GOULSTONIAN LECTURES ON THE CHEMICAL PHYSIOLOGY OF THE ANIMAL CELL.

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LECTURE III.—THE COAGULATION OF THE BLOOD.

THE subject of blood clotting is one which has attracted the attention of physiologists from the earliest times, and many have been the theories advanced to explain this phenomenon. Since the discoveries of the late Dr. Wooldridge, it has been found necessary to distinguish between the coagulation of the blood which occurs outside the vessels, and that which may occur intravascularly. It is intravascular coagulation which I shall speak of chiefly to-day. In my researches on this subject, I have throughout been helped by Mr. T. G. Brodie, M.B., Demonstrator of Physiology at King's College; and I owe him my deepest thanks for the care he has bestowed on the work.

HAMMARSTEN'S THEORY.

Before giving an account of these experiments, it will, however, be first necessary to refer to the work of others. I do not propose to go back to the older theories of blood coagulation, but I will start where textbooks, as a rule, usually finish—namely, with Hammarsten's theory. Hammarsten's theory may still be regarded as the simplest working hypothesis we have. Stated briefly, he considers that the coagulation of the blood is due to the decomposition of a substance called fibrinogen, which is dissolved in the blood plasma. This decomposition is brought about by the agency of the fibrin ferment which is the product of the disintegration of the colourless corpuscles. The most important product of this action is the comparatively insoluble material called fibrin.

CELL GLOBULIN.

My own work, some years ago, on the subject consisted in showing that these corpuscles contain or yield on disintegration, two globulins to which I gave the name of cell globulins. I spoke of these in my last lecture; one of them (cell globulin β) was found to possess fibrinoplastic properties, and, in fact, is so similar to fibrin ferment, that I felt justified in advancing the proposition that the ferment and the globulin were two names for the same thing.

Such an observation did not by any means settle the question of blood coagulation, and at the time that I published my paper it appeared impossible to reconcile these views with those of Dr. Wooldridge. The work that has been published since that time has, however, done much to show that the two opposing theories, Hammarsten's and Wooldridge's, are by no means irreconcilable, and I propose briefly to recapitulate the chief papers that have appeared.

W. L. Dickinson prepared cell globulin β , and in the main confirmed my observations regarding its properties. He, however, found that under certain circumstances it may be obtained without fibrinoplastic properties. For instance, after treatment with leech extract it may be deprived of its fibrinoplastic power without alteration in its physico-chemical qualities.

E. H. Hankin, approaching the subject from quite a different standpoint, found that cell globulin β has the power of killing certain pathogenic micro-organisms. The power is not possessed by fibrin ferment prepared in the usual way. The protective proteids or alexines are, according to this observer, of the nature of cell globulin β .

These two series of investigations show that fibrin ferment and cell globulin are not absolutely identical.

By far the most important researches, however, are those recently published by Pekelharing, especially as it is to him that the credit is due of pointing out how diverging and contradictory views on the subject may be, to a great extent, reconciled.

CALCIUM AND COAGULATION.

The first of these papers appeared in Virchow's *Festschrift*; in it he shows that the key to the problem is to be found in the facts recently made out concerning the relation of calcium salts to the coagulation process.

Many years ago Brücke demonstrated that the ash of fibrin

always contains calcium. Later (1875) Hammarsten pointed out that calcium chloride can take the place of serum globulin in aiding the action of fibrin ferment on fibrinogen. In 1887, Green found that the coagulation of various forms of plasma is much accelerated by small quantities of calcium sulphate. Later, Ringer and Sainsbury showed that the same property is possessed by other calcium salts, and to a less extent by the salts of strontium and barium.

The next to enter the field were Arthus and Pages, who made the brilliant discovery that the blood may be kept liquid by decalcification. This may be readily brought about by mixing the blood immediately it is shed with a 0.1 per cent. solution of potassium oxalate. They consider that fibrin is a calcium compound of fibrinogen, and their experiments further show that the ferment, as well as the calcium salt is necessary for the transformation of fibrinogen into fibrin.

Green, in the work already alluded to, took up the question whether the fibrin ferment exists in the blood plasma as a zymogen, which is changed into the ferment by the calcium salt. His conclusions, however, were negative. This is one of the points that Pekelharing has further investigated. He prepared from magnesium sulphate plasma and also from oxalate plasma, a substance which possesses no fibrinoplastic properties, but which by treatment with a calcium salt assumes the characters of fibrin ferment. This globulin he regards as the zymogen of the fibrin ferment; it yields an ash which contains little or no calcium, while the ferment is rich in calcium. Both zymogen and ferment have the characters of a globulin, and in fact are identical with my cell globulin β . It thus appears that this substance may exist in two modifications, one with and the other without fibrinoplastic properties.

PEKELHARING'S CONCLUSIONS.

Pekelharing considers with Arthus and Pages that fibrin is a calcium compound of fibrinogen, and that the main action of the ferment is the handing over of the calcium to the fibrinogen. He then proceeds to explain well-known facts regarding coagulation in the following ways:

1. The action of oxalates. These hinder coagulation by precipitating calcium oxalate; on account of the insolubility of this compound the calcium is not available for the conversion of zymogen into ferment.

2. The action of neutral salts. These hinder coagulation because the ferment is a globulin. The quantity of salt added to the blood is insufficient to precipitate the globulin, but sufficient to hinder those intramolecular movements upon which the specific action of the ferment depends.

