LXXIX. ON THE INFLUENCE OF THE SPLEEN UPON RED BLOOD-CORPUSCLES. I.

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SINCE the researches of Hunter [1892] on the destruction of red blood-corpuscles, an active rôle in this action has been ascribed to the spleen. The appearance of phagocytosis and haemolysis in the spleen had already been discovered by Koelliker [1847]. From this discovery resulted the researches of Virchow, Quincke and others on the transformation of blood pigments, in which for the rest the spleen was only considered as an organ of accumulation.

But it was Hunter who pointed out that the cause of the blood destruction partly lies in an action of the spleen itself. This view was based specially upon this author's researches on toluylene-diamine poisoning. As is known the action of this poison is much stronger in normal than in splenectomised animals, so that the opinion is justified that the spleen plays an interfering part in toluylene-diamine poisoning.

To determine the haemolytic power of the spleen the following four methods have been used:

1. Microscopical examination of spleen pulp in normal, experimentally modified and pathological conditions. This morphological method is not well adapted for learning the mechanism of the process observed.

2. Study of the consequences of splenectomy. We do not propose to discuss all the experiments made in this direction. The most generally accepted result of this operation is the increased resistance of the red blood-corpuscles of the animal against hypotonic salt solutions, a fact which has mainly been settled by the Dutch investigator Pel Jr. [1911].

3. Study of the action of splenic extracts. The results of this method, which appears to us as a very unreliable and inaccurate one, are moreover very contradictory.

4. Comparative examination of blood from the splenic vein and splenic artery. The latter may of course be replaced by arterial blood in general.

Destructive action of the spleen being admitted, it was obvious to investigate traces of this action in the blood of the vena lienalis. There are three principal qualities of this blood, which may be examined:

- (a) the number of erythrocytes in a cubic millimetre;
- (b) the amount of decomposition products of red cells, especially haemoglobin and bilirubin;
- (c) the resistance of red blood-cells to haemolytic agents.

The number of erythrocytes in a cubic millimetre of the blood of the splenic vein, as compared with blood from the splenic artery, is not a very exact measure of the function of the spleen. In the first place the difference which might be expected would be so small as to be within the limits of technical error. If the lifetime of a red cell is estimated at 20 days (Quincke, Bénard; Rubner consider it to be at least 70–90 days, Dekhuysen still longer) and if one supposes that the total quantity of blood of the body passes through the spleen about thirty times a day [Burton-Opitz, 1912], it is clear that of each volume of blood which passes through the organ only $1/20 \times 1/30 = 1/600$ is destroyed. So one might expect to find numbers like 6,000,000 and 5,990,000 for splenic artery and vein. This difference is too small to be determined with sufficient accuracy. Larger differences might be found, if this action of the spleen were intermittent, but there is no evidence that this is so.

Moreover the operation which is necessary for the puncture of the splenic vessels will cause a stagnation of the blood which has a great influence upon the number of erythrocytes.

Of late this method has been used again by Frey [1920]; his values for the difference between the blood from an artery of the abdominal wall and the splenic vein are so large (on an average 800,000 in a cubic millimeter) that in our opinion they must be due to some error in technique.

An increased quantity of bilirubin in the splenic vein, as compared with that in the blood from other vessels, might possibly be due to the formation of bilirubin in the spleen (Hymans van den Bergh and Snapper, Ernst and Szappanyos), it would not be conclusive proof of a haemolytic function of that organ.

The best method in our opinion is the investigation of the resistance of the red cells against hypotonic salt solutions, the so-called osmotic resistance. It is Eppinger [1920], who also stated that the osmotic resistance of the erythrocytes was decreased in the spleen, asserting that this fact is "eigentlich die einzig fassbare Tatsache die im Sinne einer zerstörenden Tätigkeit der Milz spricht, vorausgesetzt dass die Resistenzherabsetzung als ein einleitender Vorgang der Erythrozytenzerstörung aufgefasst werden kann."

