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SPATIAL ASPECTS OF THE DYNAMICS OF BLOOD CLOTTING. III. GROWTH OF THE THROMBUS *IN VITRO**

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The spatial patterns of growth of fibrin clots in thin layers of blood plasma have been studied. It was found that the growth of the clots stops or greatly slows after their thickness reaches a size close to 0.5 mm. The polymer clots growing towards each other fuse when the distance between the clotting activation centres is less than 1 mm, whereas at a distance of the order of 1.5 mm between clots a non-proliferating flat gap persists. In individual experiments the successive formation of concentric ring structures was observed. Around the clotting initiation centres as many as three fibrin rings grew separated by gaps of non-coagulated plasma. The findings agree well with the hypothesis earlier advanced by the authors on the existence of autowave mechanisms of regulation of blood clotting. © 1996 Elsevier Science Ltd. All rights reserved.

Transformation of fibrinogen to fibrin completes the cascade of enzymatic reactions of blood clotting. The molecular mechanisms of this process have for many years been the subject of close study [1–3]. Experimentally, the dynamics of the spatial growth of the fibrin clots has been studied in the greatest detail in systems *in vitro* (using microcinematography or other methods) in conditions of a complex hydrodynamic flow [4–6]. Recently, theoretical studies have appeared discussing how the molecular biochemical mechanisms determine the spatial dynamics of growth of the thrombus *in vitro* in the absence of hydrodynamic flows [7, 8]. This paper presents the results of a corresponding experimental investigation.

MATERIALS AND METHODS

In the experiments we used plasma isolated from donor blood prepared on standard haemopreservatives (Glyugitsir, CPD, CPDA-1). The end citrate concentration in such plasma is about 20 mM. The concentration of free calcium lies in the range 15–30 μM . The plasma was practically free of cells (poor plasma). The concentration of thrombocytes did not exceed $2 \times 10^{10}/\text{l}$. The plasma was stored at room temperature in containers of the Kompoplast-300 type in sterile conditions for not more than 72 h from the moment of preparing the blood.

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The samples for recalcification were taken in sterile form. Recalcification was carried out by adding 10–20 μl 1 M solution of CaCl_2 to 1 ml citrate plasma. The end concentration of free calcium was controlled with an ion-selective electrode from Orion Research, model 93-20 (reference electrode of the same company, model 90-02). The end concentration of Ca^{2+} after recalcification was 1.5–2.0 mM. In some experiments we used freshly prepared whole blood recalcified in the same way as the plasma.

The experiments were run in polystyrene Petri dishes with a diameter of 40 mm, placed in a thermostatted chamber ($t = 37^\circ\text{C}$), positioned on the stage of the SV8 binocular magnifier from Opton (Germany) with a photocap and illuminated on the side parallel to the plane of the bottom with an illuminator with an incandescent lamp. The second source of light, an illuminator with a DRSh-250 mercury lamp, was able to illuminate the contents of the dish from the top at an angle of $45\text{--}50^\circ$ to the surface. It was used to excite fluorescence in the experiments with the fluorescent substrate thrombin to visualize the formation of active thrombin. Both lamps were fitted with water thermal filters. In some experiments the recording was with a Panasonic G-120 video camera (Japan). Into the Petri dish was poured 1 ml plasma, heated for 2–5 min and then to it was added a solution of CaCl_2 and carefully mixed. The layer thickness of the plasma was 0.5 mm. Clotting was initiated by placing in the plate with the plasma glass beads (diameter 0.6 mm), the surface of which strongly activates clotting.

To investigate the distribution of active thrombin in the thrombus and its vicinities, we used the fluorescent substrate described in [9] — Boc-Ala-Pro-Arg-7-amino-4-methylcoumarin, specially synthesized in the Institute of Biological and Medical Chemistry, Russian Academy of Medical Sciences. The substrate was added in the form of a 10 mM solution in DMSO (end concentration 0.2 mM). Thrombin splits off 7-amino-4-methylcoumarin (AMC) from this peptide, which strongly fluoresces (maximum at 440 nm). To excite the fluorescence the u.v. region was cut out from the emission spectrum of the mercury lamp using the UFS-1 glass filter. Staining of the preparations for protein was carried out with crystal violet.