3. The action of "peptone." This hinders coagulation because of the great affinity it has for calcium salts. Other substances which, like soaps, have a great affinity for calcium produce the same effect when injected into the circulation. Munk found that soaps hinder coagulation, produce a great fall of blood pressure, and may in extreme cases produce death. Ringer has abundantly shown the great importance of calcium in vital processes, and no doubt the toxic action of soaps and peptones is due to the removal of lime salts from the tissues. Pekelharing finds that the action of peptone on blood pressure and blood coagulation can be counteracted by injection of calcium chloride solution into the circulation. The same is true for the blood outside the vessels, or for solutions of Hammarsten's fibrinogen. Coagulation can be prevented in all cases provided the peptone is added before the zymogen has been converted into the ferment.

4. The action of "tissue fibrinogen." Wooldridge's tissue fibrinogen is found by Pekelharing to be a mixture of nucleo-albumin and lecithin. It also contains the zymogen of the fibrin ferment, for, by treatment with a calcium salt, it is converted into fibrin ferment, and causes coagulation of a solution of Hammarsten's fibrinogen. Pekelharing considers that the calcium salts of the blood change the zymogen contained in the "tissue fibrinogen" into ferment, and that it is the ferment so formed that leads to the intravascular coagulation.

He, however, leaves it open what constituent in the "tissue fibrinogen" it is which is the zymogen.

It is this last point which he develops in his later researches. Here he confirms in the main the researches of Wooldridge, which have already been mentioned. He also finds that a nucleo-albumin can be precipitated by dilute acetic acid from blood plasma. He surmises that it originates from the white corpuscles, and that it is identical with the substance that Wooldridge obtained by cooling plasma, and doubtless also with the substance Wooldridge precipitated from serum by acetic acid and called serum fibrinogen. I may also mention in parenthesis that Dr. Shore of Cambridge has obtained a similar material from lymph. Further, Pekelharing considers that it is essentially the same substance as may be obtained from leucocytes and other cells, as in the preparation of Wooldridge's tissue fibrinogen. Lastly he

finds that what I called cell globulin β is in reality a nucleo-albumin, yielding nuclein on gastric digestion. The solubilities of the substance are the same as those of a globulin, but it is not until its nature is more deeply probed that it turns out to be something different from a globulin. He regards this substance as the same as the fibrinoplastic substance which A. Schmidt correctly described as originating from the corpuscles, but erroneously considered to be identical with serum globulin (paraglobulin). In the main Griesbach takes the same view of coagulation as Pekelharing. He has worked chiefly with invertebrate blood, and has separated from crab's blood a fibrinoplastic proteid which is a nucleo-albumin, not a globulin. He considers that this originates from the protoplasm of the amoeboid cells, and that the nuclei do not participate in the process as Lilienfeld supposes.

One can at once see how much divergence of opinion is explained by this discovery. The substances which have at various times received the names of fibrinoplastic substance, fibrin ferment, cell globulin β , serum fibrinogen, fibrinogen A, tissue fibrinogen, and so forth are all varieties of one substance, which is a nucleo-albumin; and, further, that it is in all cases a nucleo-albumin which, in co-operation with calcium compounds, brings about coagulation in the blood.

POINTS STILL UNDECIDED.

The question then arises, Does this definitely once and for all settle the long-vexed subject of blood coagulation? I am afraid it does not; it helps us a little nearer the truth, but the final solution of the problem may be a long way off yet. The observations which have been made by Mr. Brodie and myself were attempts to answer some of the points left undecided by Pekelharing's work, and I wish that it had fallen to my lot to announce that all the difficulties have disappeared, but, as we went on, what we found was that new and previously unsuspected difficulties made their appearance. I will first point out what I conceive to be the weak links in Pekelharing's chain of evidence.

1. The theory that "peptone"¹ hinders coagulation because of its great affinity for calcium salts is a very tempting hypothesis. It is supported by the fact that injection of a soluble calcium salt counteracts its action. This fact we have confirmed. We used anæsthetised rabbits in our experiments, and injected about 10 c.c. of 10 per cent. "peptone" solution into the external jugular vein. After about a minute a small quantity of blood was withdrawn from the carotid artery; this took about twenty minutes to coagulate. Then 50 c.c. of a 1 per cent. solution of calcium chloride was injected, and another sample of blood withdrawn; this clotted in a few seconds. The same result was obtained when "leech extract" was used instead of peptone; leech extract probably owes its activity to a protease, and is much more effective in hindering coagulation in the case of the rabbit than commercial peptone. The analogy of "peptone" and soap, which has already been pointed out, also lends support to Pekelharing's views. But I have heard the objection raised that other substances which undoubtedly have a great affinity for calcium do not act in this way, and I have been asked whether potassium oxalate acts like peptone. We have put this to the test of experiment, and have found that the injection of a few cubic centimetres of a weak (0.4 per cent.) solution of this salt renders the blood when it is subsequently drawn off very slow in coagulating. In one experiment a sample of blood which was drawn off before the injection clotted in less than a minute; after the injection another sample did not clot for seventeen minutes. We thus have an additional confirmation of Pekelharing's hypothesis.

2. The second difficulty may be put in the form of a question, Why do not nucleo-albumins cause coagulation to occur in extravascular as readily as they do in intravascular plasma? The amount of calcium salts is presumably the same in both. I have tried a good many experiments with salted plasma, and found, like Pekelharing, that if the nucleo-albumin is previously gently warmed with a little calcium chloride solution, it acts like fibrin ferment, and causes a rapid formation of fibrin. Calcium chloride alone does not produce this result. But if the treatment with calcium chloride is omitted

the nucleo-albumin has little or no activity in this direction; it may even hinder the coagulation, in comparison with control specimens of the same salted plasma which had been diluted with water. I feel quite unable to offer an adequate explanation of this difficulty.