French authors were the first to use the resistance determination in hypotonic salt solutions, originating from Hamburger [1904], to estimate the condition of the red blood-cells in several diseases. The value of this method however has been greatly increased by the modifications and extensions given by Brinkman [1922]. This author showed in the first place that the haemolysis in hypotonic NaCl solution is not only due to the difference of osmotic pressure, but also to the lyotropic effect of pure NaCl, the liquefaction of the cellular membrane-colloids by the Na-ions. Especially when the erythrocytes are washed several times with an isotonic NaCl solution, as is done by many authors, the cells are injured, as is shown by the diminished osmotic resistance (Snapper). This lyotropic effect may be abolished by the addition of Ca-ions to the solution in a very definite concentration. It would lead us too far to discuss the modified Ringer solution, which is used by Brinkman; we give here only the composition:

NaCl	...	...	0.7 %.	[H ⁺]=0.45 × 10 ⁻⁷ .
NaHCO ₃	...	...	0.2 %.	
KCl	...	...	0.01 %.	
CaCl ₂ 6aq.	...	...	0.02 %.	

Hypotonic solutions are obtained by diminishing only the concentration of NaCl.

If the red blood-cells are washed with this isotonic solution, the result is an increase of the osmotic resistance. This is due, as has been shown by Brinkman, to the removal of an auto-haemolytic substance from the surface of the erythrocytes, a phosphatide, perhaps lecithin¹. This same substance, if you make an emulsion of it in modified Ringer solution in an appropriate concentration, added to red blood-cells, may dissolve them.

In the surface of the blood-cells the haemolytic action of the phosphatide is balanced by the cholesterol, which is kept in solution by the first. The osmotic resistance has been shown to be dependent on the proportion cholesterol/phosphatide.

It must still be mentioned, that the modified Ringer liquid may be substituted by a mixture of primary and secondary phosphate, containing 16.30 g. Na_2HPO_4 aq. and 2.18 g. KH_2PO_4 per litre. The freezing-point of this liquid in which the erythrocytes have the same volume as in their plasma, is -0.46° . Without any theoretical consideration, the equivalence of this solution with the modified Ringer solution has been proved empirically. Its advantages are the easiness of its preparation and its stability, combined with the physiological properties of the modified Ringer solution. It was necessary to mention this liquid, because we used it in our resistance-determinations. For further particulars reference must be made to the publications of Brinkman.

After this digression we will discuss briefly the results obtained by others in the determination of the resistance of red blood-cells from the splenic vein in comparison with that of erythrocytes from arterial blood.

Gabbi [1893] finds like Hammarsten an increased resistance in the splenic vein.

Pugliese and Luzatti [1900] observed however a lowered resistance of the red blood-cells leaving the spleen.

Chalier and Charlet [1913] once more found that both minimum and maximum resistance of the blood-cells from the splenic vein (*i.e.* respectively the resistance of the weakest and of the strongest erythrocytes in regard to haemolysis by hypotonic salt solution) were higher than those of erythrocytes from the splenic artery. From their observations they conclude that the spleen only lets the strongest erythrocytes pass and so exerts a haemolytic action upon the blood. This conclusion would be more justified, if only the minimum resistance had increased, the weakest erythrocytes entering the spleen having been destroyed. How is the increase of the maximum resistance, however, from this point of view to be explained, *i.e.* the fact that the most resistant blood-corpuscles which enter the spleen, leave the organ still more resistant?

The remaining investigators, Strisower and Goldschmidt [1914], Widal and Abrami [cited by Eppinger, 1920], Banti and Furno [cited by Eppinger, 1920], Polak Daniëls and Hannema [1916], Eppinger [1920], all found a *diminished* resistance of the erythrocytes leaving the spleen. We have already quoted Eppinger's opinion on the importance of this fact.

We also have tried to study the haemolytic function of the spleen by investigating its influence upon the resistance of the red blood-cells passing through the organ. For practical reasons we took our materials from sheep at the abattoir². Immediately after the killing of the animal, some of the blood spurting out of the carotid artery was collected in a glass tube and defibrinated by shaking it with glass beads. Then as soon as possible the

¹ It may be obtained by evaporating the washing fluid and extracting the phosphatide from the dry residue, or, by direct extraction of the erythrocytes with chloroform, after they have been extracted with light petroleum, in which cholesterol and neutral fats dissolve.