EXPERIMENTAL RESULTS

Seven to 10 min after placing a layer of recalcified plasma in the Petri dish, clots (thrombi) spontaneously appeared and began to grow at different sites in it. The boundary of these formations with the liquid plasma was clearly outlined (Fig. 1). The process of the birth of new clots continued until there were regions of liquid plasma in the dish.

In the system considered, clotting may also be triggered artificially. The formation of fibrin structures initiated by particles with a coagulo-active surface occurred with exactly the same space-time dynamics as the structures initiated by centres of spontaneous origin. Clotting began at roughly the same time, the layer thickness of the clot and the growth rate on different particles being roughly identical (Fig. 1).

Figure 2 shows the change in the size of the clot with time. Two stages clearly stand out in the dynamics of clot growth. In the first, growth occurs at a practically constant rate (Fig. 2, 1–5), and in the second, after the clot has reached about 1.5 mm in diameter (taking into account the diameter of the glass bead), there is sharp inhibition of growth (Fig. 2, 6–12).

If the centres initiating clotting are situated sufficiently close together (at a distance of less than 1 mm), enlarging, the clots simply fuse, forming one common clot (Fig. 3). However, if the centres appear at a large distance from each other (more than 1.5 mm), then head-on growth of the clots ceases before their growth in other directions. The shape of the head-on boundaries of

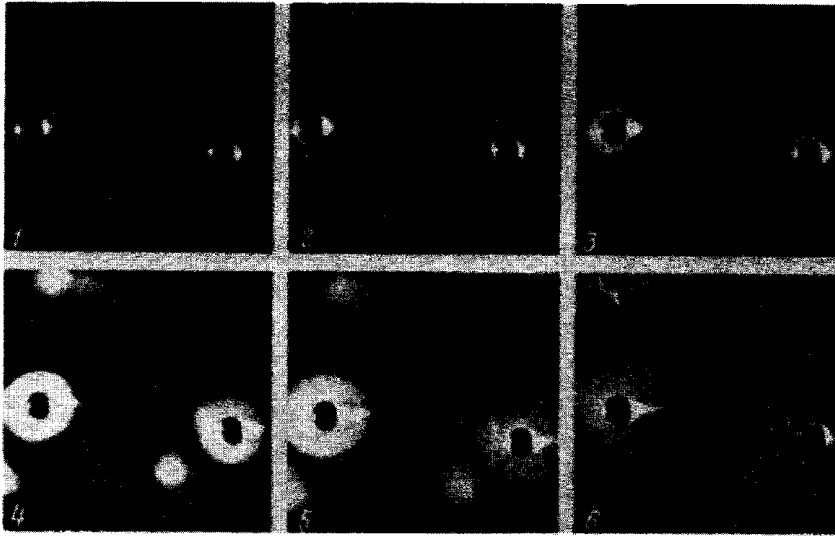


Fig. 1. Advent and growth in the plasma of fibrin clots of spontaneous origin and initiated by glass beads. Frame sequence, from left to right, from top down; time interval between frames, 3 min.