3. If fibrin ferment behaves as Pekelharing considers, its action differs from that of all other ferments with which we are familiar. The usual action of a ferment is to act as a water carrier, and having given water to the substance on which it acts, splitting into new products takes place (hydrolysis). There is, of course, no reason why ferments should not act as calcium carriers, but if we include such an action we must either extend the meaning of the term fermentation or drop the use of the word altogether in speaking of blood coagulation.

ANALOGY TO MILK CURDLING.

Of all ferment actions that of rennet in causing milk to curdle is most nearly akin to blood clotting. This resemblance has been a matter of remark from the time of Andrew Buchanan onwards. In each case we have the conversion of a soluble into an insoluble proteid, and in each case the presence of a calcium salt is necessary. Hammarsten showed that the process of milk curdling consists of two distinct stages, and more recently Dr. Ringer has confirmed this observation. Let me quote one of the most instructive of Dr. Ringer's experiments. Caseinogen free from lime salts was dissolved in sodium bicarbonate solution; addition of calcium chloride to this caused no coagulation. Addition of rennet to another portion also caused no separation of a clot; but the ferment had nevertheless caused a change in the caseinogen, for after boiling the mixture so as to destroy the ferment, the addition of calcium chloride caused the immediate formation of a curd. From this it is obvious that the first process takes place without the presence of a lime salt, but no precipitation occurs; that requires the lime salt. Probably the casein formed from the caseinogen by the action of the ferment forms an insoluble lime compound, for the curd behaves like many lime compounds in being precipitated by heat and dissolved by cold.

It appears to me quite probable that in fibrin formation there is a similar double process, the action of the calcium coming in at the end in causing the separation of the clot. But it is very difficult to prove or disprove this idea. With milk it is possible to boil it without coagulating the proteid, and one can thus destroy the ferment. With blood plasma this is impossible, for the blood proteids are coagulated by heat, and so one would destroy both ferment and fermentable substance.

On the other hand, it is quite possible that as knowledge advances we may have to drop the use of the term fermentation altogether in connection with blood clotting. This view was very strongly urged by Wooldridge, who designated his proteid matter "tissue fibrinogen" on the supposition that it formed an integral part of the fibrin, and was not merely an on-looker, as ferments generally are. The strongest fact in support of this view, in my opinion, is seen in the coagulation of invertebrate blood. Taking crustacean blood as an example, we find that the coagulation process is very closely allied to that of vertebrate blood, and there are no red corpuscles to impede one's view of what actually occurs; the crustacean fibrin formed is almost identical with ordinary fibrin, and the white corpuscles there become an actual portion of this fibrin, and cannot be recognised as corpuscles subsequently.

I shall, however, not pretend to be able to decide this question; sufficient data have not yet accumulated for a definite opinion to be pronounced one way or the other, and therefore I shall for the present retain the use of the conventional term "fibrin ferment."

INFLUENCE OF CARBONIC ACID.

4. The last difficulty standing in the way of our full acceptance of Pekelharing's theories is that it takes no cognisance of the part that carbonic acid plays in blood coagulation. Let me just enumerate a series of facts which show the influence this factor has:

(a) Venous blood when shed clots more slowly than arterial blood.

(b) When blood clots, the tension of carbonic acid in it rises. (Strassburg.)

¹ The term "peptone" includes the proteoses with which it is generally mixed.

(c) "Peptone" blood contains very little carbonic acid. (Lahousse, Blachstein.)

(d) A stream of carbonic acid passes through "peptone" plasma causes it to clot. (Fano.)

(e) Injection of nucleo-albumin causes clotting to occur chiefly in the veins. This is not universally true, for we have had specimens of nucleo-albumin which were so active as to cause immediate clotting in all the arteries, so that probably it never reached the veins at all. But, as a rule, it is the veins where the clotting is most intense. This has been very strongly insisted on by Professor A. E. Wright. He finds in the dog, where the intravascular coagulation is not so universal as in the rabbit, that those veins contain clotted blood in the regions where most carbonic acid is produced. This can be brought about by tetanising a group of muscles say in one limb.

(f) It can hardly be doubted from these and other experiments that intravascular clotting produced in various diseases, or which follows certain forms of septic poisoning, or in phlegmasia dolens, must be brought about by nucleo-albumins, which are the result of the disintegration of the blood cells, or cells of the tissues. Here also clinical evidence supports physiological experiments that it is the venous blood which is most liable to be coagulated.

We can hardly doubt from such evidence that either the quantity, or more probably the tension, of carbonic acid has an important influence in hindering or promoting coagulation. I must again confess myself unable to see the full bearing of such facts, and I also think that in the present state of our knowledge it would not be safe to attempt any theorising as to the way in which such facts are to be fitted into Pekelharing's hypothesis.

I may now pass to a more systematic account of the experiments which Mr. Brodie and I have been making during the last two years. They are very numerous, and all I shall attempt is a summary of their chief results.

They were commenced with the object of determining whether the nucleo-albumins obtained by the sodium chloride method had the same physiological action as the nucleo-albumins obtained by Wooldridge's acetic acid method, and termed by him "tissue fibrinogens." The fact that both produce intravascular coagulation formed part of the evidence that the two materials were practically the same, upon which I dwelt in my second lecture.

INFLUENCE OF LECITHIN.