² We were able to do so by the kindness of the authorities of the abattoir in Groningen.

spleen was removed, care being taken not to injure the capsule and the splenic vessels. The organ was then rapidly conveyed to the physiological laboratory. By introducing into the splenic artery a glass canula, which was connected with a reservoir, containing the modified Ringer solution, placed about 175 cm. above the working table, it was possible to obtain from the splenic vein the blood which had remained in the organ, diluted with the salt solution. The suspension of erythrocytes obtained in this manner was centrifuged immediately. These red corpuscles, which had been for some time in the spleen, longer than they are normally, but too short a time for autolysis to take place, seemed to us very well adapted for the investigation of an eventual resistance-decreasing power of the spleen. By the dilution of the blood from the spleen with modified Ringer solution, the resistance of the erythrocytes is practically not changed. The effect of washing red corpuscles with this solution, which we have already described, fails practically to be evident, if the total blood is "washed" with the liquid. To produce the same conditions, however, we have also diluted the arterial blood from the carotis and centrifuged it to get the corpuscles.

Both kinds of the corpuscles were then put in a series of tubes containing the phosphate mixture in geometrically decreasing concentrations, viz.:

9/10 isotonic (obtained by adding one vol. aq. dest. to 9 vols. of an isotonic solution).

(9/10)² isotonic (obtained by adding again one vol. of aq. dest. to 9 vols. of the 9/10 isotonic solution).

Etc.

Ten minutes after the addition of the erythrocytes, the latter were centrifuged and the degree of haemolysis read by Arrhenius' method. In 20 determinations we always reached the same result, that is to say, the blood from the spleen was *less resistant* than that of the carotis. Both the beginning and the complete haemolysis of the erythrocytes of the spleen occurred in a salt solution of higher concentration than those of the blood-corpuscles of the carotis. In a hypotonic salt solution of a definite concentration the degree of haemolysis of the blood from the spleen was always larger than that of erythrocytes from the carotis.

We do not think that *in vivo* the difference of the resistance of red blood-corpuscles from art. and vena lienalis will be as large as in our determinations, because in the latter the erythrocytes had remained for some time in the spleen. Eppinger observed *e.g.* that if the blood stagnated for 10 minutes in the spleen the resistance of erythrocytes from the vena lienalis was distinctly lower than before the stagnation. There is, however, evidence that *in vivo* also stagnation in the organ is possible.

Table I contains the determinations:

Table I.

No.	Kind of blood	% haemolysis Salt concentrations						
		Isotonic	0.9	0.9 ²	0.9 ³	0.9 ⁴	0.9 ⁵	0.9 ⁶
1	Spleen	0	0	10	30	70	100	—
	Carotis	0	0	0	0	0	0	—
2	Spleen	0	0	0	20	50	90	—
	Carotis	0	0	0	0	0	0	—
3	Spleen	0	20	40	90	100	100	—
	Carotis	0	0	0	0	30	75	—
4	Spleen	0	5	10	50	70	100	—
	Carotis	0	0	0	0	5	10	—
5	Spleen	0	0	5	20	50	90	—
	Carotis	0	0	0	0	5	—	—
6	Spleen	0	0	0	50	90	95	100
	Carotis	—	—	—	0	5	60	90
7	Spleen	0	0	5	50	100	100	100
	Carotis	—	—	0	5	30	50	70
8	Spleen	—	—	10	30	50	100	100
	Carotis	—	—	0	10	50	80	100
9	Spleen	—	5	5	20	50	80	100
	Carotis	—	0	0	5	10	50	70
10	Spleen	—	0	5	10	40	60	90
	Carotis	—	0	0	0	5	30	90
11	Spleen	—	5	20	40	60	100	100
	Carotis	—	0	5	10	30	50	70
12	Spleen	—	0	0	10	50	70	100
	Carotis	—	0	0	0	10	50	70
13	Spleen	—	0	0	10	30	60	100
	Carotis	—	0	0	0	5	20	50
14	Spleen	—	0	5	10	40	80	100
	Carotis	—	0	0	5	30	50	70
15	Spleen	—	0	5	20	50	100	100
	Carotis	—	0	0	0	30	70	90
16	Spleen	—	0	5	10	30	70	100
	Carotis	—	0	0	0	30	70	90
17	Spleen	—	5	10	30	50	80	100
	Carotis	—	0	0	0	10	40	80
18	Spleen	—	5	15	30	80	100	100
	Carotis	—	0	0	10	30	70	100
19	Spleen	—	5	10	40	70	90	100
	Carotis	—	0	0	10	40	100	100
20	Spleen	—	5	20	50	100	100	100
	Carotis	—	0	0	10	60	100	100

