THE RATE OF LOSS OF POTASSIUM FROM HUMAN RED CELLS IN SYSTEMS TO WHICH LYSINS HAVE NOT BEEN ADDED

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Prolytic cation exchanges have now been described in a variety of systems containing human red cells and lysins in hypolytic concentration (Ponder, 1947 *a*, *b*, *c*; 1948 *a*, *b*), and an Na-K exchange has also been observed to occur when the cells are suspended in isotonic or hypotonic NaCl containing no added hemolysins. In systems containing lysins, and particularly when the prolytic losses are large, the loss of K increases with time until the K concentration inside the cell is approximately the same as that in the medium outside; *i.e.*, the exponential function of time which describes the K loss approaches the line K = 1.0 as an asymptote (Ponder, 1948 *b*). The course of the K loss (and Na gain) in systems containing no added lysin, on the other hand, has not been so clearly established. There are very definite indications (Davson, 1937, Rapoport, 1947) that the losses are rapid at first and that they slow down so that a new steady state, remote from the equilibrium state at which K = 1.0, is reached; most of the experiments which point to this conclusion, however, are of short duration, and one of the points which has been brought out by the investigations of prolytic ion exchanges is that the course of the curves which relate ion exchanges to time can be ascertained only by experiments which extend over long times. If the curve for the time-course of K losses in systems containing some lysins is different from that for the time-course of K loss into lysin-free NaCl, in that the latter terminates in a new steady state while the former ends in an equilibrium, it is not certain that the time-course of the ion exchanges is not of an intermediate form in systems containing other lysins or the same lysins in smaller concentrations. Many relations are possible, and those which are characteristic of a given system require to be found by systematic exploration.

This paper is concerned with the results obtained in four kinds of system: (1) washed red cells in saline at 4°C., (2) washed red cells in saline at 25°C., (3) washed red cells in saline at 37°C., and (4) washed red cells in systems, at 4°C., 25°C., and 37°C., containing hypotonic saline, glucose, or a number of other substances the addition of which throws light on the nature of the phenomena observed. A consideration of what happens in these four types of system is sufficient to outline the principal aspects of the problem. It should be pointed out, however, that the purpose of this investigation is to describe the types of relation found between the rate of K loss and time rather than to answer the

many questions arising in connection with the forms which the relations take under different conditions.

Methods

It is essential that the extended observations of the rate of K loss from the red cells be carried out under aseptic conditions, since contamination of the system by bacteria may be tantamount to the addition of a hemolysin. A satisfactory procedure is one based on the methods used by Osgood (1939) for marrow culture. All manipulations are carried out with syringes and in 15 ml. tubes covered with vaccine caps. The caps are inserted in the tubes before sterilization, and the blood, washing fluids, etc., are introduced with sterile syringes and needles after wiping the cap with 95 per cent alcohol and inserting an air vent consisting of a needle connected by sterile rubber tubing to an air filter. The possibilities of contamination can be reduced by packing each syringe, etc., in its own pack, and by sterilizing each needle in a separate rubber-capped glass tube; by suitable arrangement of the equipment so as to constitute almost a closed system, it is possible to work without contamination in an ordinary laboratory room. The various saline solutions are sterilized in vaccine-stoppered flasks fitted with air vents and air filters.

The blood is drawn from healthy donors into sterile flasks containing a few drops of liquamin (a preparation of heparin for intravenous use). Almost all the systems with which this investigation is concerned are composed of washed cells added to saline,[1] saline plus glucose, etc., and are made by adding 2 ml. of a cell suspension of volume concentration 0.4 to 10 ml. of saline, saline plus glucose, etc. Since a small quantity of the system is needed for the preparation of the standards representing complete hemolysis and complete K loss, a 2 ml. sample can be withdrawn at only five intervals of time from each system of 12 ml. volume; these intervals require to be suitably spaced as a result of experience.

The completed systems are kept mixed by inverting the tubes containing them every 1 to 2 hours, and are oxygenated by drawing sterile air through an air filter as the withdrawals are made. These withdrawals are made with 2 ml. syringes, a needle connected with an air filter being inserted as an air vent. The systems are tested from time to time for their sterility. When determinations are made at 37°C. and also in some experiments at lower temperatures, the systems are contained in small rubber-capped Erlenmeyer flasks attached to a shaking apparatus in a constant temperature bath. By using long needles inserted through the rubber caps, sterile air or gas mixtures moistened by bubbling through warm saline can be pumped through the systems. Samples are withdrawn at intervals, as already described.

Washed cells are used in most systems, the washing being carried out at room tem-

[1] The suspension medium may be either freshly prepared NaCl (172 m. eq./litre) or a NaCl-buffer composed of a mixture of NaCl in a concentration of 10 gm./litre and various proportions of M/15 NaH_2PO_4 and M/15 Na_2HPO_4. The effect of adding buffer and controlling the pH within the range 6.0 to 7.5 is quite small, the K losses being only about 10 per cent smaller in a system buffered at pH 7.5 than they are in freshly prepared unbuffered NaCl (pH 6.0 to 6.5).

perature (25°C.). A small sample of the completed system is set aside for K analysis. This step is important, for considerable amounts of K may be lost during the washing, etc., and the initial value for the concentration gradient of K may be considerably lower in the case of washed red cells than in the case of unwashed ones. When unwashed cells are added to complete the systems, small volumes of the latter are set aside in a similar way; the cells are spun down, packed, and their K content is determined.