The next point taken up was whether the lecithin, which I regard as an impurity, but to which Wooldridge attached considerable importance, was really the active substance, or whether the nucleo-albumin itself produces the result. Our results were the following:

1. Pure lecithin injected intravenously does not produce intravascular clotting.

2. The alcoholic extract of "tissue fibrinogen" which contains the lecithin was found to contain variable amount of lecithin. It is therefore not in combination with the proteid matter in constant proportions, and is thus probably a mere mechanical admixture.

3. Injection of this alcoholic extract dried and suspended in dilute solution of sodium carbonate never produced intravascular clotting.

4. Injection of the nucleo-albumin prepared by either method, and freed from lecithin by the use of alcohol, still produces intravascular clotting. It is not so marked as before the use of alcohol, because this reagent renders the proteid matter more insoluble; in fact, one has to add a little caustic alkali to get a solution at all. Control experiments prepared with the solvent (2 to 5 per cent. solution of sodium carbonate either with or without the addition of caustic alkali) never led to intravascular clotting.

5. Chloroform has the advantage over alcohol in freeing these substances from lecithin, in that it has little or no coagulating influence on the proteid.

METHOD AND RESULTS.

Having in this way shown that the substance produces its effect *minus* its lecithin, whichever of the two methods is adopted for its preparation, the greater number of our subsequent experiments were performed with the material prepared by the sodium chloride method from the thymus. We adopted this method because it is more rapid, and the results obtained were more certain. The material prepared by Wooldridge's method often produces no intravascular clotting at all; this was recognised by Wooldridge himself, and he spoke of it as the "negative phase" in his experiments, because in the dog, which was the animal he chiefly used, the blood was not only free from clot, but it clotted with tardiness after removal from the body. I shall later on have to return to this question of the "negative phase." Our failures to produce intravascular coagulation by the nucleo-albumin prepared by the sodium chloride method have been very few, and are in part to be explained by the fact that the solution has been kept some days before it was used. In spite of thymol, solutions which are active when fresh lose their activity when kept. This difficulty we have now to a large extent obviated

by rapidly drying the solution in a vacuum over sulphuric acid. The powdered substance which contains both the proteid and the sodium carbonate used to dissolve it is readily soluble in water. The solution is viscid, but after filtration it is perfectly clear and free from solid particles. I may perhaps now describe an experiment with its ordinary results.

The animal—and we have used rabbits almost exclusively—is anaesthetised with ether and fixed in a holder in the usual way. The external jugular vein is exposed and a cannula inserted; through this the solution is injected, and about 10 c.c. of the solution—which would contain approximately 0.05 grammes of proteid—is usually sufficient to cause death. The animal dies within a few seconds; the respiration suddenly stops; there is no dyspnoea as a rule, but there may be a slight stretching movement. Another marked symptom is extreme exophthalmos and dilatation of the pupils. The *post-mortem* examination is performed as rapidly as possible, and the thrombosis found is usually limited to the venous blood, but in many cases there is extensive clotting in the arteries also; we have not often found a clot in the portal vein. The heart is still beating, the right side full of dark clot, the left usually empty—or it may also contain clot, which, however, differs from that on the right side by being bright red.

Death is thus evidently due to failure of respiration, and the question arises whether the respiratory centre is at fault, or whether the primary cause is to be sought in the lungs. The sudden stoppage of respiration and the absence of dyspnoea point to the respiratory centre, and this is confirmed by the fact that the severance of both vagi makes no difference in the experimental result.

PURIFICATION OF NUCLEO-ALBUMIN.

I must next pass to an entirely different series of experiments. I believe that our experimental methods are so far in advance of those of many other investigators, in that the solutions we employed were perfectly clear; but we sought to purify the substance we employed still further. This was done by taking the nucleo-albumin, grinding it up once more with sodium chloride, and re-precipitating it by throwing it into excess of distilled water. This was repeated two or three times. The material obtained by twice repeating the process is absolutely free from lecithin, and produces on injection the usual thrombosis; but if the material is still further purified it fails to produce any result whatever on injection; though the solution is still viscid, 200 c.c. or more may be injected, and on killing the animal by asphyxia not a trace of coagulation is found anywhere.

We have verified this very disappointing experiment half a dozen times and then sought for an explanation. Evidently in the process of purification some substance is removed from or added to the nucleo-albumin, which in consequence loses its activity. We noticed that during the processes of repeated washing there was a considerable loss of material; the nucleo-albumin collects as a scum on the surface, but, if it is allowed to stand, some passes into a condition of semi-solution in the weak saline underneath it. Injections of this, however, led to the same negative result.

On the supposition that purification had removed something from the nucleo-albumin, we proceeded to investigate what that something was. It is not lecithin; our previous experiments had shown us that lecithin is not the active substance; and moreover the substance which after one or two washings causes thrombosis, and that which after three or four fails to do so are both equally free from lecithin. The hot alcoholic or chloroformic extract of both contains absolutely no phosphorus. Is it then calcium? Again the answer is No; injection of the purified material *plus* calcium chloride is quite ineffectual in producing thrombosis; and analysis of the ash both of the purified and the unpurified substance is either entirely free from calcium or contains only imponderable traces. The only other alternative which suggested itself to us was whether purification had removed the nucleic acid. The idea that the nucleic acid is split off from the proteid proper during these processes was by no means improbable, especially as Dr. Starling called our attention to the fact that Wooldridge found that his "tissue fibrinogens" lost their activity after purification. Now repeated treatment with acetic acid (which is Wooldridge's method) would perhaps result in displacing a weak acid like nucleic acid, and there is no *a priori* reason why repeated treatment with water and sodium chloride should not do the same. Accordingly we put the hypothesis to the test of experiment. We have not yet had time to perform many analyses, but so far the result has been again negative. The so-called pure

material contains the same percentage of phosphorus as the active substance. If, therefore, we wish to avoid the unsatisfactory conclusion that what we have removed is an intangible and shadowy substance known as a ferment, it is necessary to fall back on the other hypothesis, and consider whether in the process of purification we have added something to the nucleo-albumin. A few days ago, at the Physiological Society, Dr. Haycraft suggested that by the repeated use of salt we have obtained an inactive sodium chloride compound of the proteid. This will have to stand or fall by the test of analysis, but it appears to me to be a very probable explanation.