If one calculates for every concentration the average degree of haemolysis one obtains the following table:

Table II.

Salt concentration	% haemolysis	
	Carotis	Spleen
0.9 × isotonic	0	0
0.9 ² × "	0	5
0.9 ³ × "	0	30
0.9 ⁴ × "	20	60
0.9 ⁵ × "	50	85
0.9 ⁶ × "	75	100
0.9 ⁷ × "	100	100

One may consider this as an accidental case. Fig. 1 shows the "resistance-curves" of this case (the left one of the corpuscles of the spleen, the other of the carotis corpuscles):

Fig. 1.

To deduce from the decreased osmotic resistance of the erythrocytes leaving the spleen conclusions as to the haemolytic function of the organ, it is necessary to investigate how far the phenomenon is specific. Of course it is not necessary that only the erythrocytes from the vena lienalis should have a decreased resistance; it might be also the case with blood-corpuscles leaving the liver and perhaps other organs in so far as these are concerned with blood destruction. We have contented ourselves for the present with showing that an organ like the kidney to which no haemolytic function is assigned fails to show a resistance-diminishing power. Table III shows our determinations:

Table III.

No.	Kind of blood	% haemolysis Salt concentrations					
		0.9 isotonic	0.9 ²	0.9 ³	0.9 ⁴	0.9 ⁵	0.9 ⁶
1	Kidney	0	0	5	50	75	100
	Carotis	0	0	5	60	75	100
2	Kidney	0	5	10	50	80	100
	Carotis	0	5	10	30	50	70
3	Kidney	0	0	10	50	80	100
	Carotis	0	0	10	30	70	100

We have already mentioned the opinion of Chalier and Charlet and of Gabbi, that the spleen would exert a destroying action upon the weakest blood-corpuscles, so that the result is an increased resistance of the erythrocytes in the splenic vein, as was found by these authors, and we have observed that it is difficult to explain the increase of the maximum resistance from this point of view. From our researches, however, it would follow that the spleen exerts a haemolytic influence upon all blood-corpuscles, passing through the

organ, as is proved by the diminished minimum and maximum resistance of erythrocytes from the vena lienalis. The parallel course of the curves in Fig. 1 gives a clear image of this fact. If one makes *e.g.* the assumption that the spleen produces an internal secretion, which causes an alteration of all erythrocytes passing through the organ—and perhaps also has some influence upon the bone marrow—the mode of action of the spleen becomes more intelligible. We also point to the fact that after splenectomy a general increase of the resistance of the blood-corpuscles has been found, *i.e.* an increase of the minimum and maximum resistance whilst the “breadth of resistance” (the difference in concentration between the solutions in which beginning and complete haemolysis is observed) remains the same. Pel gives *e.g.* the following table as the average of a large number of experiments:

Table III

	Normal dog	Splenect. dog
	%	%
Average concentration of NaCl, in which beginning haemolysis is observed	0.42	0.35
Average concentration of NaCl, in which just complete haemolysis is observed	0.30	0.23
Breadth of resistance	0.12	0.12

This phenomenon would also be explained in the most simple way by the assumption that after removal of the spleen the production of an internal secretion ceases, that has normally a harmful action upon the erythrocytes, which is measured by the diminished resistance.