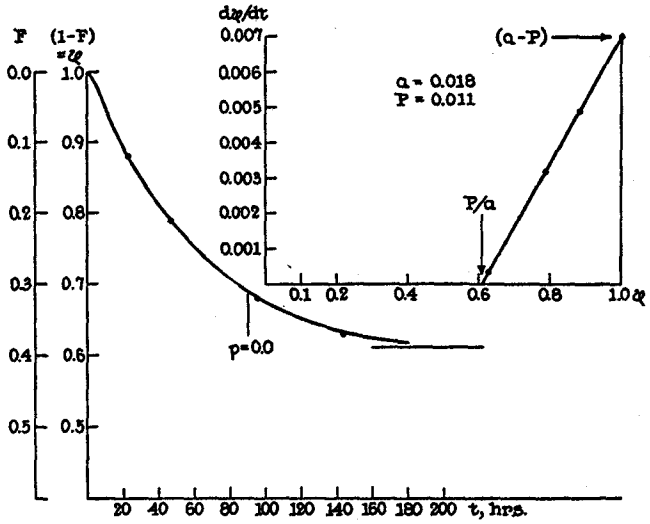

FIG. 1. Loss of K from washed human red cells into 172 m. eq./litre NaCl at 4°C. Ordinates, fraction of initial K lost from intact cells, F; φ or $(1 - F)$. Abscissa, time in hours. The point $p = 0.0$ marks the beginning of hemolysis. Inset, analysis of the curve in terms of P and a.

1. K. Losses at 4°C.

A typical curve relating the loss of K from washed human red cells into 172 m. eq./liter NaCl at 4°C. is shown in Fig. 1. The experimental values of K, the K lost expressed as a fraction of the initial K content of the cells K_0, are given in Table I together with the amount of lysis in the system ($p = 1.0$ for complete hemolysis). From the values of K and of p, the fraction of their initial K which has been lost by the intact cells of the system can be calculated: it is

$$F = (K - p)/(1 - p),$$

and the fraction of their initial K which the intact cells of the system still contain is $(1 - F)$. The values of $(1 - F)$ decrease with time, in such a way as to suggest that a new steady state with a value of $(1 - F)$ in the neighborhood of 0.6 is reached after very long times. There are several possible ways in which such a steady state might be reached and maintained (Ponder, 1948 *a*);

the cells may have a membrane which goes through a phase of permeability to K, and then becomes impermeable to K again, there may be an active ion transfer mechanism which regulates the distribution of ions such as K and Na, or the ionic distribution may be maintained by "ion-binding" processes involving the material in the cell interior rather than by a permeability process as ordinarily understood. The observations of Davson (1937) on the loss of K into hypotonic media constitute the principal experimental evidence (apart from the shape of the curve relating K loss to time) in favor of the possibility that a membrane undergoes a transient phase of increased permeability; the observations themselves and the conclusions drawn from them, however, are questionable (see section 4, below), and do not form a satisfactory basis for a theoretical treatment. The last possibility is also unsuitable as a starting point for a general formulation because it is too vague; this leaves for consideration

TABLE I

Relation of K Loss to Time at 4°C.

t	K	ϕ	F
hrs.			
24	0.12	0.00	0.12
48	0.21	0.00	0.21
96	0.32	0.02	0.31
144	0.37	0.04	0.37

the possibility that the loss of K and the attainment of the steady state are due to changes in the activity of an ion transfer process.

Suppose that $(1 - F)$ or φ, the quantity of K which the red cell contains, is determined by a process which causes K to accumulate at a rate P, and that K tends to leave the cell at a rate Q because the cell is permeable to it. For the time being, also suppose that the concentration of K in the medium surrounding the cell is small as compared with the concentration of cell K. The rate at which K leaves the cell will then depend on φ itself and on a diffusion constant a which may have the special meaning of a permeability constant and which may vary explicitly with time. The net rate of decrease $-d\varphi/dt$ is the difference between the rate of accumulation and the rate of escape,

$$-d\varphi/dt = P - a\varphi \tag{1}$$

To determine a, draw tangents to the experimental curve and plot their slopes $d\varphi/dt$ against φ (inset of Fig. 1). The result in this case is a straight line with a slope of $a = 0.018$, making an intercept $(a - P) = 0.007$ on the ordinate at $\varphi = 1.0$. The value of P is accordingly 0.011. The line also makes an intercept on the φ − axis where $\varphi = 0.61$. This gives the position of the

asymptote, $\varphi_\infty = 0.61$, which the experimental curve approaches at infinite time.[2]

In sterile systems containing human red cells at 4°C., this type of curve is obtained consistently, and the disturbing possibility that the apparent attainment of a new steady state is due to the restriction of the period of observation (Ponder, 1948 *b*) seems to be disposed of. Table II gives values of φ_∞, a, and P

TABLE II

Values of φ_∞, a, and P at 4°C.

Donor	φ_∞	a	ϕ at 144 hrs.	P
1	0.49	0.0095	0.08	0.0047
2	0.37	0.0054	0.08	0.0020
3	0.56	0.0091	0.04	0.0052
4	0.58	0.0097	0.03	0.0056
5	0.61	0.0180	0.04	0.0110
6	0.65	0.0140	0.07	0.0091
7	0.50	0.0100	0.06	0.0050
8	0.60	0.0110	0.04	0.0066
9	0.51	0.0092	0.04	0.0047
10	0.50	0.0090	0.05	0.0045
Average.....				0.0058

found in a series of observations at 4°C. on systems containing the cells of different donors.

The K losses shown in Fig. 1 were determined at intervals of 24, 48, 96, and 144 hours after the beginning of the experiment, so as to define as much of the course of the curve as possible by the position of four points. As in the case of the curves obtained in experiments on systems in which lysins were present initially (Ponder, 1948 *b*), the course of the K loss during the first few hours is therefore left undefined. These losses are very small in systems at 4°C., and it is only at higher temperatures, and even then only under special circumstances, that they become easily measured; the detailed consideration of the K losses occurring during the first few hours is accordingly better left until the events in systems at 37°C. are described. In the meantime, it is sufficient to say that at time $t = 0$ the cells of the system are apparently

[2] The solution of the differential equation (1) when a is a constant is

$$\varphi = (1 - P/a)\, e^{-at} + P/a$$

so φ_∞ also equals P/a. The value of $(1 - \varphi_\infty)$ is the same as that denoted by K_∞ in the expression

$$dK/dt = k(K_\infty - K)$$

which describes the experimental curve from the standpoint of the amount of K lost by the cell and found in the surrounding medium (Ponder, 1948 *a*, expression 1).