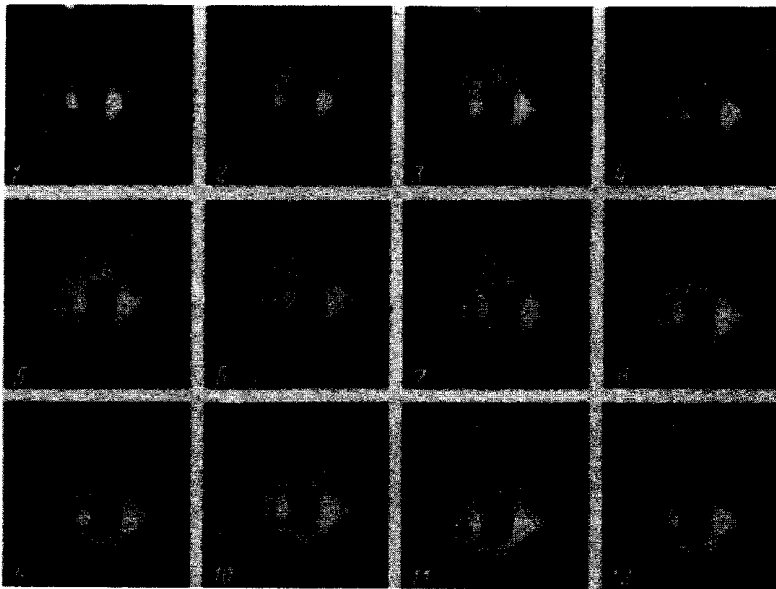


Fig. 2. Sequential stages of the formation of the fibrin clot of finite dimensions on initiation of clotting by the surface of the glass bead. Clot growth practically ceases on reaching a characteristic size of the order 0.5 mm (photograph 6). The photographs correspond to the following moments of time: first, 10 min from the moment of contact with the bead; photographs 2 to 12 are taken with an interval of 3 min.

both thrombi becomes flatter. Between the clots remains a band of the non-clotting liquid phase which continues to exist for 1 h and longer (Fig. 4).

To investigate the distribution of active thrombin in the zone of growth of the thrombus, the fluorescent thrombin substrate was added to the plasma and the growth of the clot was recorded in parallel from change in light scatter and the appearance of the activity of thrombin from

change in fluorescence. Figure 5 shows the successive patterns of development of fluorescence associated with splitting of the peptide substrate by thrombin on growth of clots from the glass beads. The zones of strong fluorescence with well-contoured margins revealing regions of appearance of active thrombin (Fig. 5, 1–3) coincided in size with the fibrin clots formed observed on switching off the source of ultraviolet. At later stages (Fig. 5, 4–9) the size of the clot did not change and the blurred boundary of the luminous zone is apparently the result of diffusion of AMC from the clot.

In individual experiments the formation of annular structures was observed. In about 10–12 min a fibrin layer formed on the surface of the coagulation-initiating glass beads (Fig. 6, 1, 2). During the following 18 min the picture did not undergo any outward changes. Then a new fibrin

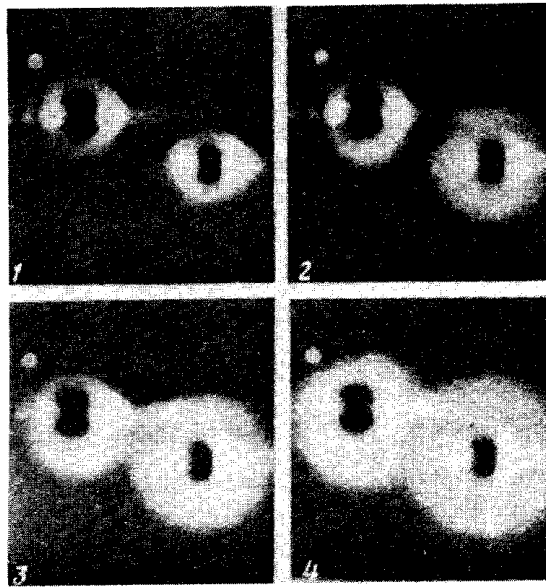


Fig. 3. Growth of fibrin clots from two closely adjacent (~ 0.6 mm) glass beads. Fusion of thrombi.

