Comparative Physiology. — *The determination of the coagulation-time of bloodplasma by means of apparatuses of* LINDERSTRØM–LANG *adapted to this purpose.* By H. J. VONK, A. STOLK and C. H. NUYTEN. (From the laboratory of Comparative Physiology, University of Utrecht.) (Communicated by Prof. G. KREDIET.)

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Numerous different methods have been indicated to determine the blood-coagulation-time. A simple one is the sucking up of blood in a long glass capillary, which is put horizontally (if possible in a thermostat), after which every other minute small pieces of this capillary are broken off. When with this breaking off a fibrin-thread arises, the coagulation is assumed to be completed (SABRAZÉS, 1906). This method is rather primitive. Even though the capillaries are put in horizontal tubes which run through a water-thermostat, they must be touched and again and again taken out of their surroundings with a constant temperature. An advantage of this method is that a small quantity of blood is sufficient.

One can also pull a white horse-hair, which has been cleaned with alcohol and ether, through a glass capillary filled with blood. If the hair comes out no longer white but red, the point where the coagulation begins has been reached. After total coagulation the hair again comes out white (VIERORDT, 1878). There are about as many advantages and drawbacks as with the preceding method.

The blowing out of blood from glass capillaries can also be used to determine the coagulation-time (WRIGHT, 1893). Also the rising in them which of course does not take place after coagulation (LÖWENTHAL).

FONIO has given a short summary of these and other methods [1]). Later WOLVIUS [2]), FESTEN [3]) and PROOST [4]) have worked out a very useful method, based on the absorption of light by the coagulating blood sample. To determine the light-absorption WOLVIUS used an extinction-meter of MOLL. FESTEN and PROOST a Pot-galvanometer of Cambridge combined with a photo-cell. The only drawback of the method according to FESTEN and PROOST seems to me that the sample is not kept at a constant temperature. An advantage is that the blood is not set in motion, as is the case with so many other methods. FESTEN and PROOST do not give figures

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[1]) A. FONIO, Die Gerinnung des Blutes, Hndb. d. norm. u. path. Physiologie, VI, 1 p. 307—411, especially p. 358—364 (1928).

[2]) J. WOLVIUS, Diss. Utrecht 1923 (Een objectieve methode ter bepaling van het verloop der bloedstolling).

[3]) H. FESTEN, Nederl. Tijdschr. v. Geneesk. **83**, I, 396 (1939).

[4]) J. B. PROOST, Diss. Utrecht 1941 (Het phosphatiden- en cholesterinegehalte van het serum tijdens senium en puerperium en het eventueel verband met den stollingstijd van het bloed).

from which it appears that the method can be reproduced on one and the same blood-sample. A certain drawback of WOLVIUS' method is that a very expensive extinction-meter is necessary for it.

In the method which we are now going to describe, use is made of part of the apparatuses which LINDERSTRØM–LANG [5]) indicated for the carrying out of his micro-titrations. Here he uses a small test-tube, the content of which is about 1 or 2 cm^3. The sharply drawn-out point of the microburette is put in the titration-liquid. The solution with which the titration is done is driven out of the microburette by screwing up a mercuric pile. As with this way of titration the liquid in the vessel cannot be shaken, LINDERSTRØM–LANG brings about the mixture by making a small glass ball, in which a little Ferrum reductum (or a small piece of iron wire) has been melted, jump up and down by means of an electromagnet provided with a mercury-interruptor. It seemed to us that this method of stirring could also be made to serve the determination of the coagulation-time of blood, at least of recalcified oxalate-plasma or of oxalate-plasma with thrombine etc. The titration-vessel may then be filled with oxalate-plasma, which can be made to coagulate by adding a solution of calcium-chloride or with a mixture of oxalate-plasma and bloodserum (which still contains a remnant of thrombine). On the latter mixture the p_H optimum of the coagulation can very well be determined, when buffers or HCl and NaOH are added.

Every 30 or 60 seconds, by switching on the magnet, we try to see whether the little ball can still be set in motion [6]). If this is no longer the case, the coagulation is ended. Permanent motion of the ball is not desirable, as this has a strong influence on the coagulation-time and often no gel arises either, but a flock of fibrin, so that the coagulation-time cannot be determined.