INFLUENCE OF LEUCOCYTES ON COAGULATION.

All observers are agreed, with the exception of Dr. Wooldridge, that the colourless corpuscles play an important part in the coagulation process, and we can hardly doubt that the fibrin ferment or nucleo-albumin which causes blood to clot when it is shed originates from the leucocytes. And here I would again emphasise the importance of comparisons between vertebrate blood and the blood of the crustacea.

Hardy² has recently shown that here certain corpuscles, which he very appropriately designates the explosive corpuscles, yield the fibrin ferment, and Griesbach³ has accentuated this point by showing that agents which, like osmic acid, prevent the explosion of the corpuscles lead to a loss of coagulability. From his long and interesting paper I take the following table that gives a summary of the way in which different reagents hinder blood coagulation.

1. Substances which fix the cells and prevent their disintegration—for example, osmic acid.
2. Substances which not only fix the cells, but also precipitate the plasma proteids—for example, salts of the heavy metals like gold chloride.
3. Substances which do not prevent cell disintegration (plasmoschism) but may increase it, but which prevent coagulation by modifying the properties of the proteid matter in the blood—for example, neutral salts like sodium sulphate and magnesium sulphate.
4. Substances which do not prevent plasmoschism, nor do they modify so far as we know the blood proteids. They act by combining with the calcium so essential for fibrin formation—for example, oxalates; and to this we must now add such reagents as peptone and leech extract.
5. Substances which do not prevent plasmoschism nor modify the blood proteids (?) nor act on the calcium salts (?), but act in some way not yet explained. As examples, the gases, carbonic acid and sulphuretted hydrogen, may be mentioned.

It struck us on reading Pekelharing's papers, and on doing our own experiments, that they did not definitely prove that the ferment is actually formed from the foreign proteid introduced into the circulation. It appeared quite possible that the ferment may at any rate in part originate from the disintegration of blood corpuscles within the blood stream. Buchner has devoted a great deal of study to the protective proteids or alexines. Like Daremberg he finds that these substances are not merely bactericidal, but also globulicidal—that is, they produce not only disintegration of bacteria, but of blood corpuscles also. Now that we know the alexines are nucleo-albumins, it appeared probable that their globulicidal properties would partly explain intravascular clotting.

On the other hand, we knew that substances like distilled water, which produces extensive disintegration of the corpuscles, never cause thrombosis. Our solutions of nucleo-albumin do produce extensive disintegration of corpuscles. Testing the matter on a slide with a drop of human blood we saw that the red corpuscles were made irregular in shape, and to a slight extent dissolved. The white corpuscles are broken up immediately, and form first a mass of adherent particles, which then separate and look just like *Blutplättchen*; but exactly the same result is obtained with solutions of the purified material, which produce no thrombosis, and very nearly as extensive disintegration is produced by the sodium carbonate solution which we use as the solvent of the proteid. Counting experiments performed on rabbits led us to the same conclusion. Injection of the nucleo-albumin produced a slight loss of red corpuscles; this is evidenced also by the slight lakiness of the blood often seen; the loss of white corpuscles is much more marked, being from 90 to 100 per cent. The loss from injection of a corresponding volume (20 to 50 c.c.) of sodium carbonate alone (5 per cent. solution) is nearly as great, 75 per cent. The loss always chiefly falls on the multi-nucleated corpuscles. We have thus something

more to reckon with than mere destruction of leucocytes, and we can hardly resist the conclusion that it is the foreign material introduced into the circulation that furnishes the active substance that produces the thrombosis.

LÖWIT'S EXPERIMENTS.

We should not have pursued the matter further if a remarkable series of papers by Löwit⁴ had not at this stage of our work fallen into our hands. He also has used rabbits in his experiments, and he finds that many reagents which, when intravenously injected cause leucocytosis, produce as an immediate primary result leucolysis or destruction of the white corpuscles, especially of the multinucleated ones. The reagents he found which acted in this way were albumose, peptone, pepsin, nucleic acid, nuclein, leech extract, pyocyanin, tuberculin, curare, urea, uric acid, and sodium urate. Löwit is a strong supporter of Schmidt's views on the preponderating influence of disintegrated leucocytes in bringing about blood coagulation. At the same time he strongly contests Lilienfeld's view that the nuclei participate in the process. He therefore asks why it is that if all these reagents cause leucolysis, that they do not cause thrombosis, and concludes that what is wanting is the calcium. He accordingly injected calcium chloride either before or after the injection of these other substances, and states that he never failed to produce intravascular clotting in this way. In our repetition of these experiments we have followed Löwit's directions very closely; we have not been through his entire list, but we have used Witte's peptone, Gröbler's peptone, leech extract, curare, urea, uric acid, and sodium urate. We have confirmed his results in so far that calcium chloride alone does not produce intravascular coagulation; the blood when shed, however, coagulates very rapidly. We found that the same result followed when the calcium chloride was used in conjunction with peptone, leech extract, and the other substances just named. With one exception in some twenty experiments we never got a trace of intravascular clotting. The one exceptional case was when we used a peptone solution that had undergone putrefaction, and the result might therefore be attributed to the nucleo-albumin in the bacteria themselves. We however failed to repeat the experiment in other rabbits with the same solution. That introduces us to another difficulty we have met with, namely, the idiosyncrasies of the rabbit tribe. A solution which produces coagulation in one animal may fail to do so in the next experiment; and it appears that the dark coloured rabbits are more prone to undergo intravascular coagulation than albinos. In connection with these same experiments, I may mention that we found that substances like leech extract, which prevent blood coagulation, are directly antagonised by solutions of nucleo-albumin; and after the injection of the first, the latter still causes intravascular coagulation.

