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Physiology. — "*Comparative researches on young and old erythrocytes.*" [1]) By J. SNAPPER. (Communicated by Prof. H. J. HAMBURGER.)

(Communicated in the meeting of June 29, 1912).

1. *Introduction.*

Of late years various investigations have been carried out with a view to ascertain if there are differences between newly formed red blood-corpuscles and those which have already circulated for some time. For this purpose the blood was examined of animals which had been made anaemic in some way or other. Since the loss of blood must be made up for by fresh red blood-corpuscles, we may depend upon it that the differences existing between the blood before and after the artificial anaemia was effected, are caused by the new formation of fresh red blood-corpuscles.

According to recent [2]) investigations there is a difference between new red corpuscles, formed after bleeding, and those formed after blood has been lost by injections with blood poisons. This difference manifested itself especially when the capacity of resistance of the blood-corpuscles was tested by means of hypotonic salt-solutions. Whilst the blood-corpuscles of an animal which had been made anaemic by poison-injections resisted the hypotonic salt-solution better than the blood-corpuscles of a normal animal, this was said not to be the case with those animals of which the anaemia had been caused by bleeding.

There are indeed reasons to assume that the regeneration after poison-injections will be stronger than after bleedings. Yet it is improbable that the young red blood-corpuscles formed after bleeding, should not be distinguished in the same way from the old erythrocytes — though it may be in a slighter degree — as those formed after poison-injections. These slighter differences too, may be of importance, however. As we know, the anaemia effected by injections of poison causes the number of blood-corpuscles to decrease so strongly that sometimes only 16 °/₀ of the original number are left. It is not improbable that after such an extreme loss of blood the regeneration may be an abnormal one. On the other hand the properties of new red blood-corpuscles, formed after a smaller loss of blood will bear a greater resemblance to the properties of young red blood-corpuscles formed under physiological conditions.

1) A more detailed account of these investigations will be published elsewhere.

2) ITAMI and PRATT, Biochem. Zeitschrift, Bd. 18.
SATTLER, Folia Haemat, 1910.

In order to discover these minuter differences it was necessary to subtilize the method. For this purpose the following considerations were held in view. In the experiments mentioned above only the maximum and minimum capacity of resistance of the young erythrocytes had been determined.

The determination of this capacity is, as is generally known, based on the following facts.

When red blood-corpuscles are suspended in a salt-solution with an osmotic pressure less than 0,9 °/₀ NaCl, they absorb water which causes them to swell. The lower the osmotic pressure of the salt-solution, the more water they will absorb. At last they burst and the haemoglobin, contained in them, enters the solution. The lower the osmotic pressure of the medium in which they can only just maintain themselves, the greater the capacity of resistance of the red blood-corpuscles. Seeing that not all the blood-corpuscles have the same capacity of resistance the *minimum* capacity of resistance of the blood is expressed by the most concentrated Na Cl-solution which already causes haemolysis. In this solution the weakest blood-corpuscles lose their haemoglobin. The *maximum capacity of resistance* of the blood is determined by the Na Cl-solution in which the haemolysis is complete, and which cannot be resisted even by the strongest blood-corpuscles. *Only these two salt-solutions have been determined in the above-mentioned investigations: as to the degree of haemolysis caused by the intermediate solutions every detail is wanting.*

By determining this degree we succeeded in discovering some qualities by which blood-corpuscles, newly formed after blood has been lost, are distinguished from the other, remaining ones. It was also found possible to study the mechanism of the regeneration more closely. The experimental method may be described as follows.

2. *Experimental method.*

The blood experimented upon was always that of rabbits. It was obtained by a slight cut in the ear and defibrinated by being beaten with 2 glass rods. A series of centrifugating tubes was filled with 5 cm^3 NaCl-solution in progressive concentrations, the difference between two successive concentrations being 0.02 °/₀. To these 5 cm^3 0.1 cm^3 of blood was added and the mixture was shaken thoroughly. Then the solutions were left exposed to the temperature of the room for a few hours; subsequently they were centrifugated. In the tube where red blood-corpuscles no longer settle at the bottom, the haemolysis is complete. In the other tubes, where blood-corpuscles have remained intact, less haemoglobin has been dissolved. *The degree of haemolysis in these tubes is expressed by the proportion between the haemoglobin concentration in these tubes and the haemoglobin concentration of the tube with complete haemolysis.* These proportions can be easily calculated by making use of the colorimetrical method first suggested by ARRHENIUS [1]). Thus a series of values is obtained expressing which percentage of the complete haemolysis is caused by each salt-concentration. (Table A). The progress of this haemolysis may be expressed by a curve, the absciss of which is formed by the NaCl-concentrations and the ordinate by the degrees of haemolysis effected by each concentration. (Fig. 1).