The figure on page 687 shows how the experiments are arranged. It represents a horizontal section of the apparatus at A, a vertical one at B. The electromagnet (1) with nucleus (2) has been fixed on a little table (3) which, by means of a serrated path (4) and screw (5) can be moved up and down and adjusted at the right height. A second table (6) which can also be put higher and lower by means of a serrated path (7) and screw (8) carries a cork-plate (9) on which are two plates of asbestos screwed together (10 and 11) between which a heating-element (12) has been fixed (13 and 14 are its supply- and outlet-wires). On the upper asbestos plate stands a *thin* crystallizing-dish (15) (Jena-glass) with a

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[5]) K. LINDERSTRØM–LANG u. H. HOLTER, Zs. physiol. Chem. **201**, 9 (1931).

[6]) Before use the small glass balls should be boiled with diluted nitric acid, after which in the used acid one reacts with KCNS to iron. If no colouring red occurs, the balls can be used.

[7]) The mercury-interruptor and the switch in front of it have been left out in the drawing.

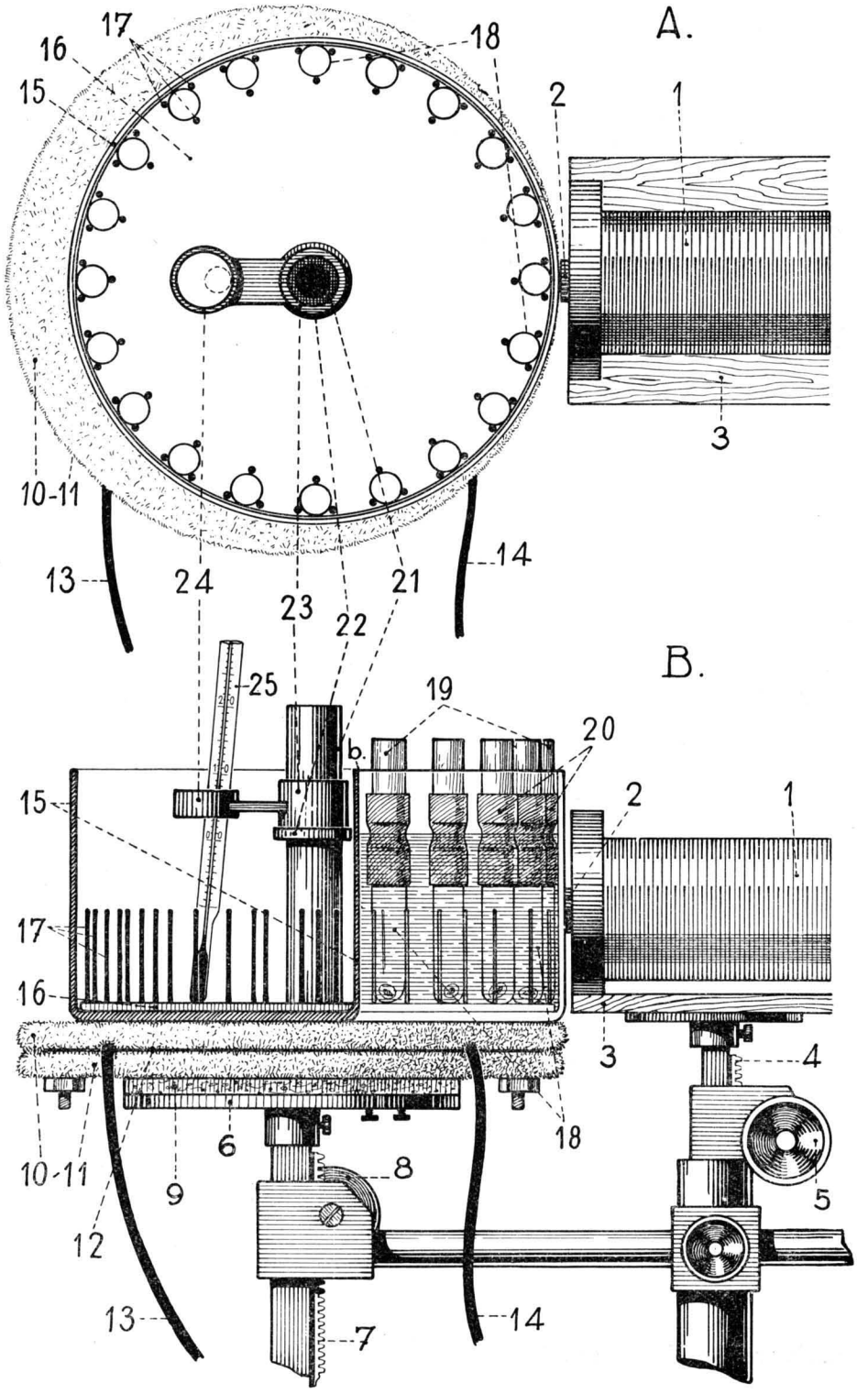
[8]) In fig. *B* on the right of *b* the crystallizing-dish is represented as filled with water, on the left of *b* empty and with the wall taken out.