in a steady state (approximating to the steady state which they would have maintained in the blood stream if left there), and that the value $\varphi = 1.0$ is itself equal to P/a_0, where P is the rate of accumulation (supposed to remain constant) and a_0 a constant regulating the rate of escape characteristic of the cells in their "normal" state in plasma in which the net escape of K is zero. What we are assuming to happen is that a increases so that K is lost and a new steady state defined by φ_∞ is reached. If the change in a is not an instantaneous one, the transition from one steady state to another occurs along a sigmoid path (Ponder, 1944), and the course of the relation between

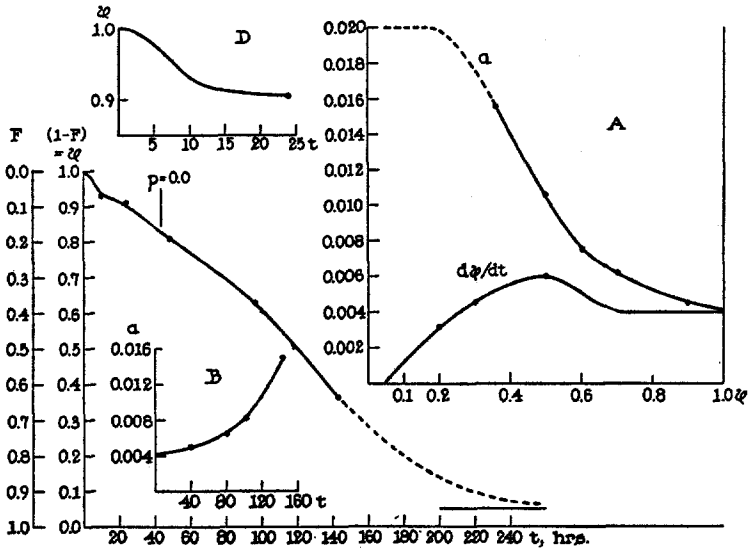

FIG. 2. Loss of K from washed human red cells into 172 m. eq./litre NaCl at 25°C. Ordinates and abscissa as in Fig. 1. Inset A, values of a, P being assumed to be zero. Inset B, values of a as a function of time. Inset D, the initial part of the curve shown on an enlarged scale.

φ and t during the first 6 to 12 hours is probably always similar to that shown in Fig. 2, inset D (*cf.* Ponder, 1947 *b*, footnote 3).

This analysis is based on the assumption that the rate of K loss from the red cell is due to a change in the value of a, the constant which regulates the rate of diffusion of K from the inside of the cell to the outside. Since what is measured is $d\varphi/dt$, the difference between P and $a\varphi$, it is equally possible that the losses of K are due to P varying explicitly with time; the same experimental curve would result, for example, from P suddenly changing from P_0 at $t = 0$ to $0.61P_0$, a remaining constant. Further, both a and P might vary with time. This analysis does not distinguish between these possibilities, its purpose being to provide a method, somewhat non-committal in the meantime, of dealing with the experimental observations. The possibility that P varies with time will

require to be considered when the effect of agents which alter metabolism, such as NaF and NaCN, is described (see section 4, below).

2. K Losses at 25°C.

A typical curve for the rate of the loss of K from human red cells into 172 m. eq./liter NaCl at 25°C. is shown in Fig. 2. This curve is plotted from experimental data (Table III) treated in exactly the same way as those from which the curve of Fig. 1 is constructed. Unlike the latter, it shows no indication of reaching any steady state other than that at which the K concentration inside and outside the cell would be equal. If the course of the K loss during the first few hours is left unconsidered in the meantime, the curve proceeds down-

TABLE III

Relation of K Loss to Time at 25°C.

t	K	p	F
hrs.			
24	0.09	0.00	0.09
48	0.19	0.01	0.18
96	0.40	0.05	0.37
144	0.68	0.12	0.64

wards almost linearly from the origin at $\varphi = 1.0$, $t = 0$ until the last experimental point is reached at $\varphi = 0.36$, $t = 144$.

Beyond this, good experimental values cannot be obtained because of the increasing amount of hemolysis. The probable course of the curve can be conjectured, however, by analogy with the course of curves for systems containing lysins (Ponder, 1948 *b*), and is shown in Fig. 2 as the dotted portion of the curve approaching an asymptote in the neighborhood of $\varphi = 0.05$; at this value of φ, the K concentrations inside and outside the cells would be equal.[3] P can accordingly be supposed to be equal to zero in the expression

$$a = \frac{P + d\varphi/dt}{\varphi} \tag{2}$$

and a can be evaluated on the assumption that the final value of P applies to the whole curve.[4] The result is shown in inset A of Fig. 2. The value of

[3] The value of φ at which the K concentrations inside and outside the cell are equal is approximately v/V, where v is the volume of the fluid phase in the cells and where V is the volume of the surrounding medium. In these systems $v = 0.56$ ml. and $V = 11.76$ ml., so $\varphi_\infty = 0.047$. If V were infinitely large, φ_∞ would be zero.

[4] It is quite likely that P is zero only when t is great and that it is positive when t is smaller. If this were so, the values of a would be larger than those calculated on the basis of P being zero; *i.e.*, the values of a shown in the inset of Fig. 2 are minimum

$d\varphi/dt$ remains comparatively constant as φ decreases, passes through a maximum, and then decreases; the value of a increases from about 0.004 at $\varphi = 1.0$ to about 0.020 at $\varphi = 0.05$.