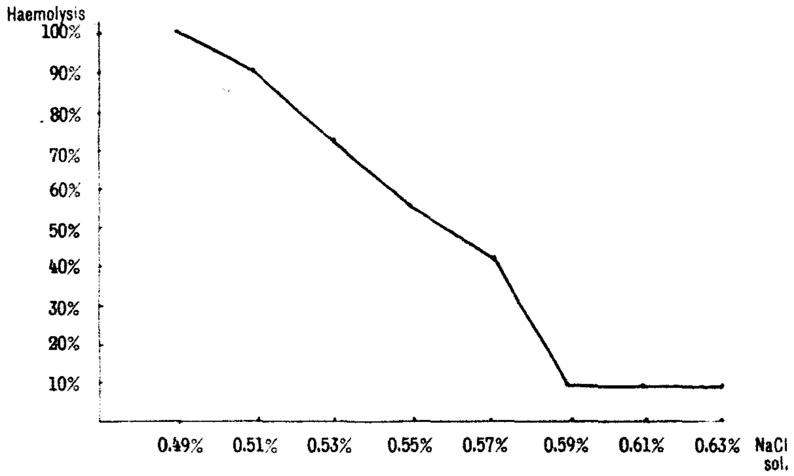

Fig. 1. Graphical representation of Table A.

TABLE A. Denoting the haemolysis caused by each NaCl concentration.

0.63 %	NaCl	solution	caused	9 %	haemolysis
0.61 »	»	»	»	9 »	»
0.59 »	»	»	»	9 »	»
0.57 »	»	»	»	42 »	»
0.55 »	»	»	»	55 »	»
0.53 »	»	»	»	73 »	»
0.51 »	»	»	»	91 »	»
0.49 »	»	»	»	100 »	»

3. *The capacity of resistance of old and new erythrocytes against diluted salt-solutions.*

The blood of an animal which has been made anaemic by bleeding, may be examined in the same way as that of a normal animal.

[1]) ARRHENIUS and MADSEN, Zeitschr. für Physikal. Chemie 1903.

After every bleeding the degree of haemolysis, caused by the different salt-solutions, is determined. (Table B). When we also represent these values graphically, the curve after every venesection is found to have moved in the direction of the lower concentrations. (Fig. 2). In the normal animal 80 °/₀ is set free at a concentration of 0.49 °/₀ NaCl. After one venesection the same solution causes 66 °/₀ of haemolysis and after 2 venesections only 25 °/₀. In other words whilst only 20 °/₀ of the blood-corpuscles of the normal animal could resist a NaCl-solution of 0.49 °/₀, the composition of the blood is changed to such an extent after two venesections, that 75 °/₀ of the blood corpuscles can still bear this concentration. Moreover, the haemolysis was complete in the case of the normal animal at 0.47 °/₀ NaCl. After two bleedings the same 0.47 °/₀ NaCl solution caused an haemolysis of 40 °/₀ only. Hence there were in the blood of the anaemic animal 100 °/₀ — 40 °/₀ = 60 °/₀ blood corpuscles which could withstand a less concentrated salt-solution than the most resistant blood-corpuscles of the normal animal. These 60 °/₀ are, therefore, blood corpuscles which were not met with in the non-anaemic animal. They have been newly formed after the bleeding; they are the new blood-corpuscles which are to replace the lost ones. *Also the young blood-corpuscles, formed after a venesection, have an increased capacity of resistance* [1]).

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[1]) About the quantity of haemoglobin new erythrocytes contain, more particulars might be discovered by counting the number of erythrocytes which remain in every concentration. If we compare this with the quantity of haemoglobin set free, then it may be decided whether the old erythrocytes contain more or less haemoglobin than the new ones. If for instance the old. that is to say the blood corpuscles with smaller resistance contained more haemoglobin, then in the more concentrated solutions more haemoglobin would proportionately be set free than in the less concentrated ones. Generally speaking, however, the values are found to agree very well.