height of about 6 cm and a diameter of 10 cm [8]). This can be shifted or turned with the hand. On the bottom of this crystallizing-dish there is a brass plate (16), on which along the edge twenty times three small brass bars (17) have been fixed, between which 20 glass tubes (18) can be put. These glass tubes (the titration-vessels of LINDERSTRØM–LANG) contain the liquid of which the coagulation-time must be examined. They are closed by glass stoppers (19), which are fastened on the tubes by pieces of rubber-tube (20). In the vertical section the small glass ball with iron nucleus is drawn in the tubes on the right of *b*. On the middle of the brass plate (16) a brass bar (21) has been attached, which serves the purpose of removing the brass plate easily from the dish. On this bar there is a stud (22), on which rests a loose ring (23), to which a second ring (24) has been fastened, which serves as holder for a thermometer (25). The dish can be turned with the hand, so that every time another tube comes to stand before the magnet-nucleus. The heating-element can be regulated by switching some carbon filament lamps of 5, 10 and 16 candle-units. Thus at a room-temperature that does not vary too much the temperature of the bath can be easily kept constant for an hour within a limit of 0.5°.

With the apparatuses of LINDERSTRØM–LANG adapted in this way the coagulation-time of bloodserum for experiments can be determined to the nearest 1 to 0,5 minutes. The technics are less satisfactory for the determination of the coagulation-time of blood itself, as the glass ball is not clearly visible. This also obtains, however, for the (up to now most exact) experiments of WOLVIUS, FESTEN and PROOST, who also used oxalate-plasma instead of blood, to which at a certain moment a certain quantity of pure calcium-chloride was added. Many experiments, which show the various phenomena on which the classical blood-coaguation theory is founded, in a simple way were carried out by us successfully in this manner.

We must state most emphatically that the test-tubes should be cleaned very carefully indeed. The coagulates should be removed from them mechanically and then the tubes should be boiled in strong nitric acid or aqua regia. After this they are rinsed many times with tap-water and finally a few times with distilled water and dried. It appeared that the inconstant values found at the beginning of the experiments were entirely due to insufficient cleaning of the tubes. As is well-known, the character of the surface strongly influences the blood-coagulation time. To this and to traces of enzyme, possibly adsorbed to the wall in former experiments, the occurring of such-like irregularities must be ascribed.

In order to investigate the accuracy of the method we determined the coagulation-time of one and the same test-liquid several times. This was mostly made up of a mixture of 1 cm^3 of oxalate-plasma and 1 cm^3 of serum (to which the same quantity of oxalate had been added). Then the thrombine is present in the serum, the fibrinogen in the oxalate-plasma. The addition of oxalate to the serum takes away the calcium in it. When joined



Apparatus to determine the blood-coagulation time.
Explanation in the text.

to the plasma this might otherwise cause the formation of new thrombine, so that irregularities might arise.

