Physiology. — "*Researches on the chemical causes of normal and pathological Haemolysis*". By R. BRINKMAN and A. V. SZENT-GYÖRGYI. (Communicated by Prof. H. J. HAMBURGER),

(Communicated at the meetings of February 23 and April 26, 1923).

I. *Isolation of the haemolytic substances of normal human blood.*

It has been known for a long time that it is possible to isolate from normal blood by means of fat-extraction methods groups of substances, which possess strongly haemolytic properties. The study of these substances must be important for the explanation of normal and pathological haemolysis, but a definite result revealing their structure and manner of action has not yet been obtained. NOGUCHI [1]), when extracting these substances supposed them to be soaps, but he only examined them in regard to immunological phenomena and his views were not supported by later investigators [2]). Others were thinking of substances with a phosphatid structure, but could not give sufficiently conclusive proofs [3]).

A more exact investigation of the chemical constitution and the physico-chemical form, in which they exist in the blood, is wanted to be able to determine the physio-pathological significance of these substances. We have started from the idea, that it must be desirable to isolate these substances in a form as pure as possible, to be able to determine their chemical and physico-chemical properties. The first condition to be fulfilled was complete extraction of the haemolytic substances. Afterwards the extracts were fractioned under the guidance of their more and more increasing activity. This activity was tested by dispersing the extracts in isotonic neutral phosphate mixture at 37° [4]).

The determination of the haemolytics was carried out in the following way:

The human blood obtained by venapunction was defibrinated and sharply centrifugalized at once The corpuscles were imbibed in fat-free filterpapers and

[1]) NOGUCHI, Biochem. Zeitschr. VI, 327, (1907)
[2]) See LANDSTEINER. Handbuch KOLLE-WASSERMANN II, 1291, (1913).
[3]) BRINKMAN, l. c.
[4]) See for the method BRINKMAN Arch. néerl. de Physiol. VI, 451, (1922).

dried at 37°. Afterwards the red corpuscles were extracted for one hour by petrolether at room temperature; in this way neutral fat and most of cholesterin are extracted without any loss of haemolytics. The following quantitative extraction of haemolytics was made in a specially constructed small apparatus for "boiling-point extraction", with always freshly destilled fluid, adaptable for small quantities. As extraction-liquid acetone was chosen in analogy to the use of this liquid in phosphatid chemistry. In order to extract the haemolytics completely with acetone a preceeding treatment with alcohol-vapour for half an hour was necessary; the than following acetone extraction dissolves all haemolytics in two hours.

To complete purification the acetone-extract was concentrated to a small volume and allowed to stand for one night in ice. In this way most of the dissolved substances are precipitated but no haemolytics are found in the precipitate. The remaining strongly active fraction has the following properties: it can be dissolved in all typical lipoid-dissolving liquids, if the reaction is slightly acid, but in an alkaline médium the haemolytics are insoluble is petrol ether. The examined substances are not precipitated by cadmium, but they are precipitated quantitatively in aquous solution by barium and in acetone solution completely by an ammoniacal solution of acetate of lead, in the presence of not less than 30 % of water

So we see, that the investigated haemolytics show the typical reactions of the higher fatty acids. The said precipetate contained no phosphorus, so that phosphatides can be excluded definitely, *and the haemolytics, which can be extracted from normal blood must be identified with higher fatty acids resp. their soaps.*

The solubilty of the Pb and Ba salts indicated, that a mixture of fatty acids must be present, containing no or one and also more than one double linkage. Further experiments must determine the constitution and procentual concentration of these substances.

Separation of the fraction of the fatty acids and of phosphatids can only be obtained by careful quantitative methods of working; it is probable, that complete separation was not got by former investigators.

In addition to these results we have examined once more the haemolytic action of pure lecithin. If was found that a praeparation of lecithin purified by the newest methods showed no haemolytic properties; the haemolytic action of common trade lecithin must be ascribed to impurities, and this also is the case if this substance is somewhat purified by the usual acetone-precipitation.