It is impossible to settle this question conclusively, the method of counting, as suggested by ZEISS-THOMA, allowing of no closer determination than with an error of 5%. This causes deviations in the agreement of the values.

By means of the haematokrit-method it can be determined whether there are any differences between the volumes of old and new erythrocytes. For this purpose the volume of the cells, left after each concentration, was compared with the number of erythrocytes left. Though here too, the values were found to agree, the method employed in counting gave rise again to important inaccuracies

Besides, an equal average volume of new and old blood corpuscles would be the more remarkable since according to unpublished investigations of this institute made by HAMBURGER and KOOY, the diameter of new blood corpuscles is greater than that of old ones.

It would follow from this that new and old erythrocytes differ in shape.

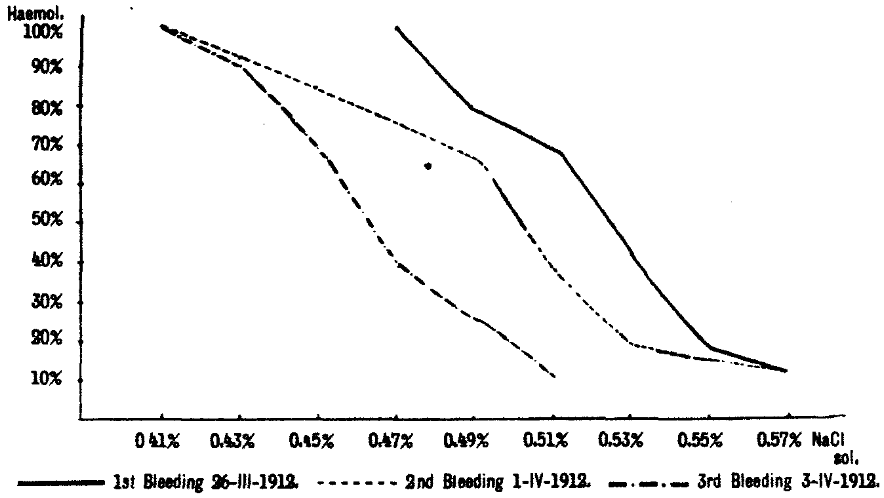

Fig. 2. graphical representation of Table B.

TABLE B, denoting the haemolysis caused by each NaCl-concentration in the normal and the anaemic animal.

	1st Venesection	2nd Venesection	3rd Venesection
At 0.57 pCt. NaCl	12 pCt.	12 pCt.	—
„ 0.55 „ „	18 „	15 „	—
„ 0.53 „ „	42 „	19 „	—
„ 0.51 „ „	67 „	37 „	10 pCt.
„ 0.49 „ „	80 „	66 „	25 „
„ 0.47 „ „	100 „	75 „	40 „
„ 0.45 „ „	—	84 „	67 „
„ 0.43 „ „	—	91 „	90 „
„ 0.41 „ „	—	100 „	100 „

(3rd Venesection, 0.51–0.43: Haemogloben was found in the solution)

4. *New erythrocytes are built up from old ones.*

The values and curves of fig. 2 may also be viewed in another way. On the first day, for instance, a solution of 0.57 % NaCl causes 12 % haemolysis, whilst at 0.55 % NaCl 18 % of the erythrocytes had disappeared. Hence there were then 18 % — 12 % = 6 % blood-corpuscles which were just unable to withstand a solution of 0.55 % NaCl. For 6 % of the blood corpuscles 0.55 % NaCl is just the minimum concentration they can bear.

TABLE C. Same rabbit as Fig. 2 and Table B.

	1st Venesection	2nd Venesection	3rd Venesection
Whilst they can still bear a slightly stronger concentration they burst at 0.57 pCt. NaCl	12 pCt.	12 pCt.	—
„ „ „ „ „ „ „ „ „ „ „ „ 0.55 „ „	6 „	3 „	—
„ „ „ „ „ „ „ „ „ „ „ „ 0.53 „ „	24 „	4 „	—
„ „ „ „ „ „ „ „ „ „ „ „ 0.51 „ „	25 „	18 „	10 pCt.
„ „ „ „ „ „ „ „ „ „ „ „ 0.49 „ „	13 „	29 „	15 „
„ „ „ „ „ „ „ „ „ „ „ „ 0.47 „ „	20 „	9 „	15 „
„ „ „ „ „ „ „ „ „ „ „ „ 0.45 „ „	—	9 „	27 „
„ „ „ „ „ „ „ „ „ „ „ „ 0.43 „ „	—	7 „	23 „
„ „ „ „ „ „ „ „ „ „ „ „ 0.41 „ „	—	9 „	10

Thus it can be established for every concentration, which percentage of the bloodcorpuscles lose their haemoglobin in one particular solution, whilst they could withstand a solution which was 0.02 % stronger.

