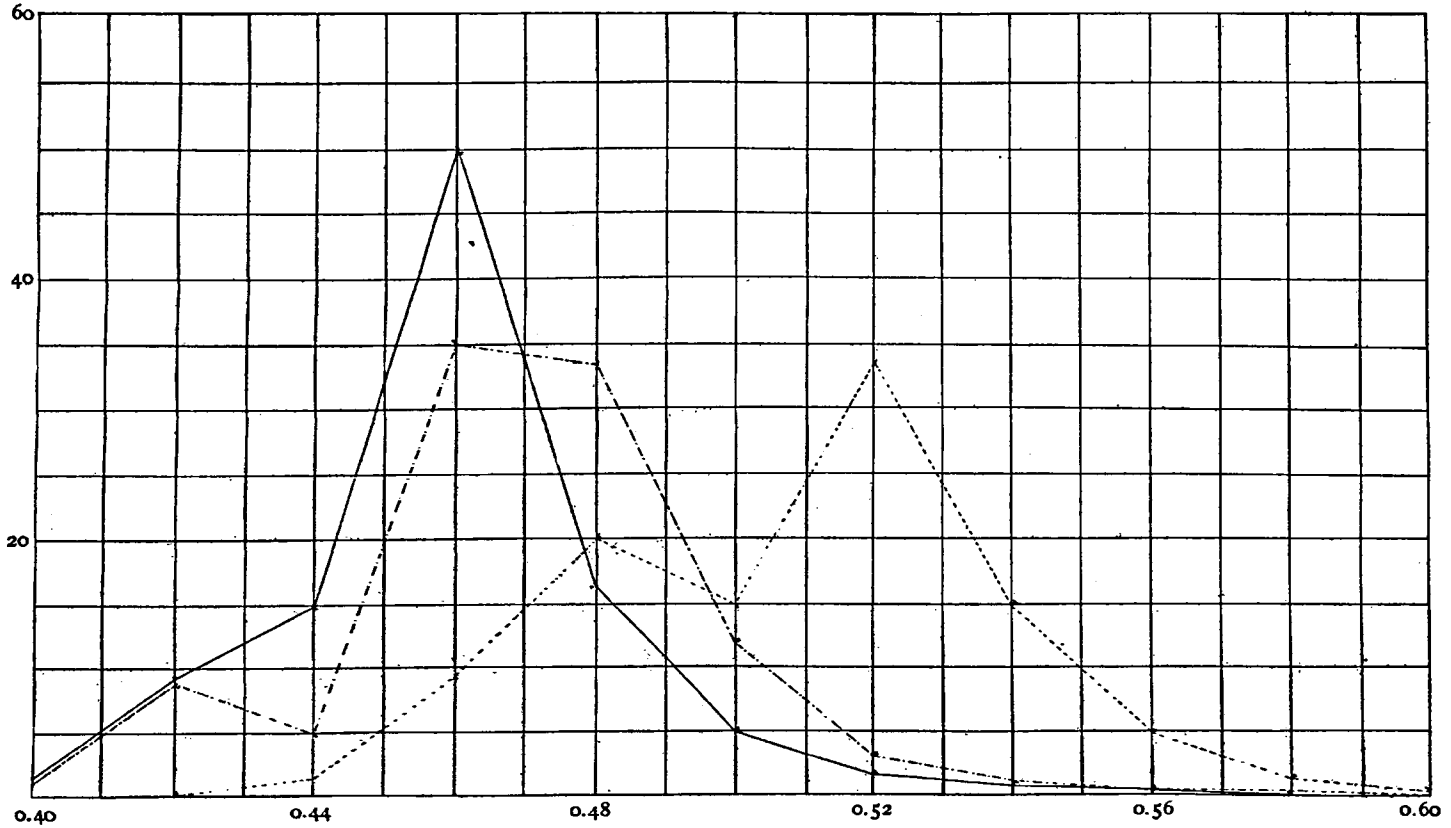

CHART I. The chart has been prepared from Table I; solid line represents 37° C.; broken line, 28° C.; and dotted line, 5° C. Percentages of sodium chloride solution are plotted on the abscissa. Percentages of erythrocytes destroyed between consecutive strengths of salt solution are plotted on the ordinate.

both directions. Chart I is the experiment of Table I represented in this way. It will be noted that Curve *a* representing the laking at 37° C. approaches nearly to the form of curve described as normal for the horse erythrocytes. Moreover, it will be seen that the form of the curve undergoes considerable modification as the temperature is lowered. To apply the reasoning of Smith and Brown to this experiment it may be observed that if the effect of lowering the temperature were to uniformly lower the resistance of the red corpuscles to water laking, the form of Curve *a* would be practically preserved but the whole curve would be moved considerably to the right on the chart. That the inner form of the curve is greatly changed while the whole curve is moved to the right indicates, not only that the general resistance to the laking effect of water is decreased as the temperature decreases, but that some groups of corpuscles are much more affected than are others. I am not prepared to discuss the particular form taken by these modified curves at present. Other experiments charted in the same way have shown internal changes in the curve of the same general nature, but the number of experiments is too few to make any attempt at generalization possible at this time.