Our work then has not confirmed that of Löwit, and shows that something else besides leucolysis must occur in order to produce thrombosis. Wright has more recently found that extensive destruction of the leucocytes follows peptone injection. A possible objection to these observations is that the leucocytes may not be actually destroyed, but they run together and get packed away in some organ like the spleen. This view is confirmed by the fact that if one performs the experiment of allowing peptone solution to run into a drop of freshly drawn blood on a microscope slide, no destruction of leucocytes is observable. But the phenomena of extravascular blood are often so different from what occurs in the vessels that it is not safe to draw inferences from one to the other. We must await further experiments before this matter can be considered settled.

NEGATIVE PHASE OF COAGULATION.

To return now to a question which I have already mentioned, namely, the so-called "negative phase" which sometimes follows the injection of nucleo-albumin. It was first observed by Wooldridge, and more recently confirmed by Wright, that if the blood is not coagulated by the injection, it undergoes clotting when shed with great slowness. Wright's hypothesis to explain this is that the nucleo-albumin injected consists of two parts, one the nuclein, which produces

² *Journ. of Physiol.*, xiii, p. 165.

³ *Pflüger's Archiv*, L. 473.

⁴ *Studien zur Physiol. u. Pathol. des Blutes und der Lymphe.* Von M. Löwit. Jena: G. Fischer. 1892.

the clotting, and the other is a peptone-like substance which hinders clotting. The negative phase is explained by supposing that the "peptone" moiety is split off in the circulation, and produces there the well known effects of the intravascular injection of "peptone." This is supported by the observation that in these animals "peptone" is found in the urine, and further supported by the experiments of Pekelharing, who demonstrated the existence of "peptone" in the blood under similar circumstances.

In a previous paper⁶ I urged that it was not proven that the "peptone" actually originates from the foreign proteid injected, because the nucleo-albumins fail entirely to give any of the peptone or proteose reactions. I think it far more probable that the peptone originates from the disintegration of leucocytes.

Peptonuria ordinarily occurs in suppurative diseases where there is a great deal of disintegration of leucocytes, and I thought one might obtain some light on the subject by the injection of non-proteid material, such as curare or urea, which produce leucolysis. In rabbits, however, one seldom sees the negative phase, and perhaps our results differ from Wright's because he has chiefly used dogs. We have searched for peptone in blood and urine by Devoto's modification of the ammonium sulphate method, and in some cases by the use of trichloracetic acid. This reagent precipitates all the proteids except proteoses and peptone. Our results are as follows:—

1. After the injection of nucleo-albumin, peptone was never found either in the blood or urine.
2. There is no difficulty by either method of recognising "peptone" after intravascular injection of small quantities of that substance.
3. After the injection of non-proteid leucolytic agents, there is also no "peptone" discoverable by Devoto's method. There were, however, in some cases traces found by the trichloracetic acid method after injection of curare and of calcium chloride.⁶

I must confess that these experiments do not throw much light on the subject, but, as far as they go, tend to support the hypothesis that the "peptone" found by other observers originates rather from the destruction of leucocytes than from the foreign material introduced into the circulation.

NUCLEO-HISTONS.

Since writing the above a paper has been sent to me by Lilienfeld⁷ which bears on this same question. He takes much the same view of the matter as Wright. He calls the substances I have called nucleo-albumins nucleo-histons, on account of the supposed resemblance between the proteid moiety of the substance and "histon," the peptone-like material obtained from nuclei by Kossel. In my second lecture I alluded to "histon;" though this is regarded by Kossel as an integral part of the nucleus, I am inclined to look upon it as a substance formed from the nuclear sap in the manipulations used in its preparation. Lilienfeld admits that his leuco-histon is coagulated by heat, and so differs from peptone. He lays it down as a general rule that substances which hinder intravascular coagulation do so either in virtue of the "histon" they contain or the histon they produce by the disintegration of leucocytes, and that substances which produce intravascular coagulation do so in virtue of the nuclein they contain or because they cause the liberation of nuclein by the disintegration of leucocytes. If both substances are liberated together the nuclein is the more powerful as a rule, and produces coagulation in histon-plasma.

From our own experiments I can fully confirm Lilienfeld's views as to the importance of nuclein. I do not regard it as at all necessary, however, to imagine that the nuclein must originate from the nucleus, as the cell protoplasm also contains nuclein in the form of nucleo-albumin.

THE BLOOD PLATELETS.

The blood platelets have long been known to have some important bearing on blood coagulation. If the clotting of a microscopic preparation of blood be watched, the fibrin strands are seen to start from groups of the platelets. What blood platelets are has been the subject of much discussion and innumerable theories. I have no wish to add another

theory, but will be content with pointing out that the experiments I described on the influence of nucleo-albumins in destroying white corpuscles, and forming from them what look like platelets, favour the theory that these little bodies are the disintegration products of the leucocytes. Lilienfeld takes the same view, and his micro-chemical method, which shows they are composed of a phosphorus-rich substance, and his further work,⁸ in which, by gastric digestion, he has separated them into nuclein and albumin, adds strong confirmation to this idea.