If, therefore, we analyse in this manner the values obtained with the anaemic animal every day, then we shall find for which percentage of the erythrocytes every salt-solution represents the minimum concentration. (See Table C). The 3 series of values obtained, may be expressed again in curves (Fig. 3). There are always one or two salt-concentrations representing for the greater number of erythrocytes the minimum concentration they can bear. On the first day these maxima are found at 0.53 % and 0.51 % NaCl, on the second day at 0.49 % NaCl, and on the third day at 0.45 % and 0.43 % NaCl.

These maxima too move in the direction of the less concentrated solutions.

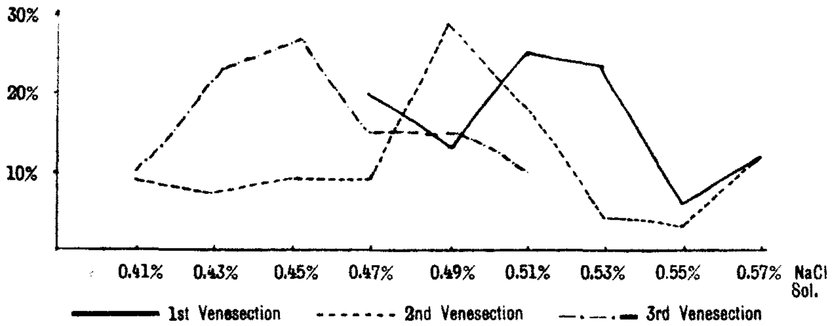

Fig. 3. Graphical representation of Table C.

It follows from this

	2nd Venesection	3rd Venesection
Bloodcorpuscles bursting just at 0.55 pCt. NaCl	equal	decreased
" " " " 0.53 " "	decreased	"
" " " " 0.51 " "	"	"
" " " " 0.49 " "	increased	"
" " " " 0.47 " "	decreased	increased
" " " " 0.45 " "	newly formed	"
" " " " 0.43 " "	"	"
" " " " 0.41 " "	"	equal

Hence the number of strong blood-corpuscles increases as the number of weak ones decreases. This increase on the one hand and decrease on the other, run so exactly parallel, that we are at once tempted to trace a connection between them.

Moreover the weaker ones are found to decrease much more strongly than can be explained by mere loss of blood only. If no regeneration were to take place, the proportion between the weaker and the stronger blood-corpuscles would not be modified at all. At the regeneration, therefore, the number of weaker bloodcorpuscles must become relatively smaller since it is just the new bloodcorpuscles which have a great capacity of resistance. Even if the entire loss of blood had been made up for, this strong decrease of the weaker ones cannot be explained. The rabbit weighed 2000 grammes, contained, therefore, $^{8}/_{100} \times 2000 = 160$ gr. of blood. After 2 bleedings of 15 ccM. (that is about 20 % of the whole) of the 80 % erythrocytes which are destroyed at 0.49 % NaCl only 25 % are found back. These weaker bloodcorpuscles decrease, therefore, much more strongly than can be explained by loss of blood only: they must be used in some way or other. Since, moreover, the increase of the stronger bloodcorpuscles runs parallel to the decrease of the weaker ones, we may with a great amount of probability assume that the *young red bloodcorpuscles are built up out of the weaker ones.*

Now it can also be explained why the young blood corpuscles develop a greater capacity of resistance as more blood is withdrawn. The weaker bloodcorpuscles decreasing very strongly after every bleeding, the old bloodcorpuscles, which in the anaemic animal serve to build up the new ones, are already much stronger than the old bloodcorpuscles which are disintegrated in the normal animal for this purpose. As the material out of which they are built up grows stronger and stronger, the young blood-corpuscles are stronger too after each venesection.

This also supplies us with one of the chief causes of the difference between erythrocytes, formed after bleeding, and those formed after poison-injections.

