DISK-SPHERE TRANSFORMATIONS PRODUCED BY LYSINS, AND THEIR REVERSAL, IN SYSTEMS OF DIFFERENT *p*H

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(*Received* 27 *August* 1946)

It is now recognized that lysis is preceded, in the case of nearly all haemolytic systems containing mammalian red cells, by a disk-sphere transformation which is at first reversible but later irreversible. While this shape change is an event which occurs during the course of reactions leading up to haemolysis, its occurrence seems to be described by relations not altogether the same as those which are applicable to the description of the haemolytic phenomena themselves. Thus it has already been shown that in the case of some lysins the shape change does not occur until just before the cell haemolyses, whereas in the case of other lysins it takes place almost as soon as the cells are added, although lysis does not occur for minutes or even hours. One concludes from this that the reactions which produce the shape changes are not identical with those which bring about the lysis, but have a certain degree of independence of the latter. The purpose of this paper is to describe two other differences which distinguish the shape transformation from the phenomenon of lysis which usually follows it. The first of these is that a change in *p*H does not usually affect the shape change and the subsequent lysis in the same way, for in the case of most lysins haemolysis is inhibited by an increase in *p*H in the range 6·0 to 8·5, whereas the shape transformation occurs more readily at high *p*H's than at low ones. The second is that substances which inhibit the haemolytic process may or may not transform the spherical form into the disk, an observation which suggests that the shape transformation is not simply an early manifestation of the effects of the same processes which eventually bring about haemolysis.

I. THE SHAPE IN ISOTONIC CITRATE-PHOSPHATE BUFFERS AT VARIOUS *p*H's

The cells of 8 ml. of freshly obtained heparinized human blood are washed three times with 1 % NaCl, and are then suspended in isotonic citrate-phosphate buffers of various *p*H so that each ml. of suspension contains 10^8 red cells. Citrate-phosphate buffers are selected because red cell shape is so well maintained in 3 % sodium citrate, and because the requisite *p*H range can be obtained by varying the proportions of $M/15$ KH_2PO_4 and $M/15$ Na_2HPO_4. In preparing the suspension media, the buffers are prepared first and then mixed with equal volumes of 3 % sodium citrate: it is the *p*H of these mixtures which are given in the Tables.

The cell suspensions, in citrate-phosphate buffers at *p*H 6·0, 6·6, 7·3 and 8·3, are added to 1 % NaCl in the proportion of 0·5 ml. of suspension to 2 ml. of

1% NaCl. The shape of the cells is ascertained by examining them immediately in uncovered drops on plastic slides: this examination is repeated at intervals, the cell-buffer system being kept at 25° C. throughout.

Discoidal red cells in dilute suspension can be distinguished macroscopically from spherical cells if the contents of the tube containing the system are first mixed, and if the tube is then given a sudden flip in order to set up currents in the fluid in it. For a second or two, a watered-silk appearance is observed when the cells are discoidal, this being caused by the flat cells being carried along in the currents and reflecting light in different directions as they move. Crenated disks give the same effect in a less striking way; spheres and crenated spheres give no such effect at all. By repeatedly comparing the shape, as judged by this macroscopic method, with the shape as seen microscopically, considerable skill can be gained in determining shape from the appearance of the system in the tube, and the approximate lysin concentrations in which disks become spheres can be found in this way. For more exact observations in the region of these concentrations, the cells should always be examined under the microscope so as to verify the results obtained macroscopically.

The results of experiments designed to ascertain the shape of human red cells in isotonic citrate-phosphate buffers at various *p*H's are given in Table 1, which shows that there is a tendency for the discoidal form to crenate and ultimately to become spherical at the higher *p*H's.* Information of this type is essential for the interpretation of the results of the experiments in §§ II and III.

Table 1. *Shape of washed human red cells in citrate buffers after various times of standing at 25° C.*†

Observed	*p*H 6·0	*p*H 6·6	*p*H 7·3	*p*H 8·4
At once	Disks	Disks	Disks	Disks with slight crenation
After 30 min.	Disks	Disks	Disks	Crenated disks and spheres
After 1 hr.	Disks	Disks	Disks	Crenated spheres
After 5 hr.	Disks	Disks	Crenated disks	Spheres

† The *p*H given is that of the citrate-phosphate buffer, and not that of the completed system. The *p*H of the cell suspensions are 6·0, 6·5, 7·0 and 7·9 respectively.