Since φ decreases with time and a increases as φ decreases, a can also be shown as a function of time (Fig. 2, inset B). This way of looking at the situation suggests that the difference between the type of relation found at 4°C. and that found at 25°C. is that the constant a, which is equivalent to a permeability constant in the way in which it regulates the rate of escape of K from the cells

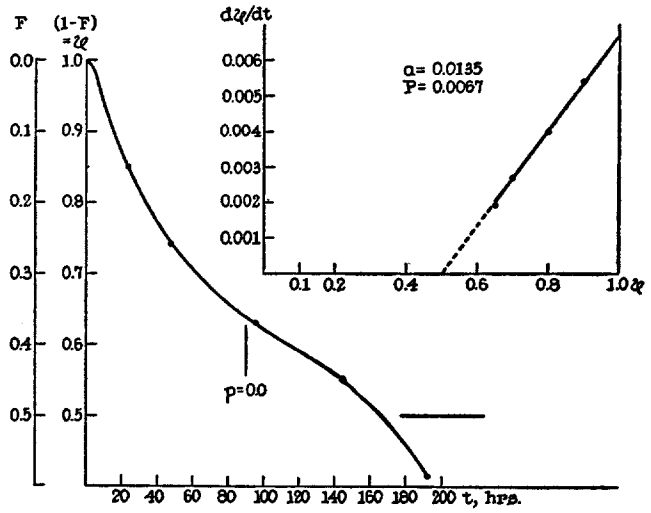

FIG. 3. An "intermediate" type of curve, obtained at 4°C. Ordinates and abscissa as in Fig. 1. Inset, analysis as in Fig. 1.

of the system, increases with time in systems at 25°C. instead of remaining substantially constant as it does at 4°C. Taking into consideration that the systems at 25°C. show much more hemolysis than corresponding systems at 4°C. do, the most likely cause of the continuous increase in a observed at 25°C. is an autolytic process. The effect of this reaction seems to be (a) to increase the value of a as time progresses, and (b) to decrease the value of P from its initial value to zero, probably as a function of time.[5]

values. In the meantime there is no way of examining this possibility further; what is needed is an independent method for finding P (or a) as a function of φ or of t. Studies of red cell metabolism might conceivably provide the necessary information. Until this is forthcoming, the experimental curves can be analyzed only in a qualitative sort of way.

[5] In the case of systems containing lysins such as resorcinol, the curve relating the loss of K to time is one for which P is zero, or nearly zero, and a constant and large,

While the assumption that the value $P = 0$ applies to the entire curve can be made for the purpose of discussing the probable nature of the relations, it is not tenable for the purpose of an analysis, in unequivocal terms, of the curves obtained at 25°C. (see footnotes 3, 4, 6).

Intermediate Types of Curve.—The conclusion that the curve relating φ and t at 4°C. is an essentially different type of curve from that obtained at 25°C suggests that there may be curves which are intermediate in type. These are somtimes met with in experiment, and one of them is illustrated in Fig. 3. This curve (human red cells 172 m. eq./liter NaCl at 4°C.) starts off as if proceeding to an asymptote at $\varphi = 0.50$ ($a = 0.0135$, see inset of Fig. 3). After 100 hours, the curve bends downwards and there is little doubt but that its further course is similar to that of the curve shown in Fig. 2. The simplest explanation of this stiuation is that there is negligible autolysis in the system up to 100 hours, and that the effects of autolysis become appreciable thereafter.[6]

Returning to the relation between φ and t observed at 25°C. and shown in Fig. 2, observations[7] made during the first 24 hours show that the curve is a really composite one such as that of Fig. 3, *i.e.* that it begins as a sigmoid curve which approaches an asymptotic value of φ much greater than zero (Fig. 2, inset D), the effects of autoly-

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but in these systems the lysin is present in relatively high concentration from the time $t = 0$. In the case of the autolytic systems now under consideration, there is no lysin present at $t = 0$, the lysin appearing and increasing as the time becomes greater. It is therefore very likely that P as well as a is a function of time.

[6] The change in the course of the curve may also be due to the accidental introduction of bacteria. The relation between φ and t in contaminated systems is often of the type shown in Fig. 2 rather than of the type shown in Fig. 1, even at 4°C.

[7] Flame photometer determinations have to be carried out very carefully when the initial portions of these curves are being investigated, for the K losses are small during the first few hours. The analysis of the initial portion of the curve at 25°C. (Fig. 2, inset D) shows that the curve starts off as an exponential approaching the asymptote $\varphi_\infty = 0.85$ with a constant value of a of 0.057. This gives $P = 0.048$, a higher value than that usually found at 4°C., and so the temperature coefficient of both P, the measure of the activity of the accumulation process, and a, the permeability constant, is positive.

The loss of K during the first hour or two is less than one would expect from the subsequent course of the curve, the relation between φ and t being sigmoid instead of uniformly convex to the t-axis (*cf.* Ponder, 1947 *b*, footnote 4). This is presumably the result of the change in P or in a which causes the system to proceed to a new steady state not being instantaneous, but taking place over the course of an hour or two. It is this portion of the curve which is most likely to suffer distortion, particularly at the higher temperatures, as a result of the liberation of K from rapidly disintegrating white cells and platelets (*cf.* Sheppard and Martin, 1948). Washing the cells reduces the number of white cells to about 50 per cent, and the number of platelets to about 10 per cent, of their initial values, and so this source of error is not as important as it would be in experiments with whole blood.

sis becoming apparent only after this initial period. This is a state of affairs similar to that described by Davson (1937), whose curves, at 40°C., seem to flatten off to asymptotes during the 8 hours to which his observations were limited.

There is some justification, indeed, for regarding the intermediate type of curve, such as that shown in Fig. 3, as being the most general expression of the relation between φ and t. Looked at from this point of view, the curve shown in Fig. 1 constitutes one extreme case in which no lysin is present in the system at $t = 0$ and in which no autolysin ever appears, whereas the exponential curves with a = constant and $\varphi_\infty = 0$ (Ponder, 1948 *b*) constitute the other extreme case, in which a lysin is present in the system from the time $t = 0$, and in which the effects of the lysin are substantially instantaneous.

Demonstration of Presence of Autolysins.—The presence of autolytic substances or of substances which increase autolysis can be shown by replacing the supernatant fluid of a system of human red cells in saline at 4°C. with some of the supernatant fluid from a system of human red cells in saline which has stood for 48 hours at 25°C. The rate of K loss in the first system, which is kept at 4°C. both before and after the substitution of the supernatant fluid from the second system, is compared with the rate of K loss in a system, also kept at 4°C. throughout, the supernatant fluid of which has been left undisturbed.