Owing to the abnormally strong decrease of the number of blood-corpuscles after poison-injections, the newly formed cells could not but become very strong — much stronger than after a few bleedings.

5. *Also in the blood the regeneration greatly surpasses the loss.*

Finally a conclusion may be arrived at as regards the degree of the regeneration. After 2 venesections about 20 % of the blood of the rabbit had been withdrawn. At the third bleeding 60 % bloodcorpuscles were found with a greater capacity of resistance than the strongest of the normal animal. After 20 °/₀ blood has been withdrawn at least 60 °/₀ new cells are formed. In this case too, the rule of WEIGERT, applicable in general pathology, holds good: *the regeneration surpasses the loss by far.* Only with regard to blood it cannot be deduced from the number of bloodcorpuscles, because for each new bloodcorpuscle an old one has to be disintegrated. Hence the absolute number of bloodcorpuscles per m.M^3. can increase but slowly.

Yet this strong regeneration of the blood-corpuscles too, has probable a beneficial effect upon the organism. Though there is no difference as regards size or haemoglobin-percentage, MORAWITZ has pointed out the fact that while blood in normal circumstances can bind chemically hardly any oxygen, the anaemic blood consumes rather large quantities of O_2 [1]). Hence the new blood-corpuscles differ qualitatively from the old ones, which appears besides from their increased capacity of resistance.

6. *Effect of the serum on haemolysis.*

a. *The serum is replaced by 0.9 °/₀ NaCl.*

Before drawing the conclusions, mentioned above, *it was necessary to determine the effect which the serum has upon haemolysis.* Mostly we read that the serum contains substances which impede haemolysis [2]), for when the blood-corpuscles have been washed with 0.9 °/₀ NaCl-solution, their capacity of resistance has decreased. If these substances were really present, it might have a considerable effect upon the capacity of resistance of anaemic blood. In anaemic blood there is relatively more serum than in normal blood: the greater quantity of serum would impede haemolysis more strongly, and this might give an impression of a greater capacity of resistance.

It is indeed found that *the capacity of resistance of the blood is lessened when it is washed with 0.9 °/₀ NaCl.* (See Fig. 4).

b. *The serum is replaced by 4 °/₀ glucose.*

That this is not due, however, to the effect of the serum having disappeared, but probably to osmotic changes, follows from the fact that washing with an isotonic glucose solution (4 °/₀) does not modify the capacity of resistance (See Fig. 4).

[1]) MORAWITZ, Archiv f. exper. Pathol. u. Pharmacol. Bd 60.

[2]) GROS. Ztschr. f. exper. Pathol. u. Pharmacol. Bd. 62.
SATTLER l.c.

Seeing that glucose cannot enter the blood-corpuscles, no ions can leave them. A solution leaving intact the osmotic equilibrium does not modify the capacity of resistance. Hence the removal of the serum by washing the blood-corpuscles need not alter the capacity of resistance.

The serum contains, therefore, no substances which impede haemolysis.

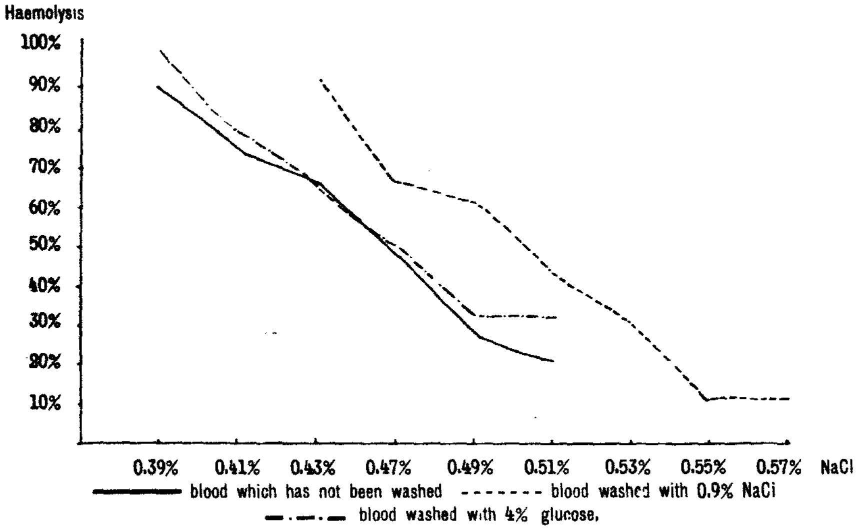

Fig. 4. Graphical representation of Table D.

