THE EFFECT OF SAPONIN ON THE OSMOTIC HEMOLYSIS OF CHICKEN ERYTHROCYTES[1]

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I

Although the hemolysis of erythrocytes by lytic agents such as saponin has received much attention, few investigators have studied the effect of these agents prior to hemolysis. Ponder (1937) reported a decrease in the fragility of rabbit erythrocytes which had been exposed to sub-lytic concentrations of lysins. This effect, he believed, was similar to the action of narcotics (cf. Jacobs and Parpart, 1932). Davson and Danielli (1938) reported that saponin caused no loss of K^+ from erythrocytes in either sub-hemolytic or hemolytic concentrations.

The present experiments were performed to determine what effect saponin would have on the penetration of small molecules prior to the time that the membrane became permeable to the hemoglobin molecule.

II

Chicken blood was obtained by cardiac puncture and then defibrinated. One half a cc. of blood suspended in 5 cc. of Ringer-Locke required approximately 0.2 cc. of 1 per cent saponin in Ringer-Locke to cause slight hemolysis in two hours. A control suspension was similarly prepared, omitting the saponin. The slight difference in total volume (0.2 cc. in 5.5 cc.) did not introduce any error, since the method used to measure permeability is not influenced by variations in the number of cells within a wide range.

In this way stock suspensions of control and experimental cells were prepared. In most experiments 0.2 cc. of these suspensions were added to 10 cc. of an isosmotic solution of the substance whose rate of penetration was to be measured. Permeability, fragility, and swelling measurements were made at a temperature of $37° \pm 0.5°$ C., using the photronic cell technique usually employed in this laboratory (cf. Hunter, 1936; Hunter and Pahigian, 1940). In every experiment sufficient

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$NaHCO_3$ was added to insure complete clearing of the suspension (Stringer et al., 1940; Hunter et al., 1940). Permeability measurements were made until the experimental cells were found to hemolyze more rapidly than the controls when placed in the isosmotic solutions of penetrating substances. At this time the experimental solution was diluted with Ringer-Locke and centrifuged. In some of the experiments the supernatant fluid after centrifugalization was colorless and all of the packed cells were red, which indicated that the saponin had not caused any hemolysis. In others, the supernatant fluid was red and some of the cells were white. This indicated that the saponin had been allowed sufficient time to destroy the membranes of some of the cells and liberate the hemoglobin. Experiments in which this had happened served to indicate that the cells whose permeability was being studied had been exposed to hemolytic concentrations of the lysin, but the process had been stopped by removing the lytic agent before many of the cells had been hemolyzed. After the cells had been centrifuged, the supernatant fluid containing the saponin was discarded and the cells were resuspended in a volume of Ringer-Locke sufficient to give a suspension containing approximately the same number of cells per unit volume as the control. Additional washing with Ringer-Locke was unnecessary, as these cells would remain unhemolyzed for several days. These resuspended cells exhibited the same permeable properties that they had had immediately preceding the centrifugalization. The control cells were not centrifuged in every experiment, since this treatment had no marked effect on their permeability.

Some of the molecules penetrated the cells so rapidly at 37° C. that measurements could not be made using the photronic cell technique. In these cases the rate of hemolysis was measured by eye at room temperature (about 23° C.). The complete hemolysis curve could not be obtained in this way, but it was possible to make comparisons by measuring the times for a given percentage of the cells to hemolyze.

Permeability measurements were begun as soon as the control and experimental suspensions were prepared. A comparison of the effect of saponin on the permeability to glycerol and monoacetin was made using the photronic cell apparatus, while comparisons using the other method were made between various rapidly penetrating lipoid-soluble and insoluble molecules. Since it usually required at least one hour before the saponin produced any marked effect, there was sufficient time to make several measurements before the cells were centrifuged.

After the suspensions had been centrifuged and the cells resuspended in Ringer-Locke, hemolysis measurements were made using a number

of different substances. Centrifuged cells were used to obtain the swelling and fragility data. In some experiments, higher concentrations of saponin were used which produced slight hemolysis in 10–15 minutes. Cells obtained in this way gave the same results as those which had been exposed to lower concentrations of the lysin.