With the knowledge, that the haemolytics of lipoid blood extract are higher fatty acids it is possible to isolate them in a simpler way. This may be done by the following method: the dried blood, sucked in filterpaper (5 cc. of blood) is treated with absolute alcohol for one hour in the boiling-point extraction apparatus. The extract

is concentrated to 5 cc. and than 5 cc. of a solution is added, which contains 0,2 n. Na_2CO_3 and 0,2 n. NaOH (in water). After five minutes the mixture is thoroughly shaken with 5 cc. of petrol ether; in this way neutral fats and cholesterin are eliminated completely and phosphatids for the greater part. The remaining alcoholic extract is acidulated with 0.5 cc. of HCl conc. and shaken with 2 × 5 cc. of petrol ether; afterwards 1 cc. of benzol is added to the alcoholic extract, and this is once more shaken with 5 cc. of petrol ether. The three petrol ether fractions thus obtained contain practically all normal haemolytics.

If this extract is dried and the residue emulgated in neutral isotonic phosphate mixture, then the amount of fatty acids obtained from 1 cc. of blood and emulgated in 1 cc. of phosphate solution may be diluted to $^1/_{64}$ and is still capable to haemolyse 1 % of blood corpuscles completely in half an hour at 37°.

CONCLUSION.

It is possible by means of lipoid extraction to isolate from normal blood substances wich are strongly haemolytic. These substances solely consist of higher fatty acids. A simple method is indicated for their quantitative extraction.

II. *The form in which strongly haemolytic fatty acids are contained in normal blood.*

In the previous communication it was stated, that a rather large quantity of intensively haemolytic higher fatty acids can be isolated from normal blood. It will be obvious that in normal blood this action must be completely on or nearly completely prevented; the mechanism of this inactivation is not definitely known. In this relation the formation of a protein-fatty acid compound was generally supposed, but we did not know if these combinations could exist in the blood plasma and if their haemolytic character has disappeared in this way. The knowledge of this inactivation must be important for the analysis of normal and pathological haemolysis, because insufficiency of the inactivation-mechanism must be dangerous to the corpuscles.

In order to investigate in which way the fatty acids are bound in the blood, we have made use of the high degree of capillary activity of these compounds; and this in the first place because this

surface activity is a property to which combining power and haemolytic action are intimately related, and secondly because the surface tension of small a amounts of blood can be measured accurately and easily by the torsion balance method [1]).

The surface-tension of a neutral highly diluted solution of fatty acids is much lower than the static surface tension of blood or serum. This fact already indicates that the fatty acids of blood cannot occur in the free state but must be bound somewhere. The minutest trace of free fatty acid must reveal itself immediately by a marked decrease of static surface tension, but the capillary-activity of the protein-fatty acid compounds also is, as far as we know, so intensive, the a decrease of surface tension plasma-air should be observed if an added fatty acid was bound as protein-compound.

The possible combination of protein and fatty acids may supposed to be primarely chemical or adsorptive; the last form would be probable by the intensive surface activity of these substances.

In the following table it is shown how the surface tension serum-air changes when small quantities of a diluted neutral emulsion of fatty acids are added.

Surface tension of fresh human serum	52	Dyne c.M.
+ 0.001 N oleic acid in neutral emulsion	52	„
+ 0.002 N „ „ „ „	52	„
+ 0.003 N „ „ „ „	52	„
+ 0.004 N „ „ „ „	51	„
+ 0.005 N „ „ „ „	47	„
+ 0.006 N „ „ „ „	45	„
+ 0.007 N „ „ „ „	42	„
+ 0.008 N „ „ „ „	41	„
+ 0.009 N „ „ „ „	39	„
+ 0.010 N „ „ „ „	39	„

It is seen, that 0,004 N. oleic acid may be added to serum without change of surface tension; when more acid is given, a gradual decrease of surface tension takes place till the value of ± 40 dynes/cm. has been reached. A further lowering is only to be seen, if large amounts of fatty acid are added. We will not delay upon the explanation of the gradual decrease of tension, but try to investigate the mechanism by which the plasma can preserve its original tension. Any marked lowering of this tension must be considered abnormal.