We have tried to ascertain more particulars about the resistance-diminishing power of the spleen. Assuming the action of an internal secretion, it was obvious that in the first place the surface layer of the erythrocytes would be altered by such an action. From the researches of Brinkman and Miss van Dam [1921] we know however the importance of the proportion cholesterol/phosphatide in the surface layer for the osmotic resistance. A decrease of this proportion means a diminution in the osmotic resistance. To test the assumption that only or principally the surface layer of the erythrocytes is altered by the action of the spleen it is only necessary to investigate the resistance of the blood-corpuscles of the vena lienalis, when this surface layer has been removed by washing the erythrocytes with modified Ringer solution. We have made these determinations by washing the blood-corpuscles obtained in the manner described on p. 757 and also the erythrocytes from the art. carotis three times with the modified Ringer solution and then determining the resistance in the same way as described above. In all our determinations we found a considerable difference between the two kinds of corpuscles, before washing, which disappeared however almost entirely after this process. Table IV contains the values of these determinations:

Table IV.

No. ¹	Kind of blood. Three times washed with	% haemolysis. Salt concentrations						
		Isotonic	0.9	0.9 ²	0.9 ³	0.9 ⁴	0.9 ⁵	0.9 ⁶
1	Spleen	0	0	0	0	5	20	—
	Carotis	0	0	0	0	0	5	—
2	Spleen	0	0	0	0	20	50	—
	Carotis	0	0	0	0	0	0	5
3	Spleen	0	0.	0	0	5	20	—
	Carotis	0	0	0	0	0	5	—
4	Spleen	—	0	5	10	30	50	100
	Carotis	—	0	0	10	30	50	100
5	Spleen	—	0	0	0	5	10	40
	Carotis	—	0	0	0	5	10	40
6	Spleen	—	0	0	10	40	80	100
	Carotis	—	0	0	10	40	80	100
7	Spleen	—	0	0	20	50	70	100
	Carotis	—	0	5	20	30	70	100
8	Spleen	—	0	0	10	30	60	90
	Carotis	—	0	0	20	30	50	90
9	Spleen	—	0	0	0	10	40	100
	Carotis	—	0	0	0	10	30	70
10	Spleen	—	0	0	10	40	80	100
	Carotis	—	0	0	20	50	80	100

Table V contains the average values which are represented also in Fig. 2.

Table V.

Salt concentration	% haemolysis	
	Washed spleen Corpuscles	Washed carotis Corpuscles
0.9 × isotonic	0	0
0.9 ² × "	0	0
0.9 ³ × "	5	5
0.9 ⁴ × "	20	15
0.9 ⁵ × "	45	35
0.9 ⁶ × "	90	85

Fig. 2.

¹ The resistance of the unwashed corpuscles of these cases may be seen respectively in Nos. 1, 2, 3, 4, 11, 13, 14, 15, 16, 17, 18 of Table I.

The resistance of the arterial erythrocytes does not increase in the same degree as that of the blood-corpuscles of the spleen, so that after the washing the difference between the two kinds of blood-corpuscles has been reduced considerably, as is shown by Table V and Fig. 2.

There remains a small difference; this is perhaps partly due to the defibrinating of the blood from the art. carotis (the resistance is increased hereby somewhat); moreover arterial erythrocytes are always somewhat more resistant than blood-corpuscles from venous blood (Hamburger). The same difference exists between the resistance of erythrocytes from the vena renalis and art. carotis.

From our experiments we draw the conclusion that the influence exerted by the spleen upon the red blood-cells is confined to the adsorbed surface layer of the latter, *i.e.* to the layer which may be removed by washing with a modified Ringer solution. As we have not found any conception in the literature about the mode of alteration of the erythrocytes themselves in the spleen, except the following of Jacobsthal [1921], it seemed worth while to quote it. "Ich habe mir die Vorstellung gebildet, dass die Abnahme der Blutkörperchenresistenz beim haemolytischen Ikterus sich so entwickelt, dass die die Milz passierenden Blutscheiben jedesmal nicht haemolysiert, sondern an ihrer Lipoidhülle sozusagen nur *angenaht* werden."