III

The data have been analyzed in several ways. In Table I are listed the average number of seconds required for 80 per cent hemolysis (one-half the total deflection) of the experimental and the control cells when placed in isosmotic solutions of the various substances studied. Since the change in hemolysis time depends on a number of factors such as the concentration of saponin, and the time allowed for its action, there

TABLE I

The effect of saponin on the osmotic hemolysis of chicken erythrocytes.

Substance	Average time in seconds for 80% hemolysis		Per cent change	Number of observations
	Control	Experimental		
*Water	18	7	−61.1	1
*Ethylene glycol	47	38	−19.2	10
*Diethylene glycol	75	39	−48.0	3
Triethylene glycol	130	87	−33.1	8
Urea	148	112	−24.3	6
Thiourea	193	148	−23.3	7
Glycerol	193	104	−46.1	38
Malonamide	868	273	−68.5	11
Erythritol	1125	292	−74.0	1
*Diacetin	34	23	−32.3	5
Monoacetin	144	63	−56.3	31
*Acetamide	38	22	−42.1	3
*Propionamide	38	22	−42.1	5

* Measurements made by eye.

is great variability observed in this value. To reduce this variability, only the figures obtained after the cells had been centrifuged are recorded. The differences in the percentage change which remain result from the fact that the saponin acted for a longer time in some experiments than in others. The table merely indicates that the permeability to a number of different substances has been greatly increased. The data for those substances which are starred were obtained from measurements made by eye. Figure 1 presents a representative control and experimental curve.

Having established the fact that treatment with saponin could increase the permeability of the cell membrane without causing hemolysis,

the data were analyzed in an attempt to demonstrate any differential effects. The possibility that an increase in permeability to lipoid soluble-molecules might appear first was considered. These data are presented in Table II. Once again there is considerable variability, but the evidence indicates that the experimental treatment increases the permeability