The constancy of surface tension indicates that the researched

[1]) BRINKMAN and VAN DAM. Münch. med Woch. 1550 (1921).

fatty acid compound can not lower the surface tension of water to less that 52 dynes/cm. By this observation the hypothesis of inactivation of fatty acids by protein solely is proved to be insufficient. Therefore we had to think of other possible compounds und found a sufficient explanation for constancy in the formation of calcium soaps. The existence of this proces of inactivation was found in the following way:

A. If the Calcium of serum or blood is precipitated by addition of oxalate of ammonia, the surface tension can not be held constant if small quantities of oleic acid are added. This is to be seen in the next table.

Surface tension of fresh human oxalate plasma	49 d. c.M.
+ 0.001 N oleicacid in neutral emulsion	47 "
+ 0.002 N " " " "	45 "
+ 0.003 N " " " "	42 "
+ 0.004 N " " " "	40 "
+ 0.005 N " " " "	38 "

The same results are obtained, when NaFl plasma is used.

B. A salt-solution containing the same amount of Ca as Plasma can maintain its tension above 50 dynes on addition of a neutral emulsion of oleic acid at 37°, to the same extent as plasma can. This holds for a solution of $CaCl_2$.6 Aq. 0,05 % as well as for a solution composed of NaCl 0,7 %, $NaHCO_3$ 0,2 % KCl 0,02 %, $CaCl_2$.6 aq. 0.05 % and H_2CO_3 till $[H^{\cdot}] = 0{,}4.10^{-7}$ is reached.

The following table gives the surface tension of the said salt solution if small amounts of oleic acid were added very gradually.

Surface tension of the balanced salt-solution	74 d. c.M.
+ 0.0001 N oleicacid in neutral emulsion.	54 "
+ 10 × 0.0001 N " " " "	53 "
+ 10 × 0.0001 N " " " "	52 "
+ 10 × 0.0001 N " " " "	52 "
+ 5 × 0.0001 N " " " "	50 "
+ 5 × 0.0001 N " " " "	39 "

If the surface tension of the saline shall not be lowered unter the plasma tension, it is necessary to add the oleic acid very gradually, and to leave the mixture for a half hour at 37° after each addition; only in this way it is possible to obtain a form of Oleate of Ca, whose capillary activity is low enough. But this condition is fulfilled in vivo.

The question now arose, wether this mechanism of inactivation would be equally important for the normal fatty acids of the blood as it proved to be for oleic acid. It is certain, that about one third part of the blood-calcium is present in the colloidal state; when we

consider the insolubility of Ca soaps it is possible, that the indiffusible part of the plasma Ca will consist wholly or partially of soaps. It is easily to be shown, that complete precipitation of the blood Ca is followed by a marked decrease of surface tension.

Surface tension of one cc. of freshly taken human serum is 53 dynes/cm; when 0,3 ccm. of a saturated solution of oxalate of ammonium is added, it decreases to 50, 48, 46 dynes/cm. The action of NaFl is similar.

Further if at little acid is added to the plasma, the fatty acids must be liberated from the eventually existing Ca soap compound. This proved to be the case; the amount of HCl necessary to lower the surface tension of serum from 52 to 45 dynes is exactly equivalent to the potential alkalinity of that serum. So it is probable that in normal blood also a great deal of the fatty acids are circulating in the form of Ca compounds. Direct chemical analysis will have to bring further evidence.

Till now we only examined the inactivation of oleic acid; the saturated fatty acids appear to be bound in the same way, but the physiologically important highly unsaturated linolenic acid give Ca salts, which lower the surface tension of the balanced salt solution to 38 Dynes. In accordance with this the blood or plasma it is not capable to maintain its surface tension if a small amount of isotonic neutral emulsion of linolenic acid is added, contrary to what occurs when oleic acid is given. This is seen from the following experiment.