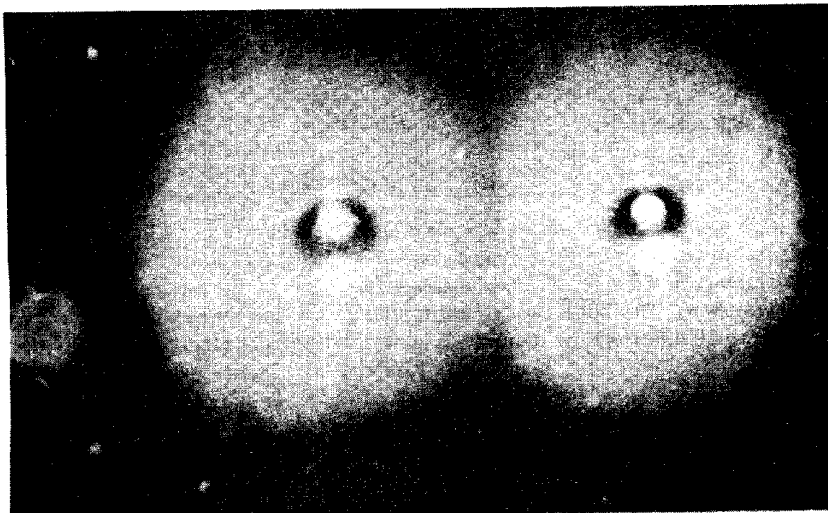


Fig. 4. Result of the interaction of two growing fibrin clots the centres of which are far apart (> 1.5 mm). It will be seen that the thrombi did not completely merge. The photograph corresponds to 40-min incubation.

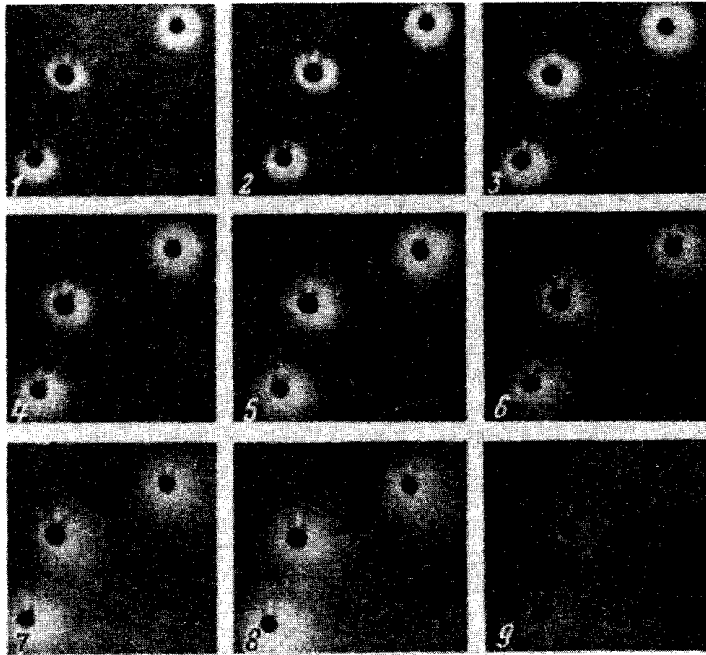


Fig. 5. Distribution in space around the centres of initiation of clotting (glass beads) of the fluorescent product (AMC) arising from the corresponding substrate in the zones of work of thrombin (photographs 1–4) and subsequent slower blurring of the boundaries of the luminous zone apparently due to the diffusional distribution of the previously formed AMC. The photographs correspond to the following times: 1, 10; 2, 13; 3, 16; 4, 19; 5, 21; 6, 24; 7, 27; 8, 30; 9, 90 min.