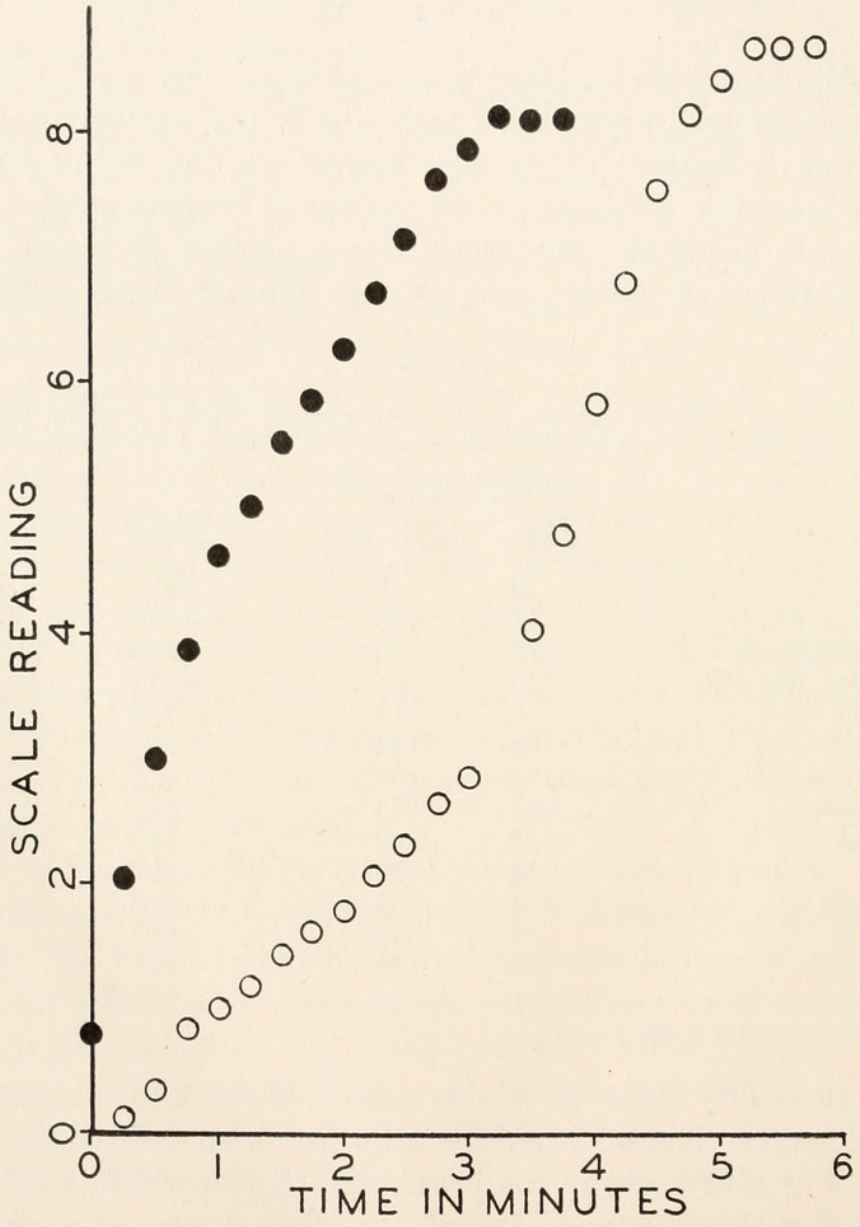

FIG. 1. The effect of saponin on the osmotic hemolysis of chicken erythrocytes in glycerol. ○—control; ●—experimental. (Scale reading represents degree of hemolysis.)

to lipoid-soluble and lipoid-insoluble molecules similarly. The time at which the increase is first noted and the degree of change are the same for the two types of substances.

Although the increase in permeability to lipoid-soluble and lipoid-insoluble molecules apparently occurs at the same time, it was thought

TABLE II

Data comparing the effect of saponin on the osmotic hemolysis of chicken erythrocytes in lipoid-soluble and lipoid-insoluble molecules after a short exposure to the saponin.

		Percentage change		
Date	Glycerol	Monoacetin	Ethylene glycol	Diacetin
3/6/40	−42.2	−62.2		
3/8/40	−13.8	−22.7		
3/27/40			− 7.2	−15.2
3/29/40			−18.3	−12.3
3/30/40	−11.1	− 4.5	+ 0.5	− 2.4
4/6/40			−16.2	−20.9
4/8/40	−18.0	− 9.5		

TABLE III

Data comparing the effect of saponin on the osmotic hemolysis of chicken erythrocytes in lipoid-soluble and lipoid-insoluble molecules after a long exposure to the saponin.

Glycerol			Monoacetin		
Time in seconds for 80% hemolysis		Percentage change	Time in seconds for 80% hemolysis		Percentage change
Control	Experimental		Control	Experimental	
200	50	−75.0	205	110	−46.3
215	142	−33.9	205	150	−26.8
175	24	−86.3	175	26	−85.2
195	115	−41.0	195	95	−51.3
195	95	−51.3	195	68	−65.1
131	18	−86.3	175	20	−88.6
125	50	−60.0	85	50	−41.2
106	80	−24.5	140	28	−80.0
165	100	−39.4	110	95	−13.6
154	152	− 1.3	130	112	−13.8
255	60	−76.5	155	38	−75.5
210	40	−81.0	130	90	−30.8
185	140	−24.2	130	40	−69.2
185	55	−70.3	130	110	−15.4
237	157	−33.8	175	25	−85.7
237	80	−66.2	205	135	−34.1
237	25	−89.5	205	40	−80.5
237	35	−85.2	205	23	−88.8
237	135	−43.0	146	82	−43.8
237	188	−20.7	146	130	−11.0
237	120	−49.4	146	70	−52.0
215	35	−83.7	127	13	−89.8
215	26	−87.9	127	13	−89.8
215	130	−39.5	127	37	−70.9
Average 192	86	−55.2	157	67	−57.3

possible that after the membrane had become considerably altered a difference in rate of penetration might appear. The data in Table III are presented as evidence on this point. These were obtained after the saponin had completed its action on the cell and had been removed by centrifugalization. The results indicate that under similar treatment the permeability to a lipoid-soluble molecule such as monoacetin is in-

TABLE IV

Data comparing the effect of saponin on the rate of penetration of a small and a large lipoid-insoluble molecule.