The following results were obtained. A coagulation-time of 11 min. was found in 7 experiments at 23° for a mixture of 1 cm^3 of oxalate-plasma and 1 cm^3 of serum. 8 min. was found 6 times for 2 cm^3 of plasma and 3 cm^3 of serum. Differences below 1 minute could not be observed in this way (switching of the magnet every other minute). A few times the differences appeared to be greater than 1 min., but this was the case with a long coagulation-time (serum and plasma, brought together in a proportion 2 to 3, were both 1 day old, so that the activity of the thrombine becomes considerably weaker, probably by destruction of the enzyme). Thus we found: 58¾, 60¾, 58¾, 59¾ and 59¾ min. Average 59,5 min. The mean error of a single determination was then (rounded off) ¾ min., that of the average $^3/_8$ min. In spite of the somewhat larger dispersion, the determination, as for percentage, becomes more exact when the time is longer.

In experiments with serum and oxalate-plasma joined together, in various proportions of the quantities of the components, a minimal coagulation-time is found. The cause of this is the following. When there is a constant quantity of oxalate-plasm, at first the coagulation-time will become shorter as the quantity of added serum increases, because the quantity of the enzyme is enlarged. As it is only the plasma that contains fibrinogen, the concentration of the fibrinogen decreases as the quantity of added serum increases, so that the formation of a gel will be delayed and finally this will not appear at all. The following experiment shows this:

No.	Quantity of ox. plasma	Quantity of serum	Temp.	Coag. time
1	1 cm^3	0.5 cm^3	22°	50 min,
2	1 cm^3	0.7 cm^3	22°	30 min.
3	1 cm^3	0.9 cm^3	22°	26 min.
4	1 cm^3	1.0 cm^3	22°	22 min.
5	1 cm^3	1.5 cm^3	22°	18 min.
6	1 cm^3	2.0 cm^3	22°	14 min.
7	1 cm^3	2.5 cm^3	22°	13 min.
8	1 cm^3	3.0 cm^3	22°	15 min.
9	1 cm^3	3.5 cm^3	22°	15 min.
10	1 cm^3	4.0 cm^3	22°	16 min.

Tube no. 7 shows the minimal coagulation-time. It appeared however, that after 24 hours the coagulate could be shaken out of the tube without difficulty, while this was least easy with the coagulates in number 3 and 4. So we shall be able to determine the coagulation-time in the most exact manner when the proportion of the mixture is 1 : 1. For the more solid the congelation that has been formed, the more unmistakably shall we be able to determine the moment when the motion of the ball ceases.

The method of WOLVIUS, where the absorption-curve is completely fixed and measured, is undoubtedly the most exact. But it requires very expensive apparatuses. A further advantage is that it can also be carried out at a constant temperature and that the liquid is not set in motion, except when we mix with calciumchloride. The methods of FESTEN and PROOST also have the latter advantage. But they don't work at a constant temperature, which is a drawback especially for a longer period of time, while our method does work at a constant temperature. As for accuracy, they are about equal to our method. The motion of the glass ball every other minute has with our method a certain influence on the coagulation. On the other hand, a special mixture of the liquids by a spatula, needed for the optical methods, is superfluous here. This drawback is removed, however, for comparative experiments, when the coagulation-times do not differ too much. (If one wishes to determine the absolute coagulation-time by means of our method, one can make three similar experiments. In the first tube one lets the ball move every other minute. Only when the liquid in this tube has coagulated one lets the ball in number 2 jump every other minute. When the coagulation of this tube is completed, then that in no. 3. In this way one approximates the true coagulation time.)

Further, our method has the advantage that a great number of determinations can be made at the same time. So those who have the titration-apparatuses of LINDERSTRØM–LANG at their disposal, can make them very suitable in a very simple way, for the determining of the blood-coagulation time. The description of this method seemed important to us, as in many modern investigations (e.g. of ASTRUP c.s. [9]) rather subjective methods are still used to determine this coagulation-time (observation of the first fibrinous flock or moving of the tube with the hand).

Summary.

A method is described to determine the blood-coagulation-time, founded on an application of the way in which in the micro-titrations of LINDERSTRØM–LANG the mixture of the liquids takes place.

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[9]) ASTRUP, GALSMAR, VOLKERT, Acta physiol. Scand. **8** (1944).