When the supernatant fluid is left undisturbed, the curve relating φ and t resembles that shown in Fig. 1; *i.e.*, φ is a simple exponential function of t with an asymptote in the neighborhood of 0.5. After a long time, such as 144 hours, φ is accordingly found to be about 0.5. When the supernatant fluid of the system is removed at the end of 48 hours and replaced with an equal volume of supernatant fluid from another system which has been kept at 25°C. for 48 hours and in which lysis has begun,[8] the curve relating φ and t turns downwards like that shown in Fig. 2, so that at the end of a long time such as 144 hours it has a value in the neighborhood of 0.1 instead of in the neighborhood of 0.5. This shows that autolysins or substances which favor autolysis develop in the system kept at 25°C., and that these substances are transferrable to the system kept at 4°C., in which autolysis does not usually occur in the times under consideration.[9]

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[8] The supernatant fluid contains the K lost at the end of 48 hours by the cells of the system kept at 25°C., and the addition of this K (usually $F = 0.2$ to 0.3) to the system at 4°C. has to be allowed for in calculating the subsequent course of the curve for the system at 4°C.

[9] The possible nature of autolysins such as these has been discussed elsewhere (Ponder, 1948 *c*), the two most interesting possibilities being that they are substances related to lysolecithin, produced by enzymatic action on the red cell lipids themselves, and that they are substances of the fatty acid class resulting from tissue autolysis. Following a suggestion made by Bianchi (1946), I have added 1 in 2000 quinine to the systems in an attempt to poison the lysolecithin-producing system; greater rather than smaller K losses occurred at all temperatures, probably because 1 in 2000 quinine is a lysin in a hypolytic concentration. NaCN, however, slightly decreases both K loss and spontaneous lysis; it is possible that it does so by inhibiting an enzyme system.

3. *K Losses at 37°C.*

A typical curve for the loss of K from human red cells into 172 m.eq./liter NaCl at 37°C. is shown in Fig. 4, plotted from data (Table IV) treated in the same way as those from which the curves of Figs. 1, 2, and 3 are constructed. So far as the observations made between 2 and 24 hours are concerned, the

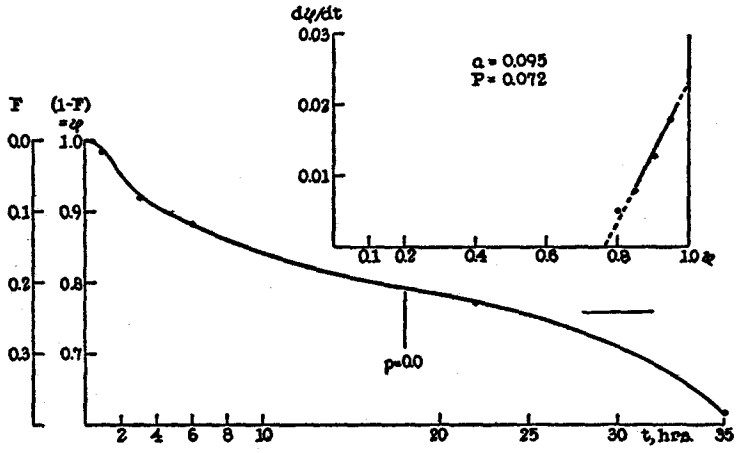

FIG. 4. Loss of K from washed human red cells into 172 m. eq./litre NaCl at 37°C. Ordinates and abscissa as in other figures. Inset, analysis in terms of P and a.

TABLE IV

Relation of K Loss to Time at 37°C.

t	K	p	F
hrs.			
1	0.02	0.00	0.02
3	0.08	0.00	0.08
6	0.12	0.00	0.12
22	0.22	0.03	0.23
36	0.38	0.13	0.29

curve relating φ and t is an exponential approaching the asymptote $\varphi = 0.76$; it therefore resembles the curve at 4°C. shown in Fig. 1 and the initial portion of the curve at 25°C. shown in Fig. 2, inset D. If observations are continued for more than about 24 hours, the curve begins to bend downwards to become a curve of the "intermediate" type just described. Analysis of the part between $t = 2$ and $t = 24$ gives $\varphi_\infty = 0.76$, $a = 0.095$, and $P = 0.072$. These values of a and of P are higher than those usually observed at 25°C., which again are higher than those usually observed at 4°C.

The initial portion of the curve is sigmoid. Between $t = 0$ and $t = 2$, it is

concave to the t-axis; after $t = 2$, it becomes convex, and so the relation between φ and t is of the type which would be expected if initial values of P_0 or of a_0 were to change, not quite instantaneously, to the new values which determine the position of the new asymptote $\varphi_\infty = 0.76$. If there were enough experimental points, this region of the curve could be analyzed by the same method as that used in the case of the curve in Fig. 2 or of the curve for the NaF system shown in Fig. 5.

TABLE V

Values of φ_∞, a, and P at 37°C.

Donor	φ_∞	a	P
1	0.86	0.125	0.108
2	0.82	0.116	0.095
3	0.76	0.095	0.072
4	0.76	0.097	0.070
5	0.69	0.072	0.049
6	0.55	0.055	0.028
7	0.68	0.082	0.056
8	0.65	0.079	0.051
9	0.70	0.100	0.070
10	0.66	0.091	0.061
Average.....			0.066

Table V gives values of φ_∞ and of a found in a series of observations at 37°C. on systems containing the red cells of different donors.

4. Systems Containing Substances Which Affect the Rate of Respiration, Metabolism, Etc.

Given a cation-permeable red cell which maintains a difference between the K content of its interior and the K content of the medium surrounding it by means of a metabolic process, it will be clear from the foregoing that a loss of K will occur if the constant a increases or if the rate of accumulation P decreases. The systems in which resorcinol, guaiacol, or n butyl alcohol are present at $t = 0$ (Ponder, 1948 *b*) are probably instances of cases in which the final result is brought about by an increase in the "permeability constant" a.[10] The

[10] If the curves for the three concentrations of resorcinol, 0.016 M, 0.032 M, and 0.048 M, all reach the asymptote K = 1.0 or $\varphi_\infty = 0.0$ in a simple exponential manner, P/a must approach zero in all cases. This may be the result of a decrease of P to zero or of a large increase in a, P remaining relatively constant; the point can be settled only by making simultaneous determinations of metabolic activity. Again, however, some doubt must arise as to whether the curve for the system containing 0.016 M resorcinol, for example, does not approach an asymptote a little higher than $\varphi_\infty = 0.0$, in which case the value of P/a for the system would be greater than zero and P might have a real value; see also the section of "immobile K," below.

analysis does not distinguish between the many possible combinations of variation in a and P, but some further insight into the course of events may be obtained by adding substances, such as fluoride, cyanide, iodoacetate, etc., which are known to affect red cell metabolism.