Glycerol			Malonamide		
Time in seconds for 80% hemolysis		Percentage change	Time in seconds for 80% hemolysis		Percentage change
Control	Experimental		Control	Experimental	
131	18	−86.3	670	204	−69.6
125	50	−60.0	578	126	−78.2
165	100	−39.4	805	644	−20.0
154	152	− 1.3	729	675	− 7.4
255	60	−76.5	1080	180	−83.3
237	157	−33.8	1080	75	−93.1
237	80	−66.2	920	540	−41.3
237	25	−89.4	920	210	−77.2
237	35	−85.2	920	83	−91.0
237	135	−43.0	920	110	−88.0
237	120	−49.4	920	150	−83.7
200	110	−45.0	730	390	−46.6
230	110	−52.2	810	330	−59.3
190	35	−81.6	570	60	−89.5
180	65	−63.9	480	65	−86.5
330	320	− 3.0	1190	1070	−10.1
340	315	− 7.4	1260	1080	−14.3
330	285	−13.6	630	270	−57.1
330	260	−21.2	690	210	−69.6
125	45	−64.0	400	120	−70.0
125	45	−64.0	640	150	−76.6
Average 221	120	−45.7	807	321	−60.2

creased the same amount as the permeability to a lipoid-insoluble molecule such as glycerol.

Finally, a comparison was made of the effect of the experimental treatment on the penetration of a large molecule such as malonamide and a small molecule such as glycerol. In this case there appeared to be a consistent difference, but in order to be certain, an additional series of experiments was performed. All of the data are included in Table IV. The last ten sets of readings were from the second series of

experiments. These data indicate that the rate of entrance of malonamide is increased more by the experimental treatment than the rate of entrance of glycerol.