II. SPHERING AND LYSIS PRODUCED BY VARIOUS LYSINS AT VARIOUS *p*H's

Four identical series of dilutions of the lysin (e.g. saponin) in powers of 2, are set up in a series of tubes, each finally containing 2 ml. of the diluted lysin. The range over which the dilutions have to be made so as to include both the dilution in which complete lysis occurs and that in which perfect sphering occurs has to be found by trial, but can be inferred from the values in Table 2 for the five substances

* These results differ from those described by Teitel-Bernard (1932) because the systems and the conditions of observation are not the same as in his experiments. His systems contained plasma diluted about 1 in 40, which is sufficient to prevent sphering at *p*H 8·4. Further, the zone of crenation described between *p*H 7·0 and 6·0, corresponding to the intracellular crystallization of haemoglobin with its accompanying birefringence, was the result of observations made after some hours and not, as in the case of the experiments described here, after 30 min. only.

distearyl lecithin, sodium tetradecyl sulphate, sodium taurocholate, saponin and digitonin. These last three substances are both dissolved in and diluted with 1 % NaCl. The distearyl lecithin is prepared as a sol in 1 % NaCl; the tetradecyl sulphate is first dissolved in methyl alcohol in the proportion of 5 mg./ml., and is added to and diluted with 1 % NaCl immediately before use.

Table 2. *Minimum quantities in γ required for sphering and for complete lysis of* 5×10^7 *washed human red cells in citrate-phosphate buffers at the end of 30 min. at 25° C.*

Lysin or sphering agent	*p*H 6·0		*p*H 6·6		*p*H 7·3		*p*H 8·4	
	Lysis	Spheres	Lysis	Spheres	Lysis	Spheres	Lysis	Spheres
Lecithin	—	15	—	5	—	3	—	0·5
C-14 sulphate	7	2	8	1	9	0·5	10	0·1
Sodium taurocholate	200	25	200	12	300	4	350	2
Saponin	12	10	12	10	12	10	16	2
Digitonin	5	4	5	4	6	5	6	1

To each tube in the first series of dilutions of each lysin is added 0·5 ml. of the red cell suspension in citrate-buffer at *p*H 6·0, prepared as in § 1, above. Similarly, 0·5 ml. of the cell suspensions at *p*H 6·6, 7·3 and 8·3, prepared as in § I, is added to each tube in the second, third and fourth series respectively of the dilutions of each lysin. The contents of the systems are mixed and allowed to stand for 30 min. at 25° C. The smallest quantity of each substance required at each *p*H to produce (*a*) complete lysis and (*b*) perfect sphering is then determined by examining the systems first macroscopically and then microscopically.

The results of a typical experiment of this kind are shown in Table 2.

III. REVERSAL EFFECTS

A series of systems are prepared containing 5×10^7 human red cells per ml. in citrate-phosphate buffer at *p*H 7·3 together with distearyl lecithin, sodium tetradecyl sulphate, sodium taurocholate, saponin, or digitonin in sufficient quantity to produce perfect sphering in 30 min. at 25° C. (column 2 of Table 2). The systems should be at least 20 ml. in volume. At the end of 30 min., 1 ml. portions of a system are transferred to small tubes, and various 'reversing substances' are added to reconvert the spherical cells into disks.

The reversing substances used in these experiments were two components of plasma (which itself converts spheres into disks if added before the stage of the prolytic sphere is reached), serum albumin and cholesterol, as well as one of the agents which has already been described (Ponder, 1942) as producing a reversal of the lecithin shape transformation, benzene. It may be stated at once that the other principal inhibitory components of plasma, lecithin and serum globulin, do not produce reversal of the shape change brought about by any of the five lysins used in these experiments.

The serum albumin (kindly sent to me by Dr E. J. Cohn as a 25 % solution) is made up as 0·1 and 0·01 % solutions in 1 % NaCl. The cholesterol is prepared as

a sol in water with a cholesterol content of 500 γ/ml. (Lee & Tsai, 1942), and is diluted 1 in 10 with 1% NaCl just before use. The benzene is used as a saturated solution in saline (1 ml. = 780 γ).

The procedure is to take 1 ml. of a system containing a sphering agent or lysin, to examine the cells so as to be sure that they are spherical, and then to add a small amount of a reversing agent, e.g. 0·1 ml. of 0·01% serum albumin, and to ascertain if it converts the cells immediately into disks. If it does not, further small amounts of the reversing agent are added until the volume of the system is increased to about 1·5 ml.; if the cells are still spherical, another 1 ml. sample of the system is taken and 0·1 ml. of the reversing agent in higher concentration, e.g. 0·1% serum albumin, is added. If this is not enough to turn the disks into spheres, larger amounts and if necessary higher concentrations of reversing agent are added and the effect on shape, if any, observed. Care should be taken to control the observations by adding the same amount of 1% NaCl, in order to be sure that a mere dilution of the system does not turn the spheres into disks.