The effects of these substances will be described as they occur at the two temperatures 4° and 37°C., since curves at intermediate temperatures are difficult to interpret for the reasons already set forth (section 2, above).

1. Fluoride.—Fig. 5 shows the effect on the K loss at 4°C. of replacing half the NaCl of the system with 0.172 M NaF. The curve for the NaCl system approaches the asymptote $\varphi_\infty = 0.54$ exponentially with $a = 0.019$, $P = 0.010$,

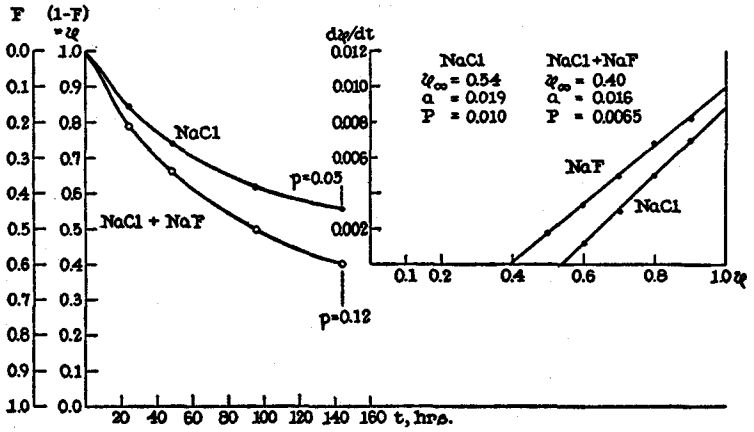

FIG. 5. Effect of 0.086 M NaF on the K loss from washed human red cells into 172 m. eq./litre NaCl at 4°C. Ordinates and abscissa as in other figures. The amount of lysis in the systems at the end of 144 hours is given by the two values of p. Inset, analysis of the curves in terms of P and a.

while the curve for the NaCl + NaF system approaches $\varphi_\infty = 0.40$ with $a = 0.016$, $P = 0.0065$. This result is compatible with the rate of accumulation P being reduced by the presence of fluoride to about 65 per cent of its value in the NaCl system.

Fig. 6 shows the effect on the K loss at 37°C. of replacing half the NaCl of the system with 0.172 M fluoride. The increase in the rate of K loss in the NaCl + NaF system is now very conspicuous, the curve relating φ and t approaching the asymptote $\varphi_\infty = 0.005$ (equilibrium between K inside the cell and K outside) and φ being as small as 0.2 even after 12 hours. Analyzing this curve in the same way as the curve in Fig. 2 is analyzed, a series of values of a corresponding to various values of φ can be found (Fig. 6, inset A, curve marked a). The value of the constant rises from its initial value of 0.02 to a new value of 0.2 as φ falls from 1.0 to 0.7, and the curve marked a in Fig. 6, inset B, shows how the value of a varies with time. Within the first 5 hours it increases from

0.02 to 0.2, a change which would correspond to a relatively slow effect of NaF in increasing the value of a.

This, however, is not the only or even the most likely possibility, for NaF may produce its effect by reducing metabolic acitvity and the rate of accumulation P. Suppose that a remains constant and that P varies explicitly with time; then, from (1),

$$P = a\varphi - d\varphi/dt \tag{3}$$

i.e., P can be evaluated by drawing tangents to the experimental curve and subtracting their slopes from $a\varphi$. To decide on the value of a which ought to be

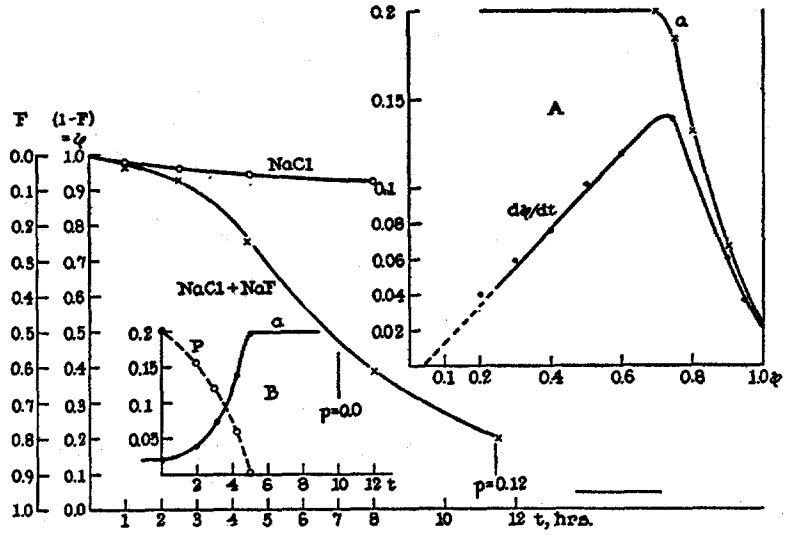

FIG. 6. Effect of 0.086 M NaF on the K loss from washed human red cells into 172 m. eq./litre NaCl at 37°C. Ordinates and abscissa as in other figures. Inset A, values of a, P being assumed to be zero. Inset B, variation of a with time, and of P with time, a being assumed to remain constant.

assumed, let us suppose that $P = 0$ for the lower part of the curve, *i.e.* that the effect of NaF is to reduce the rate of accumulation to zero after it has been acting for 5 hours or more; the constant value of a is then $(d\varphi/dt/\varphi) = 0.2$. The curve starts at $\varphi = 1.0$, which is equal to P_0/a; the initial value of P_0 at $t = 0$ is accordingly 0.2. From this value it decreases to zero during the first 5 hours of the experiment (Fig. 6, inset B, curved marked P and drawn through circles), the decrease corresponding to a relatively slow effect of NaF in decreasing the rate of accumulation P.