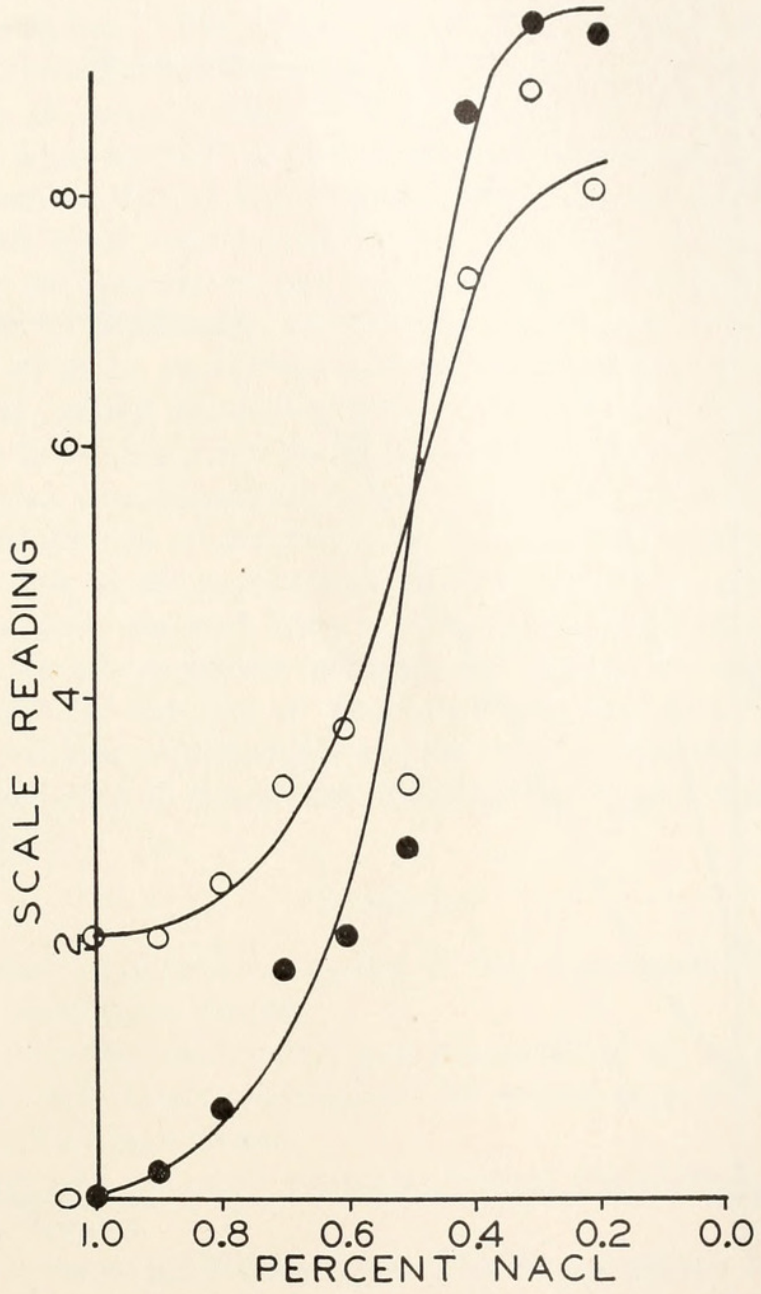

FIG. 2. The effect of saponin on the fragility of chicken erythrocytes. ○—control; ●—experimental (Scale reading represents degree of hemolysis.)

A decrease in the time for hemolysis does not necessarily indicate an increase in permeability (cf. Hunter and Pahigian, 1940). In order to test for a possible change in cell fragility, the following experiments were performed. Twenty cu.mm. of blood were added to 10 cc. of NaCl solutions of concentrations from 1.0 per cent to 0.2 per cent. The

pH of these solutions was adjusted by the addition of two drops of $NaHCO_3$ to each tube. A typical pair of curves is presented in Fig. 2. These data indicate that there is little, if any, change in the volume at which the experimental cells hemolyze.