This procedure is repeated with the systems containing each of the five sphering agents, always in just sufficient concentrations to produce sphering in 30 min. at 25° C., and with each of the three reversing agents, the cells being suspended in the first instance in citrate-buffer at *p*H 7·3. The whole experiment is then repeated with cell suspensions at *p*H 6·0 and 6·6, the quantities of sphering agent being changed so as always to be the minimum amount for sphering in 30 min. at 25° C.; these amounts require to be determined beforehand by constructing a table such as Table 2. There is no point in repeating the experiments at *p*H 8·3, for at this *p*H the cells are almost spherical in the citrate-buffer itself, and the quantities of the sphering agents required to produce sphering are very small.

I have not been able to demonstrate any dependence on *p*H of the reversing effects, and so Table 3 shows the quantities of the three reversing agents required at *p*H 7·3 only.

Table 3. *Quantities of reversing agents, in γ, required for the reversal of sphering of 5×10^7 cells after* 30 *min. at* 25° C. *and at p*H 7·3

Sphering agent	Amount per 5×10^7 cells	Serum albumin	Cholesterol	Benzene
Lecithin	3	150	No reversal	550
C-14 sulphate	0·5	15	75	780
Sodium taurocholate	4	50	No reversal	780
Saponin	10	25	No reversal	No reversal?
Digitonin	5	100	No reversal	No reversal?

In the case of each of the five lysins, a reversal of the disk-sphere transformation is brought about by serum albumin in quantities of from 15 to 150 γ. Only in the case of the lysin sodium tetradecyl sulphate does cholesterol produce reversal; in systems containing the other lysins it is ineffective at all *p*H's between 6·0 and 8·3. The uncertainty attached to the effects of benzene in systems containing saponin and digitonin is due to the concentration range which separates the production of

spheres from the lysis of the cells being so small as to make it very difficult to pick up reversal effects with the amounts of benzene which can be introduced into the systems; these are limited by the solubility of benzene in saline.

IV. DISCUSSION

Three principal conclusions, all of interest in connexion with the phenomena of the shape transformations, can be drawn from the data in Tables 1, 2 and 3.

(1) *The mole ratios.* By converting quantities into moles, two additional tables can be constructed from the data. The first of these (Table 4) shows the number of

Table 4. *A.² of red cell surface, per molecule of lysin or sphering agent. Values calculated from the values in Table 2*

Lysin or sphering agent	*p*H 6·0		*p*H 6·6		*p*H 7·3		*p*H 8·4	
	Lysis	Spheres	Lysis	Spheres	Lysis	Spheres	Lysis	Spheres
Lecithin	—	67	—	200	—	350	—	2000
C-14 sulphate	60	210	53	420	89	800	42	4200
Sodium taurocholate	4	32	4	67	3	180	2	360
Saponin	110	130	110	130	110	130	80	670
Digitonin	330	420	330	420	270	330	270	1670

Table 5. *Ratios of reversing agent to sphering agent, calculated from Table 5*

Sphering agent	Serum albumin	Cholesterol	Benzene
Lecithin	0·57	—	110
C-14 sulphate	0·13	110	3600
Sodium taurocholate	0·10	—	800
Saponin	0·35	—	600
Digitonin	0·36	—	1500

A.² of red cell surface, per molecule of lysin or sphering agent, in systems in which (*a*) perfect sphering, and (*b*) complete lysis, occurs. The values in A.² are minimum values, for it is assumed that all the lysin or sphering agent in the system is distributed over the red cell surface; this is almost certainly not the case, considerable quantities being left free in the suspension medium (Ponder, 1946).

A point which stands out clearly is that some substances can produce the disk-sphere transformation, and can even bring about lysis, when they are present in such small quantities that they cannot cover the cell surface with a monolayer. There seems to be no foundation for the idea that a lysin must form a monolayer at the surface before it can bring about lysis (Gorter, 1937, cf. Ponder, 1941). The anionic detergent sodium tetradecyl sulphate, for example, produces sphering when there is only one molecule per 420 A.² at *p*H 6·4 and per 800 A.² at *p*H 7·3, even if all of it is imagined to be monodisperse and present at the red cell surfaces. Since the dimensions of the molecule are about $20 \times 4{\cdot}5 \times 4{\cdot}5$ A., there is not enough of it to form a monolayer. The same remarks apply to the sphering produced by lecithin at *p*H 7·3.