Surface tension of fresh human serum	53 dynes p. cm.
+ 0.001 N linolenic acid emulsion	47 „ „
+ 0.002 N „ „ „	46 „ „
+ 0.003 N „ „ „	46 „ „
+ 0.004 N „ „ „	44 „ „
+ 0.005 N „ „ „	43 „ „

Although linolenic acid also is in plasma subject to considerable capillary inactivation, this process is not so complete, that the surface tension can be maintained absolutely constant. This fact must be explained by the capillary activity and solubility of the linolenate of Ca.

By these circumstances the higher unsaturated fatty acids circulating in the blood must have a great biological importance, because their Ca inactivation is failing. Therefore these acids must be bound by plasma colloids or corpuscles with decrease of interfacial tension. If now the inactivation of fatty acids extracted from corpuscles is compared in serum and in salt solution with the process described, it appears that these substances have the same properties as saturated

acids, and oleic acid have, but that a very small fraction is present which acts in the plasma as would do linolenic acid. Addition of fatty acids extracted from blood lowers the surface tension of serum from 53 dynes to 49,5 dynes; when more extract is added, the tension remains as constant as if oleic acid were added. The extracted fatty acids lower the surface tension of the serum to that of total blood, for corpuscles also can decrease the surface tension of serum to 50 dynes. So the tension of *blood* is not decreased by extracted fatty acids. If it may be concluded, that a small fraction of highly unsaturated fatty accids is absorbed normally to the corpuscles, this must be verified by further investigation.

In a following communication we will describe the influence which the investigated mechanisms of inactivation have on normal and pathological haemolysis.

SUMMARY.

By means of determination of surface tension of blood and serum it was shown, that the normal fatty acids of the blood or also those added on purpose are bound in the form of Calcium soaps, by which mechanism their capillary activity is decreased considerably. It is very probable, that this formation of a Calcium compound is the cause of disappearance of haemolytic properties of stearic acid, palmitinic acid and oleic acid in serum. The inactivation by means of Ca is not present in the case of linolenic acid; by this circumstance the haemolytic character of this substance of serum will be much greater.

III. *Experimental anaemia caused by injections of linolenic acid.*

In a previous communication it was pointed out, that higher fatty acids in the blood generally are circulating as Ca compounds, and thus have lost their marked capillary activity. It was stated however, that the Ca soaps of the higher unsaturated fatty acids i.e. of linolenic acid, do not loose their capillary activity, and that by this reason we have to expect much greater haemolytic action in vivo of this substance.

It was shown, that linolenic acid is an intravital haemolytic substance, of great activity and that there is no direct inhibition of the action of linolenic acid in the plasma. It was known for a long time, that injection of the saturated fatty acid or of oleic acid can not cause a distinct intravital haemolysis, probably by the mechanism of Ca inactivation, described formerly. In the case of linolenic acid

the injection is followed by marked haemolytic symptoms, as appeared from the following experiments.

Intravenous injection. A rabbit of 3620 gr. is injected in the auricular vein 0.250 gr. of linolenic acid dispersed in 10 ccm. of isotonic phosphate mixture. After 10 minutes the surface tension of the blood, which otherwise is 54,5—55,5 dynes/cm is decreased to 50 dynes and the serum is coloured lightly reddish. If now the surface tension is measured with regular intervals, it is seen, that the surface tension can not rise to the normal value but always has a value of 50 dynes approximately. The haemoglobinaemia is increasing more and more. After twenty minutes a strong haemoglobinuria is observed, and the rabbit makes a very sick impression. One hour after injection the animal dies with symptoms of utmost anaemia and dyspnoe.

In this way we could prove by several experiments, that a rabbit is killed by intravenous injection of ± 100 mg. of linolenic acid per Kg. under symptoms of very strong haemolysis. If smaller quantities of linolenic acid are given intravenously, the rabbit is not killed at once, but a chronic haemolysis with severe anaemia, urobilinuria, etc. sets on. When the linolenic acid is given intravenously, there is always a certain chance, that a little too large dosis of linolenic acid will lead to a direct mortal haemolysis.