Thus we are brought once more back to the subject of the disintegration of leucocytes, and I will conclude what I have to say on the subject of blood coagulation by a few general remarks on that question.

It was Schmidt who originally taught the importance of the destruction of leucocytes as the main cause of coagulation. These corpuscles are exceedingly prone to undergo changes when exposed to altered conditions; but a considerable change has come over our notions on the subject since that view was first promulgated. Rauschenbach, one of Schmidt's pupils, recognising that a number of corpuscles do not disintegrate, spoke of α leucocytes, which are acted upon and disintegrated by the plasma when the blood is shed, and β leucocytes, which remain unaltered. Some very remarkable experiments published within the last few months by Dr. Sherrington,⁹ who has succeeded in keeping leucocytes alive outside the body for several weeks in oxalate plasma, bear very strongly against the idea that they are specially prone to disintegrate.

The term disintegration does not, however, mean complete destruction and disappearance of the corpuscles in question. It signifies merely retrograde changes that end in the death of the corpuscles. Haycraft regards these changes as being due to mechanical stimulation of living and naked protoplasm by foreign solid matter, for if the fluid after being shed is surrounded by a fluid like oil coagulation is prevented. By watching the corpuscles under the microscope he has shown that they become flattened and irregular; they then lose their granules, or these retire to one part of the cell, leaving the rest clear. Such changes are better marked in the coarsely granular corpuscles than in the finely granular ones. He regards the shedding out of the fibrin ferment which accompanies these changes rather as a process of metabolism than of disintegration. I am inclined to adhere, however, to the old word disintegration, as what goes on is something different from what occurs in normal metabolic processes. As I pointed out some years ago, the disappearance of the granules from the cells inevitably suggests a comparison between the process and secretion, in which the shedding out of a ferment by secreting cells is accompanied by the disappearance of the granules which indicate the existence of its zymogen or mother substance. This idea has since been verified by the discovery of the zymogen of fibrin ferment, and so strongly has the resemblance to secreting cells been recognised that some have gone so far as to speak of the coarsely granular or eosinophile cells as unicellular glands having a different origin and function from the neutrophile or finely granular cells, which appear to be the principal phagocytes.

Hankin¹⁰ has arrived at the conclusion that the alexines or protective proteids which confer bacteria-killing power on blood serum originate from the eosinophile cells; and this bears upon our present question because of the similarity, or it may be identity, between fibrin ferment and the alexines.

There is, however, evidence that cells which are not eosinophile yield fibrin ferment. The explosive cells in crustacean blood are hyaline, not eosinophile. (Hardy.) According to Sherrington, too, the eosinophile cells are not fibrinoplastic.

I think it would be premature to lay down any definite rule as to which group of corpuscles fibrin ferment comes; and, further, seeing that substances which produce coagulation can be obtained from cells as diverse as those in the thymus, brain, liver, testis, and kidney, it seems very probable that both kinds of leucocytes in the blood may give rise to fibrin ferment; they both consist of protoplasm, and

⁶ The leucolytic action of calcium chloride was proved by microscopic experiments with human blood, and by counting experiments after intravascular injection of 50 to 100 c.c. of a 1 per cent. solution (the strength we have used in all our experiments) in rabbits.

⁷ *Verhandl. d. physiol. Gesellsch.*, Berlin, 1891-2, No. 16.

⁸ *Chem. Central.*, 1892, ii. 82.

⁹ *Internat. Physiol. Congress.*, Liège, 1892.

¹⁰ *Centralbl. f. Bacteriol. u. Parasitenkunde.* Bd. xii, Nos. 22 and 23.

the main proteid constituent of all protoplasm is nuclealbumin.

PRACTICAL BEARINGS.

During these lectures I have from time to time stepped aside from my subject proper, in order to point out certain practical bearings which I considered to be of importance. I wish that I had had time, and, still more, the necessary experience to have done so more fully. If, however, any grains of usefulness are scattered through what I have said, there is no doubt that others will pick them up, and work out their practical side. In connection with blood coagulation and thrombosis I need not stop to point out the immense importance of a correct knowledge of this subject to the pathologist, and I will content myself with two remarks only on its practical aspects.

The first of these is to point out the great resemblance between the phenomena observed in the animals in the experiments described, and those cases of pulmonary thrombosis which are occasionally seen in the human subject. One can hardly doubt that here we have to deal with poisoning by the same material. Professor Gerald Yeo was witnessing some of our experiments a few days since, and he described to me a case he had had in King's College Hospital. It was one of amputation of the breast for cancer, and the wound was perfectly aseptic. But ten days after the operation, without any warning, the patient died suddenly with great dyspnoea. Examination of the brain revealed no lesion, but a firm fibrinous clot was drawn like a tree with its branches from the pulmonary artery at the necropsy.¹¹ The dyspnoea in this and similar cases points to the cause of death in the lungs themselves rather than in the respiratory centre; this difference is easily explicable on the grounds that the dose of poison enters the circulation more slowly than in our experiments, where it is injected.

The second practical point which I wish to emphasise is a word of warning. When I have seen animals die in a few seconds, as though by a lightning flash, after a dose of 5 cubic centimetres of this poison, which could not have contained more than a few centigrammes of the material, I feel very loth to urge that any practical use of such substances should be made in the human subject, either for the stoppage of hæmorrhage, or for the production of a clot in aneurysm. The cure would in such instances be worse than the disease.