An intelligible enough result is arrived at by looking at the situation in either of these two ways, and there is the further possibility that both a and P, and

not only one of them, may vary explicitly with time. This treatment of the experimental data is therefore more of an inquiry into the possibilities than an analysis, and the next step is obviously to obtain an independent measure of the metabolic activity upon which P is supposed to depend. It would be a great advance if one could establish an understandable relation between the rate of metabolism (perhaps of glycolysis alone) and the events described by a curve such as that marked P in Fig. 6, inset B, because only by doing so is there any likelihood that the interlocked effect of variations in a and in P can be separated from each other.

The effect of fluoride on the K loss increases with the NaF concentration, at first rapidly and then more slowly. When human red cells are used, there is no evidence that the NaF effect goes through the maximum at 0.07 M observed by Davson (1941) in systems containing rabbit red cells.

"Immobile K".—It should be emphasized that in systems containing NaF at 4°C. φ tends towards an asymptote appreciably higher than $\varphi = 0.05$, at which the K content of the cells would be equal to that of the external medium. Some 20 to 40 per cent of the cell K seems to have a diffusibility much smaller than that of the rest of the cell K. A similar conclusion can be arrived at by considering the curves for K loss into NaCl or NaCl-buffer, where the asymptotic value of φ usually lies between 0.4 and 0.6. Translated into values of P, these values require that the cells accumulate about 0.6 per cent of their initial K per hour, *i.e.* about 0.6 m.eq./liter per hour; this is about 10 per cent of the accumulation which would be expected to occur from the glycolytic mechanism *at 37°C. working with 100 per cent efficiency*, and so is too large to be probable for a system which has stood at 4°C. for 100 hours or more, and which contains no added glucose. Even when glycolysis has been greatly reduced by the addition of NaF, the value of φ may still be between 0.2 and 0.4; it therefore seems likely that part of the cell K is held inside the cell by forces which are not dependent on metabolism (*cf.* Mazia, 1940). It is an attractive idea that these forces are related to the orderly arrangement of materials in the cell surface and interior, for the unexpectedly high position of the asymptote which suggests that some of the cell K is "immobile" is not observed when the systems contain lysins (Ponder, 1948 *b*; see also footnote 8) or in systems containing NaF at 37°C. It is only observations at low temperatures which suggest that a fraction of the cell K is "immobile;" if it should prove to be, the variables to be considered in the description of the curves for K loss as a function of time will be not only the accumulation rate P and the diffusion constant a, but also a quantity (φ_∞ in the absence of metabolism) which defines how much "immobile K" the cell contains and which may vary with temperature and other factors affecting cell structure.[11]

[11] Another way of accounting for the observations is to suppose that, after a long time (*e.g.*, 100 hours), the value of a becomes much smaller than it was previously. This is essentially Davson's idea that the membrane of the cell goes through a phase of permeability to K and then becomes impermeable. It does not seem likely in view of the increasing hemolysis observed as the cells are allowed to stand for longer

2. Iodoacetate.—The effect of sodium iodoacetate in concentrations between 0.02 per cent and 1.0 per cent on the course of K loss is small for the first 5 to 10 hours at 37°C.; after that time the K loss becomes rapid and is nearly complete at the end of 24 hours. As Wilbrandt (1937) has already observed, the effect is delayed as compared with the NaF effect; after 5 hours at 37°C., for example, the value of φ may be 0.8 in a system containing 0.02 per cent iodoacetate, while it would have fallen to the neighborhood of 0.3 in a system containing 0.5 per cent NaF.[12]

At 4°C., concentrations of iodoacetate between 0.02 per cent and 1.0 per cent produce an increase in the rate of K loss, the curve usually obtained being one which approaches $\varphi = 0.05$ as an asymptote. Only occasionally is the asymptote situated at a higher value (as it regularly is in systems containing NaF). Iodoacetate in these concentrations, however, is quite lytic, *e.g.* $p = 0.2$ after 144 hours in a system containing 0.02 iodoacetate, and so it is almost certain that the results are complicated by a modification of the cell structure.

3. Cyanide.—The addition to the system of M/1000 NaCn (buffered at pH 7.5) does not produce any increase in the rate of K loss either at 4°C. or at 37°C. The rate of loss, indeed, tends to be smaller in systems containing NaCN because of a slight reduction in the amount of spontaneous hemolysis.

4. Sugars.—Addition of sufficient glucose to produce a 200 to 400 mg./100 ml. concentration in the system has very little effect on the course of K loss at 4°C. At 37°C., K is taken up by red cells from media into which it has been lost at lower temperatures, provided that glucose is added and that a glycolytic mechanism is active (Harris, 1941; Maisels, 1948). The kinetics of this uptake remain to be studied.

In the systems with which this paper is concerned, *i.e.* in systems in which 0.8 ml. of red cells loses about 30 per cent of its K into 11.2 ml. of isotonic NaCl during 96 hours at 4°C., the rate of uptake of K by the cells at 37°C. in the presence of added glucose (200 mg./100 ml.) is just about great enough to balance the loss which occurs

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and longer times. A possibility which has to be considered more seriously is that fluoride produces irreversible injury to the cells, and that what appears to be an effect on an accumulating mechanism and a reduction in P is really the result of this injury and an increase in the constant a. Still another way of accounting for the observations is to suppose that there are some 30 per cent of red cells which are altogether impermeable to K at low temperatures; *i.e.*, that the K-Na exchanges observed take place in a part of the population only (*cf.* Ponder, 1947 *a*). Up to now, no experimental method has been found for excluding this as a possibility.