A severe chronic haemolytic anaemia is produced by the subcutaneous, or better intramuscular injection of the acid. In this case the greater part of the injected substance seems to be inactivated and the following discase develops itself.

A rabbit of 3450 gr. in good state of health. Number of red cells 5,400,000. Haemoglobin 60 (Sahli). The form of the red cells in plasma is purely biconcave; a very small degree of anisocytosis, no polychromatophilia, absence of normoblasts. The serum is colourless and does not contain bilirubin. No uroblin or urobilinógen in the urin. The surface tension of the blood is 55,4 dynes at 19°.

The rabbit is injected every day with 200 mgr. of linolenic acid intramuscularly. After the first injection the surface tension of the blood decreases to 51—52 dynes, and remains so during all the experiment. 2—3 days after beginning of the treatment an intensive urobilinuria sets on and does not disappear during the course of injections. The blood picture shows from the third day a more and more increasing anisocytosis and polychromatophilia, while the number of irregularly shaped cells and sphaeric cells is rising. After five days the number of red corpuscles was lowered to 2.500.000; after that the first regeneration showed itself with numerous normo-

blasts, and strong anisocytosis and polychromatophilia. The number of red cells at this time was 3.700.000, the haemoglobincontent 55. So the index had increased distinctly and this increase remains very marked during the course of injections.

Twelve days after the first injection the number of red cells had decreased again till 2.900.000 (Haemoglobin (45), and the second period of regeneration began. Now the blood picture demonstrated the typical symptoms as they are found in distinct pernicious anaemia. Especially macrocytosis, poikilocytosis, strongly disshaped corpuscles, polychromatophilic megalocyts and normoblasts were striking. Bilirubinaemia could only be traced in the rabbit in cases of strong acute haemolyses. In the more chronic forms this phenomenon is not observed, urobilinuria being very marked however.

The rabbit is emaciated and makes a sick impression. If the injections are stopped in the beginning, the anaemia may be cured; if the treatment is continued, the typical pernicious symptoms will last.

So there is no doubt, that intramuscular injection of linolenic acid causes a chronic haemolytic anaemia in a short time, the red picture of which is showing all typical marks of pernicious anaemia. The picture of white cells has not yet been researched till now. We shall have to make a more exact analysis of this anaemia by linolenic acid, but it may be stated already, that linolenic acid is a very severe haemolytic substance. As it was found in the previous communications, we must ascribe this intravital action to the fact, that the Ca soaps of higher unsaturated fatty acids are capillary active and haemolytic, contrary to the Ca soaps of palmitinic acid and oleic acid.

Now this acid, forming an important percentage of the phosphatid fatty acids, it is practically certain, that the formerly used praeparation of trade-lecithin could effect the described haemolytic action by the rather large content of linolenic acid. Linolenic acid is a substance, which is found in the biochemistry of fat and phosphatid metabolism and it is probable, that this acid is circulating in normal blood.

In fact we were able to demonstrate by means of specific extraction, that in the 0,6—0,7 mgr. of fatty acids, which are found in one ccm. of normal human blood there is always present a small fraction consisting of higher unsatured fatty acids.

It appeared further, that all other fatty acids of the blood are inactivated by serum, in regard to capillary active and haemolytic action, but this small fraction of higher unsaturated fatty acids can

not be inactivated completely, so that we must ascribe a great importance as a physiologically haemolytic substance to the normal circulating linolenic acid. We will try, to determine the concentration of linolenic acids in the severe human anaemias.

SUMMARY.

Intramuscular injection of 200 mg. of linolenic acid per day in the rabbit is followed in a short time by chronic haemolytic anaemia. The blood picture shows a striking resemblance to that in pernicious anaemia.

Intravenous injection of ± 100 mgr. of linolinic acid pro Kg. causes a letal haemolysis.