The three following alterations in the surface layer of the red blood-corpuscles in the spleen are possible:

1. Amount of phosphatide the same; amount of cholesterol decreased.
2. Amount of phosphatide increased; amount of cholesterol the same.
3. Amount of phosphatide and cholesterol both increased, the former however in the highest degree.

In all these cases the proportion cholesterol/phosphatide would diminish and this means a decrease of osmotic resistance. It seems that of these three possibilities the third one holds true. It has appeared to us that the erythrocytes leaving the spleen contain more cholesterol than the erythrocytes from the art. carotis¹. It is therefore necessary that the phosphatide should undergo a considerable augmentation. We want to point out here already that this increase may be as well of a qualitative as of a quantitative nature. The specific influence of the phosphatide and cholesterol of the surface of the erythrocyte upon its resistance is undoubtedly due to the physico-chemical properties of these substances. If one makes an emulsion of lecithin *ex ovo* in an isotonic modified Ringer solution, this emulsion has a low surface tension and a great haemolytic power. If, however, cholesterol is added in increasing small amounts, the surface tension and the viscosity of this emulsion rise gradually and at the same time the haemolytic power decreases. The haemolytic power of an emulsion of lecithin is dependent on the *quantity* of the substance per cc. of the solvent, but apparently small *qualitative* alterations of the lecithin also have an enormous influence upon its haemolytic power.

¹ Publication of these investigations will follow.

This has been shown by Delezenne and Fourneau [1914], who withdrew oleïc acid from lecithin by means of cobra venom and in this way obtained a substance, called by them "désoléoléïcithine" or "lysocythine," which had a very strong haemolytic power. It is possible that *in vivo* also analogous chemical changes play a part in haemolytic processes. Perhaps it will be possible to settle the nature of the actual quantitative or qualitative changes in the phosphatides in the erythrocytes in the spleen and elsewhere by further researches.

To judge the value of our opinions, developed after investigations on the sheep's spleen, for normal and pathological physiology (Eppinger's Hypersplenie) of the human spleen, it will be necessary to repeat the determinations of the resistance described by us in man. This is possible where splenectomy is performed. Till now only one case has been investigated in this respect. For these figures we are indebted to Dr H. H. de Zoo de Jong, curator of the clinic of Prof. L. Polak Daniëls, University Hospital, Groningen. Splenectomy was performed in the case of a haemolytic anaemia. Some blood was taken from the vena lienalis and the red blood cells compared with erythrocytes from the peripheral blood in regard to their osmotic resistance before and after washing with a modified Ringer solution. Table VI contains the determinations:

Table VI.

		% haemolysis			
		Before washing		After washing	
Salt concentration		Peripheral	Spleen	Peripheral	Spleen
	$\frac{1}{2}$ isotonic	20	30	0	0
0.9 ×	"	35	65	40	40
0.9 ² ×	"	70	80	70	70
0.9 ³ ×	"	80	85	80	80
0.9 ⁴ ×	"	95	95	90	95
0.9 ⁵ ×	"	100	100	100	100

The difference between peripheral blood and blood from the vena lienalis would perhaps have been more evident if the resistance had also been determined in solutions stronger than $\frac{1}{2} \times$ isotonic. Then the beginning of haemolysis would also have been determined.

For the rest it is clear that in this case the investigations on the sheep's spleen are wholly confirmed. It will, however, be necessary to repeat these determinations in regard to man if possible.

CONCLUSIONS.

The spleen has the power of *diminishing* the *osmotic* resistance of the erythrocytes. These are prepared hereby for haemolysis, which partially takes place in the organ itself.

Erythrocytes from the vena lienalis, which have been washed with an equilibrated salt solution, *do not show* this decreased osmotic resistance. This fact proves that the point of attack of the haemolytic power of the spleen lies in the removable surface layer of the erythrocytes. The alteration in this layer must consist in a decrease of the proportion cholesterol/phosphatide.

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