[12] From 5 to 10 per cent of the washed human red cells in systems containing Na iodoacetate become resistant to hypotonic hemolysis, remaining intact as spheres in a tonicity of 0.2 at 25°C. To prepare standards representing 100 per cent hemolysis, a small amount of K-free lysin, such as Na tetradecylsulfate, should be added.

into glucose-free NaCl at 37°C. Only rarely is it greater; these systems are accordingly unsuitable for the direct measurement of K accumulation, although they are excellent for the measurement of the rate of K loss. Since the rate of K loss at 37°C. is about 1 per cent per hour, the rate of uptake must be of the same order. Maisels (1948) gives uptake rates of about 1.5 per cent per hour for systems containing unwashed red cells and much more K in the supernatant fluids. It will be noticed that these values are considerably less than the 7 per cent per hour which corresponds to the average value of P at 37°C. (Table V). The latter, however, is a value calculated in relation to a supposedly steady state, whereas the former are average values for a process which is probably continually slowing down; the experimental values of 1.0 to 1.5 per cent per hour are accordingly likely to be minimal values.

5. *Hypotonicity.*—Davson (1937) observed that the red cells of all species examined (rabbit, ox, horse, guinea pig, pig, man) lose K when placed in hypotonic NaCl at 40°C.; at lower temperatures, the losses were smaller. The rate of loss (per cent of initial cell K lost in 120 minutes) was found to increase with decrease in tonicity, and the curve relating K loss to time was found to tend towards an asymptotic value far removed from that at which there would be an equilibrium between the K inside the cell and that outside; *i.e.*, to resemble the initial part of the curve shown in Fig. 4 of this paper. Davson made the suggestion that the cell membrane undergoes a transitory increase in permeability as the cell swells to a certain critical volume at which the loss of K becomes rapid; this critical volume would be less than the critical volume for hemolysis, and would be related in some way to the stretching of the cell surface. With the loss of K, the cell would shrink away from this critical volume, the surface would no longer be stretched, and the initial impermeability of the membrane to K would be restored; in this way the loss of a certain fraction of the cell K, and no more, might be explained.[13] Davson's observations extended over relatively short times (2 to 8 hours); they were made before the extent to which K is lost into isotonic media was appreciated, and the losses into hypotonic media were not systematically compared with those occurring into isotonic media.[14]

[13] The evidence which Davson gives in favor of this explanation is indirect, and involves the assumption that Na does not enter the cell as K leaves it. Analysis of the K and Na contents of the swollen cells, after they have stood at 25°C. in a medium of tonicity 0.6 for 48 hours, during which time about 20 per cent of the cell K is lost, shows that a K-Na exchange has taken place with the final concentration of K + Na equal to about 0.6 times the initial concentration. Davson also considered the possibility that the new steady state reached in the systems might be the result of a metabolic process, but rejected this mechanism on grounds which would not now be thought adequate.

[14] Over and above the K losses under consideration, losses as large as 9 per cent of the initial cell K may occur when red cells are centrifuged (Davson and Danielli, 1938).

(*Footnote continued on following page*)

In protracted experiments with sterile systems at 4°C., the rate of loss of K from human red cells into hypotonic NaCl is usually less than the rate of loss into isotonic media. At the same time, the value of φ_∞ is greater, *e.g.* $\varphi_\infty = 0.60$, $a = 0.011$ for $T = 1.0$, but $\varphi_\infty = 0.65$, $a = 0.009$ for $T = 0.7$. At 37°C. there is no significant difference between the K loss into isotonic NaCl and into hypotonic NaCl of a tonicity $T = 0.6$ or 0.7, a typical result being $\varphi_\infty = 0.76$, $a = 0.097$ in each case.

SUMMARY

Curves describing the loss of K from human red cells as a function of time can be interpreted in terms of an equation which treats the K content of the cell (φ) as the result of an accumulation process occurring at a rate P and an outward diffusion process regulated by a constant a. The equation is useful for describing the observations and for exploring the mechanisms which may be responsible for the K losses, although it cannot be used for analyzing the experimental data in a strict sense in the absence of independent metabolic data because P and a may both be functions of time. The applicability of the equation is illustrated by its use in connection with experimental curves showing K loss as a function of time at 4°, 25°, and 37°C. for systems containing human red cells in isotonic NaCl or NaCl-buffer.

At 4°C., the K loss follows an exponential curve approaching an asymptote in the neighborhood of $\varphi = 0.50 \pm 0.15$. The corresponding value of P implies that the cells are able to accumulate about 0.6 per cent of their initial K per hour under these conditions.

At 25°C., the K loss starts exponentially but becomes roughly linear with time after 24 to 48 hours. The change of form is probably due to the appearance of autolysins in the system. Curves of a similar mixed or intermediate form may be obtained even at 4°C. if the observations are sufficiently extended and if spontaneous hemolysis becomes appreciable.

At 37°C., the K loss is exponential for the first 24 to 36 hours, the curves approaching asymptotes which, translated into terms of P, indicate that the cells can accumulate about 7 ± 3 per cent of their initial K per hour. After this time autolysis begins to affect the shape of the curves, the rate of K loss increasing rapidly.

When the suspension medium is made hypotonic by the addition of water, between 2 and 5 per cent of the initial K is lost within 10 minutes at 25°C. (Ponder and Robinson, 1934), only about 0.5 per cent being lost during the same time into isotonic NaCl. These losses would occur, in these experiments, before the time denoted by $t = 0$. It will be noticed that the initial K concentration K_0 is determined in the cells of a sample of the completed system, and is almost always less than the K concentration in the unwashed red cells of the heparinized blood.

The effect of adding fluoride or iodoacetate is to lower the position of the asymptote to which the curves proceed; *i.e.*, to decrease the accumulation rate P, to increase the diffusion constant a, or both. Cyanide has almost no effect. Hypotonicity has little effect on the rate of K loss at 37°C.; at 4°C., the rate of loss is somewhat less in hypotonic NaCl.

The observation that the K loss in systems at 4°C. and containing as much as 0.086 M NaF does not become complete, but proceeds exponentially towards an asymptote between $\varphi = 0.2$ and 0.4, suggests that 20 to 40 per cent of the cell K is much less diffusible than the remainder at low temperatures and in the absence of lytic substances. A similar conclusion is suggested by the form of the curve for K loss into saline at 4°C., an accumulation rate of 0.6 m. eq./litre of cells/hour at the end of 100 hours or more being improbably great for a system at such a low temperature and containing no added glucose.

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