Examination of the tables, or better the chart, makes it apparent that the differences between the older results obtained by Hamburger and those here presented are accounted for by a technical departure, that of developing the whole resistance curve, instead of relying on the point of beginning hemolysis for the information sought. It is true that the differences so pronounced in the center of the curve are generally maintained at either end. But at the ends of the series the total quantity of corpuscles of which effective use is made in the test, would be from one-fourth to two or five per cent. of the whole, depending on the closeness with which the end-point is determined, and the percentage difference in hemolysis relied on would be from one-fourth to two or five per cent. These small differences in amount of hemolysis, while recognizable when controlled by the striking differences at the center of the curve, would probably be neglected as within the probable limits of experimental error, if taken alone. If to correct the fault of using too small a number of corpuscles in the regions of beginning hemolysis

a larger absolute quantity of corpuscles were used in the series, the effect would be chiefly to extend the range of hemolysis. This would put the point where laking was first noticeable in a higher concentration of salt solution, but would not increase the absolute amount of laking at the end-point sufficiently to make the method more accurate. At the middle of the series, on the other hand, over forty per cent. of the total number of erythrocytes employed may be affected by a change of two-hundredths of one per cent. of salt in solution or its equivalent in changed corpuscle resistance.

How is the increase in hemolysis with decreasing temperature to be accounted for? Two factors must be considered, the erythrocytes and the saline solution as well as the relation between the two. The relation between erythrocyte and surrounding medium has usually been considered in this connection to be analogous to the relation between a cell with semipermeable membrane, having contents of certain osmotic tension and immersed in a medium of somewhat similar osmotic tension. So long as the osmotic tensions within and without the cell are equal the membrane would be under no strain. After finding no change in the amount of hemolysis with changing temperature Hamburger reasoned that the supposed fact was in harmony with these physical conditions. The fluids within and without the cell would be altered together, in their osmotic tensions, as the temperature changed; there would consequently be no occasion for fluid to pass in either direction. This is certainly correct for the case in which the osmotic tensions within and without the cell are equal. But equality can never be the case under the conditions as at present considered. The intra-corpuscular tension is usually stated to be a little above or below the equivalent of nine-tenths per cent. of sodium chloride solution according to the animal from which the blood is derived. The percentage of salt solution in which the laking is most effectively studied is just above or below five-tenths per cent. sodium chloride, or somewhat more than half of the intra-corpuscular equivalent.

According to the law that osmotic pressure of substances in solution increases directly with the absolute temperature, the absolute increase when the temperature is raised from 5° C. to 35° C. would be to 308/278 of the pressure of the solution with which we

started. With a .50 per cent. salt solution the increase expressed in terms of percentage salt solution would be .056; with a .90 per cent. solution as a starting point the absolute increase expressed in the same terms would be .098. If simply this law were in operation, therefore, an increase in temperature within the limits used should increase the relative concentration of the corpuscular contents as compared with that of the surrounding medium by an amount equivalent to the difference between the above increases. Expressed in terms of salt solution concentration this difference would be .041 per cent. This would, if acting alone, cause an increase in hemolysis with increasing temperature. The difference would be about equal to that which we actually find to occur in the opposite direction.

Other purely physical factors operating in the internal and external fluids are the degree of electrolytic dissociation and the changes in volume. The differences due to these changes would be relatively slight and may be safely disregarded.

Considering the purely physical factors involved has then served to emphasize the value of the difference as experimentally determined. The factor remaining and the one to the properties of which we must look for an explanation of the difference in degree of hemolysis is the cell membrane. The properties of this membrane are almost unknown at present. It has been shown by a number of methods not to be perfectly impermeable to various ions. If this partial permeability were less at lower temperature the tendency for an equilibrium to be established between the contents of the cell and the surrounding medium would be less effective in protecting the cell against the results of sudden osmotic pressure changes in the latter. The membrane can hardly be of the rigid character of the cellular membrane of plant cells. It must rather be of viscous or semi-gelatinous nature kept in place by its lack of solubility in the fluids and substances with which it is in contact. If on cooling, the rigidity of the membrane were increased after the manner of gels in general it would become more friable, less elastic and the quantity of water needed to swell the cell to the breaking point would be less.

It is not worth while to speculate further along this line, how-

ever, especially in view of the probability that the above-mentioned analogy is not a perfect one. When we are considering only physical changes in the surrounding medium it may be entirely pertinent to regard the cell as a definite membranous sack enclosing fluid contents. But when the question is approached from the point of view of the erythrocyte it is much more probable that we have to do with a mass of colloids and electrolytes in more or less close physical and chemical apposition. The conditions in this mass must be very complicated and it would be impossible at the present time to draw reasonable conclusions as to the manner of action of any condition or substance which affects the whole mass.

So far I have commented only on the changes which take place with temperature variation between the limits of a point somewhat above freezing and the normal body temperature. It will have been noted by those who have examined the tables presented that they include some observations at temperatures of from 42° C. to 43° C. At these temperatures I have always found an increase in hemolysis with horse erythrocytes. The hemolysis with rabbits, calf and guinea-pig erythrocytes on the contrary has continued to decrease up to 42° C. to 43° C. Whether the observed differences are constant and represent a definite species character may be determined only by future observations.

SUMMARY.

Decreasing the temperature from 37° C. to 5° C. perceptibly and regularly increases hemolysis in hypotonic sodium chloride and cane sugar solutions, when the erythrocytes of a number of the common mammals are considered. The measurements were carried out with Smith's modification of the method of Hamburger. If following the original method of Hamburger one relies on the point of beginning hemolysis as an index of corpuscle resistance, the facts are not brought out clearly. The effect is in the opposite direction from that which would prevail if the laws governing change of osmotic pressure with change of temperature were the influential factors. The results possibly depend on some change in the permeability or consistence of the erythrocytic protoplasm considered as a semi-permeable membrane.