THE ANTI-CHOLERA VACCINATION:

AN EXPERIMENTAL CRITIQUE.

Read before the Pathological Society of London.

By E. KLEIN, M.D., F.R.S.

HAVING for some time been engaged for the medical department of the Local Government Board in investigating the antagonistic action of various pathogenic bacteria, I have during the last year extended these investigations to the anti-cholera vaccination, as described by M. Haffkine of the Pasteur Institute. Haffkine, by an extensive series of experiments, has shown that if a large dose of choleraic comma bacilli, grown on and then removed from the surface of solidified nutrient agar, be injected (as a suspension in sterile beef bouillon) into the peritoneal cavity of a guinea-pig, a fatal result is produced in, or within, twenty-four hours, the peritoneal cavity containing fluid exudation full of the living comma bacilli. R. Pfeiffer¹ had already shown that this result is produced by injecting intraperitoneally large masses of the cholera bacilli, either living or after having been previously killed by heat or drying, by chloroform, ammonium sulphate, alcohol, or other means, and he justly concludes from these experiments that the comma bacilli themselves contain in their protoplasm a poisonous principle. The fatal result in the guinea-pig thus obtained with large doses of the cholera bacilli was shown to be due to acute severe peritonitis, and it was further shown that the same result is obtained by the same methods with the vibrio of Finkler and the vibrio of Metschnikoff, so that also these two species include in

their substance a similar poisonous principle. Notwithstanding these facts, Pfeiffer regards the intracellular poison of the cholera bacilli as "the primary cholera poison."

Haffkine starts from these experiments, and finds that by transmitting, by intraperitoneal injection, the (peritoneal) exudation of a guinea-pig dead after the above injection through a succession of guinea-pigs (twenty to thirty generations), an exudation fluid is ultimately obtained of which the cultivation on agar possesses a high degree of virulence, "virus fort," so much so that that a small dose of this cultivation injected intraperitoneally into the guinea-pig produces a fatal result in eight to twelve hours, the fatal dose of this "virus fort" being in amount only a-fifth, a-sixth, or less of that required of the original cholera culture. Repeated subcutaneous injections of small doses of this "virus fort" produce in the guinea-pig a transitory constitutional illness; at the seat of inoculation there appears a soft oedematous swelling, which, after a few days, diminishes in size, becoming at the same time firmer, and is either totally absorbed or more generally produces a local necrosis and ulceration of the skin, which ultimately completely heals up. Guinea-pigs which have thus been twice subcutaneously inoculated with small doses of the "virus fort," generally attenuated by the addition of phenol, prove themselves refractory—that is, "vaccinated" against the intraperitoneal injection of a fatal dose of the "virus fort," a dose that invariably kills control guinea-pigs in eight to twelve hours. I have made a considerable number of experiments on guinea-pigs in a similar direction; the results of these I now propose, with the sanction of the Medical Department of the Local Government Board, to communicate to the Society. These results may be arranged in the following order:

1. Experiments of intraperitoneal injection were made with beef bouillon suspension of various microbes, grown on the surface of solidified nutrient agar; the amount used for each animal being in all cases one-fifth to one-half of a culture tube; each culture tube presenting an agar surface of 6 centimetres by 2 centimetres. The microbes used were: (a) the cholera bacilli; (b) the vibrio of Finkler; (c) the bacillus coli; (d) the proteus vulgaris; (e) the bacillus prodigiosus; and (f) the bacillus of typhoid fever.

2. In all cases the intraperitoneal injection of one-fifth to one-half of a culture was followed after a few hours (4 to 8) by distinct illness; the temperature is lowered, the animals are quiet, do not move and do not feed; after about 8 to 12 hours, sometimes earlier, this condition is more pronounced; next day, that is within 20 to 24 hours, the animals as a rule are found dead. On *post-mortem* examination there is found intense peritonitis with pseudo-membranes on the omentum and liver; the intestine and particularly the serous covering of the stomach are much congested. The small intestine is either relaxed and filled with sanguineous mucus, or is more or less contracted. The peritoneum contains more or less turbid, slightly sanguineous, fluid exudation filled with the microbes used for the injection. The heart's blood also contains the microbes, as cultivations prove; the colonies obtained by such cultivations are always fairly numerous, but of course not so numerous as those of the peritoneal exudation. It must be insisted on, that in all this there is absolutely nothing to warrant the assertion that the results produced by the cholera bacillus are comparable to cholera. The symptoms of the disease and the pathological changes produced in the guinea-pigs by the intraperitoneal injection are for all the above species exactly the same, but it ought to be mentioned that the cultivations of bacillus coli, bacillus of typhoid, and bacillus prodigiosus act more virulently than those of the cholera vibrio or the vibrio of Finkler, since the dose of the latter, in order to be fatal, has to be larger than that of the former, and the appearances produced by the same amount of culture, though identical in character, are more intense in the case of the bacillus coli and typhoid, and bacillus prodigiosus, than in that of the vibrio of cholera and of Finkler respectively.

3. The same result is also produced by the intraperitoneal injection of sterilised culture (exposure to 62° to 65° C. for 10 to 12 minutes), the amount required to produce a fatal result being, however, a little larger than that required of the living bacilli.

4. As only the bacteria themselves (scraped off from the

¹¹ For numerous similar cases see Sir J. Fayer's *Clinical and Pathological Observations in India*, pp. 94 et seq.

¹ *Archiv. Hygiene und Infektionskrankheiten*, vol. x1, part 3, p. 393.