TABLE D. When the blood has been washed with 0.9 pCt. NaCl-solution, the same NaCl-concentration effects more haemolysis than it does in blood which has not been washed.

Blood, washed with a 4 pCt. glucose-solution has *no* decreased capacity of resistance.

	Blood which had not been washed	Blood washed with 0.9 pCt. NaCl	Blood washed with 4pCt. glucose
At 0.57 pCt. NaCl	—	11 pCt.	—
„ 0.55 „ „	—	11 „	—
„ 0.53 „ „	—	31 „	—
„ 0.51 „ „	21 pCt.	44 „	33 pCt
„ 0.49 „ „	27 „	61 „	33 „
„ 0.47 „ „	47 „	66 „	50 „
„ 0.45 „ „	66 „	92 „	66 „
„ 0.43 „ „	73 „	—	80 „
„ 0.41 „ „	91 „	—	100 „

Haemoglobin had been set free

7. *In the osmotic disturbance, caused by washing with* 0.9 °/₀ *NaCl, the ions of Ca play a prominent part.*

This disturbance of the osmotic equilibrium of the blood-corpuscles, caused by washing with 0.9 °/₀ NaCl, is not effected if we wash with 0.9 °/₀ NaCl + 0.1 °/₀ $CaCl_2$. What was pointed out before in the case of leucocytes, viz. the importance of ions of Ca[1]), is also found to apply to the erythrocytes. Though only traces of Ca are found in the erythrocytes, yet their capacity of resistance is considerably modified if osmose causes these few ions to disappear.

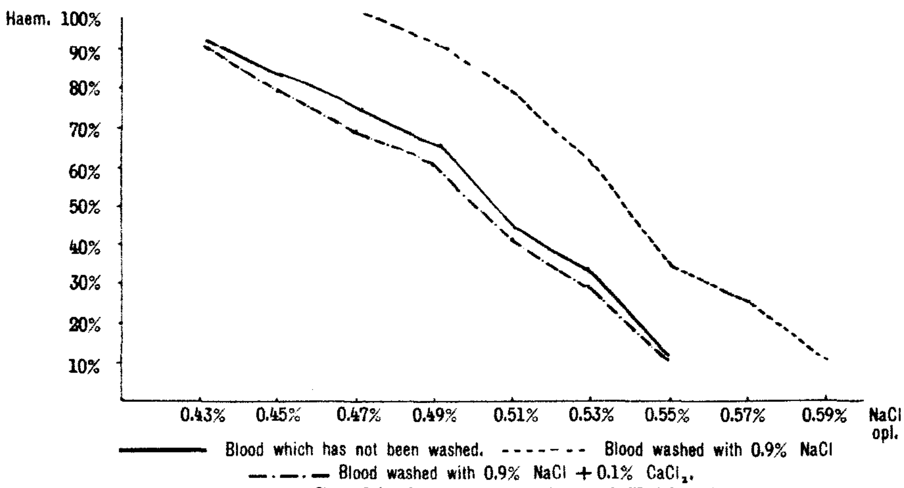

Fig. 5. Graphical representation of Table E.

TABLE E. Blood which has been washed with a 0.9 pCt. NaCl-solution has a decreased capacity of resistance
Blood which has been washed with a 0.9 pCt. NaCl + 0.1 pCt. $CaCl_2$-solution has *no* decreased capacity of resistance.

	Blood which had not been washed	Blood washed with 0.9 pCt. NaCl	Blood washed with 0.9 pCt. NaCl + 0.1 pCt. $CaCl_2$	
At 0.59 pCt. NaCl	—	10 pCt.	—	Haemoglobin had been set free.
„ 0.57 „ „	—	25 „	—	
„ 0.55 „ „	11 pCt	35 „	10 pCt.	
„ 0.53 „ „	33 „	60 „	28 „	
„ 0.51 „ „	44 „	78 „	40 „	
„ 0.49 „ „	66 „	90 „	60 „	
„ 0.47 „ „	74 „	100 „	68 „	
„ 0.45 „ „	83 „	—	78 „	
„ 0.43 „ „	92 „	—	90 „	
„ 0.41 „ „	100 „	—	—	

[1]) HAMBURGER and HEKMA Biochem. Zeitschrift Bd. III and Bd. VII.