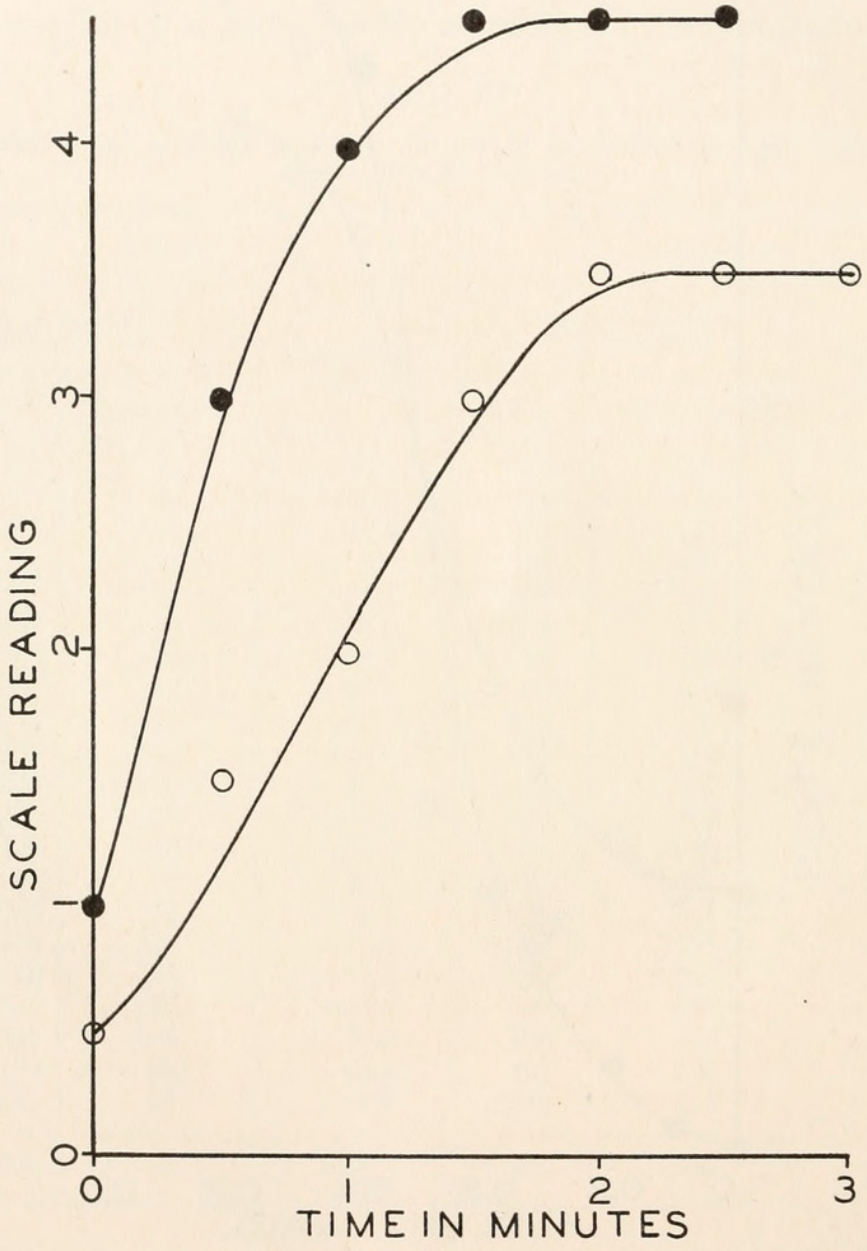

FIG. 3. The effect of saponin on the rate of swelling of chicken erythrocytes. ○—control; ●—experimental. (Scale reading represents amount of swelling.)

As a final test, swelling experiments were performed. Figure 3 presents a typical pair of curves obtained when control and experimental cells were added to an hyperosmotic solution of 0.3 M glycerol in Ringer-Locke. It can be seen that the experimental cells swell more rapidly than the controls. These data, then, definitely indicate an increase in permeability resulting from the experimental treatment.

IV

Schulman and Rideal (1937) presented evidence which indicated that the lytic action of saponin resulted from its reaction with cholesterol in the cell membrane. This would suggest that the experimental cells in the present investigation had altered membranes as a result of the action of saponin on some portion of the membrane in which lipoids were involved. The fact that the rate of penetration of lipoid-insoluble molecules, as well as that of lipoid-soluble molecules, was increased, would indicate that lipoid molecules in the membrane were in some way associated with the channels through which both types of molecules pass. A recent series of experiments by Ballentine and Parpart (1940), in which the effect of lipase on the cell surface was investigated, gave similar results. By making chemical analyses, these authors concluded that the lipase split fatty acids from the phospholipids in the cell surface. They suggested that these phospholipids were " an important structural unit in determining the rate of penetration in the aqueous channels."

As a result of the experiments in which lipase was used and those in which saponin was used, there is evidence to indicate that some of the lipoids in the cell membrane influence the passage not only of lipoid-soluble molecules but also of lipoid-insoluble molecules. It has been demonstrated that phospholipids are one type of molecule involved and the data contained in the present investigation suggest that cholesterol may be another.

Summary

1. Chicken erythrocytes exposed to low concentrations of saponin have their membranes altered.

2. By removing the saponin and resuspending the cells in Ringer-Locke, they will remain unhemolyzed for several days, even though the membranes have been altered.

3. These cells are more permeable to both lipoid-soluble and lipoid-insoluble molecules.

4. The penetration of both types of molecules appears to be affected equally.

5. The rate of penetration of a large molecule such as malonamide is increased more by this treatment than the rate of penetration of a smaller molecule such as glycerol.

6. The fragility of these cells is not increased by this treatment.

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