Table 5 shows the value of the ratio

$$\frac{\text{Number of molecules of reversing agent needed to produce reversal}}{\text{Smallest number of molecules of sphering agent needed for sphering}}$$

for the five sphering agents and the three reversing agents. In the case of serum albumin (M.W. taken as 70,000) the ratio varies from 0·10 to 0·57, i.e. it is of the same order of magnitude for all the sphering agents. In the case of benzene, on the other hand, the ratio varies from 110 to 3600, which means that benzene is a very much poorer reversing agent than is serum albumin and that its effect of the spheres produced by the various sphering agents is not at all the same, even allowing for the fact that the determinations are made to the nearest power of 2 only. In the single case in which cholesterol produces a reversal of the disk-sphere transformation, the ratio has the value of 110; like benzene, cholesterol is therefore a much poorer reversing agent than serum albumin, as well as being one whose effect is less general. Serum albumin, indeed, seems to occupy a unique position which may be related to its being the 'anti-sphering substance' for the disk-sphere transformations which occur, as a result of a *p*H change, between glass slides and cover-glasses (Furchgott, 1940 *a*, *b*; Furchgott & Pondér, 1940).

(2) *p*H *dependence of shape change and of lysis.* The data in Table 2 show that the *p*H dependence of the sphering produced by four of the five substances examined (distearyl lecithin does not produce lysis except in very high concentrations and then only partially) is not at all the same as the *p*H dependence of the haemolysis which follows it. The amount of lysin required to complete the haemolytic process increases with increase in *p*H, but the amount required to bring about the disk-sphere transformation decreases with increase in *p*H. The shape changes in the systems containing lysins are no doubt contributed to by the tendency towards spontaneous crenation and sphering at the higher *p*H's (Table 1), but the conclusion remains that the processes which bring about haemolysis are affected by *p*H changes in a different way from those processes which result in the shape change, even although the latter are usually associated with the early stages of the former.

(3) *Reversal of shape transformation and inhibition of lysis.* The observation that a reversal of the disk-sphere transformation produced by lecithin occurs when a large amount of saline is added to the system (Ponder, 1936) suggests that the reversing agents may act by reacting with the lysins and reducing their concentration. This simple explanation is not tenable in view of the fact that cholesterol, a powerful inhibitor of lysis by the anionic detergents, sodium taurocholate, saponin, and digitonin, does not bring about a shape reversal except in the case of the spheres produced by the C-14 detergent. The effect of the reversing agents on shape must therefore be regarded as something specific, and as distinct from their effect on the concentration of free lysin in the system.

SUMMARY

1. Although sphering almost always occurs at some time before a mammalian red cell haemolyses, the shape change has to be placed in a variable position on the time scale to which the progress of the lytic reaction is referred. In the case of some lysins, e.g. saponin, the shape change is a late event, while in the case of others, e.g. sodium tetradecyl sulphate, it is an early event on the same time scale.

2. In the case of four lysins (sodium taurocholate, sodium tetradecyl sulphate, saponin and digitonin) the amount of lysin required for complete haemolysis increases with increasing *p*H, but the amount required to produce sphering decreases with decreasing *p*H.

3. Serum albumin produces a reversal of the disk-sphere transformation brought about by distearyl lecithin, sodium taurocholate, sodium tetradecyl sulphate, saponin and digitonin. Benzene has a weak reversing effect when the spheres are formed as the result of the action of the first three substances mentioned, and cholesterol produces reversal of the shape change only when it is brought about by sodium tetradecyl sulphate. The simple explanation that the reversing agents act by reacting with the lysins and reducing their concentration is not tenable, and the effect of the reversing agents must be regarded as something distinct from their inhibitory effect on the lysin in the system.

4. Some of the substances used can produce the disk-sphere transformation and can even bring about lysis when they are present in such small amounts that they cannot cover the red cell surface. Either the effects produced by each molecule of sphering agent or lysin extend to neighbouring regions on the cell surface and perhaps also into the interior, or sphering and lysis occur as the result of changes at spots on the surface.

REFERENCES

FURCHGOTT, R. F. (1940*a*). *J. Exp. Biol.* **17**, 30.
FURCHGOTT, R. F. (1940*b*). *Cold Spr. Harb. Symp. Quant. Biol.* **8**, 224.
FURCHGOTT, R. F. & PONDER, E. (1940). *J. Exp. Biol.* **17**, 117.
GORTER, E. (1937). *Trans. Faraday Soc.* **33**, 954.
LEE, J.-S. & TSAI, C. (1942). *Quart. J. Exp. Physiol.* **31**, 281.
PONDER, E. (1936). *J. Exp. Biol.* **13**, 298.
PONDER, E. (1941). *J. Gen. Physiol.* **25**, 247.
PONDER, E. (1942). *J. Exp. Biol.* **19**, 220.
PONDER, E. (1946). *J. Gen. Physiol.* **29**, 203.
TEITEL-BERNARD, A. (1932). *Arch. roumain. Path.* **5**, 389.