Conversely the capacity of resistance does not change if the Ca is prevented form leaving the blood-corpuscles though all the other metal-ions should disappear.

8. *Also washed new erythrocytes have a greater capacity of resistance than washed old erythrocytes.*

At any rate the objection to the results of the examination of anaemic blood is removed: the fact that anaemic blood contains more serum than normal blood can have no effect upon the capacity

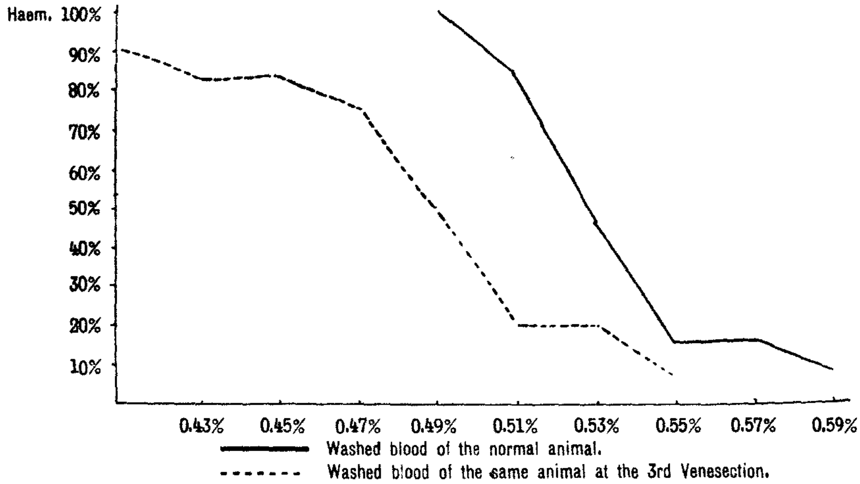

Fig. 6. Graphical representation of Table F.

TABLE F. Denoting the course of haemolysis in the normal and the anaemic animal after the blood-corpuscles have been washed with 0.9 pCt. NaCl.

	Normal blood (washed)	Anaemic blood (washed)
At 0.59 pCt. NaCl.	8 pCt.	—
„ 0.57 „ „	16 „	—
„ 0.55 „ „	16 „	7½ pCt.
„ 0.53 „ „	46 „	20 „
„ 0.51 „ „	83 „	20 „
„ 0.49 „ „	100 „	50 „
„ 0.47 „ „	—	75 „
„ 0.45 „ „	—	82 „
„ 0.43 „ „	—	82 „
„ 0.41 „ „	—	90 „

Haemoglobin had been set free.

of resistance as the serum contains *no* substances which impede haemolysis.

Moreover it may be observed that *also the washed blood-corpuscles of an anaemic animal possess a greater capacity of resistance than the washed blood-corpuscles of the same animal in a normal condition.* See Table F and Fig. 6.

Summary.

The colorimetrical determination of the haemolysis, (ARRHENIUS) caused by diluted NaCl-solutions, suggests a means to compare the qualities of blood-corpuscles, differing as regards their capacity of resistance.

With the aid of this experimental method the following results were obtained.

1. New erythrocytes resist diluted NaCl-solutions better than old ones.

2. It must be assumed that new red blood-corpuscles are built up out of the old ones.

3. After venesections the regeneration greatly surpasses the loss. (Rule of WEIGERT).

4. Washing the blood-corpuscles with a NaCl-solution of 0,9 °/₀ renders them less capable of resisting diluted NaCl-solutions.

5. The conclusion drawn from this by several workers that this phenomenon is caused by the removal of unknown substances, found in the serum, which substances impede haemolysis, is incorrect. Experiments have shown that if a 4 °/₀ glucose-solution is used instead of a NaCl-solution 0,9 °/₀, this decrease in capacity of resistance does not manifest itself.

6. The phenomenon, mentioned sub 4, should rather be viewed in the light of an osmotic disturbance, the principal factor of which is the loss of Ca, suffered by the blood-corpuscles. Indeed the capacity of resistance is not modified if 0,1 °/₀ $CaCl_2$ is added to the NaCl-solution.

7. New erythrocytes, washed with NaCl-solution 0,9 °/₀, have a greater capacity of resistance than old ones which have been treated in the same way.

May 1912. *Groningen, Physiological Laboratory.*