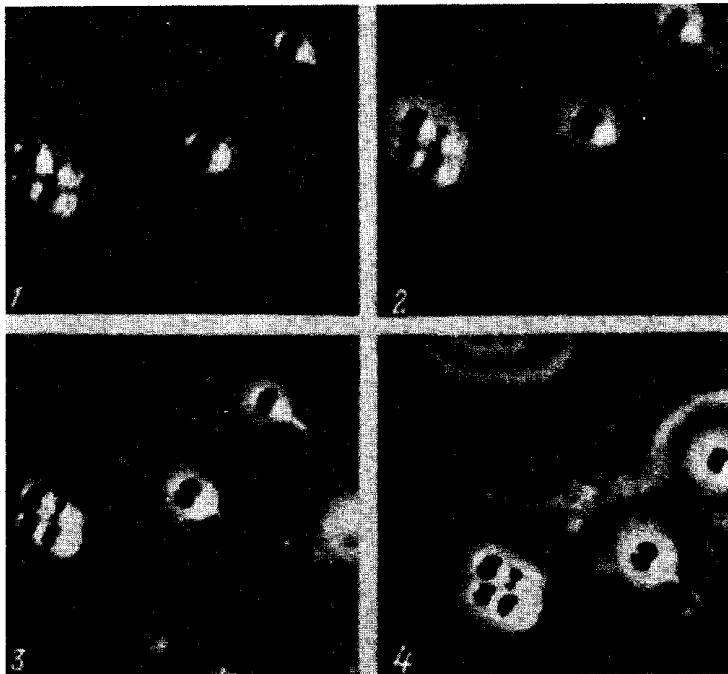


Fig. 6. Dynamics of formation of ring structures. Clotting in the plasma was initiated by adding glass beads. The photographs correspond to the following incubation times: 1, 8; 2, 14; 3, 32; 4, 50 min.

layer began to grow at a certain distance from the first. The plasma in the zone separating the layers remained liquid. Growth of the second layer was just as fast as that of the first. It stopped after the layer had reached roughly the same thickness as the first (Fig. 6, 3). Then a third layer formed (Fig. 6, 4). The intensity of the second and following layers was often low. Only the first clot could be seen from light scatter. However, if 1 h and longer after the start of clotting the uncoagulated plasma was decanted and the protein remaining on the surface of the dish stained, one could observe ring structures of small amplitude (Fig. 7). The experimental conditions necessary for the formation of such structures now need to be specified in greater detail.

The character of the growth of the thrombus somewhat changes if the Petri dish is covered with a lid immediately after the glass beads are placed in it. The initial stages of the process are very similar to the above described clot growth in an open dish. However, after reaching a critical size, growth does not cease but only greatly slows, continuing without interruption (Fig. 8). On head-on spread of such fronts they merge. Although in this case there is uninterrupted growth, all the features of the process of inhibition described above take place though in highly lubricated form. Although the two colliding fronts fuse, the density of the thrombus between them is low. The zones of collision of different clots are clearly distinguishable in the photographs even 30 min after their union (Fig. 9).

DISCUSSION OF RESULTS

As Fig. 1 shows, the final layer thickness of the polymerized clot in practice does not depend on the size and material of the activating particles. This indicates that the dynamics of clot growth is chiefly determined by the molecular-kinetic properties proper of the biochemical system of regulation of clotting and not by the initial triggering signal.

Insensitivity to the initial conditions is characteristic of many of the previously discovered structures in essentially non-equilibrium self-organizing systems. As is known, such typical characteristics of autowave regimes as the natural frequency of the auto-oscillations and the speed of the autowave do not depend on the initial amplitude and phase [9–11]. In the system considered, we apparently come up against a new internal characteristic of non-equilibrium systems (auto-size) which characterizes the spatial scale predetermined by the kinetic structure

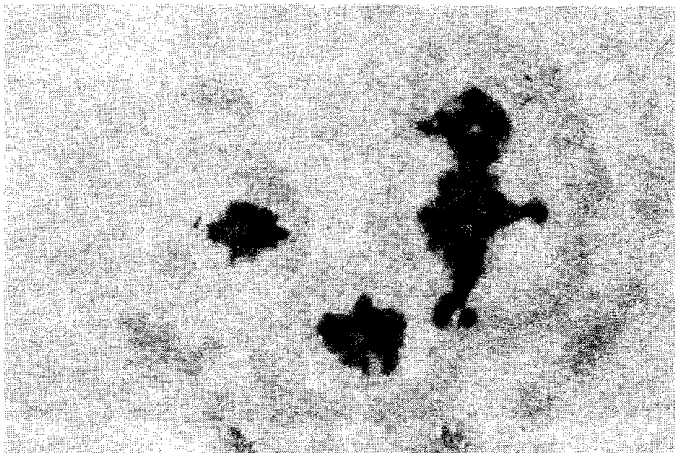


Fig. 7. Ring structures manifest only after staining for protein of the clots formed in the Petri dish.

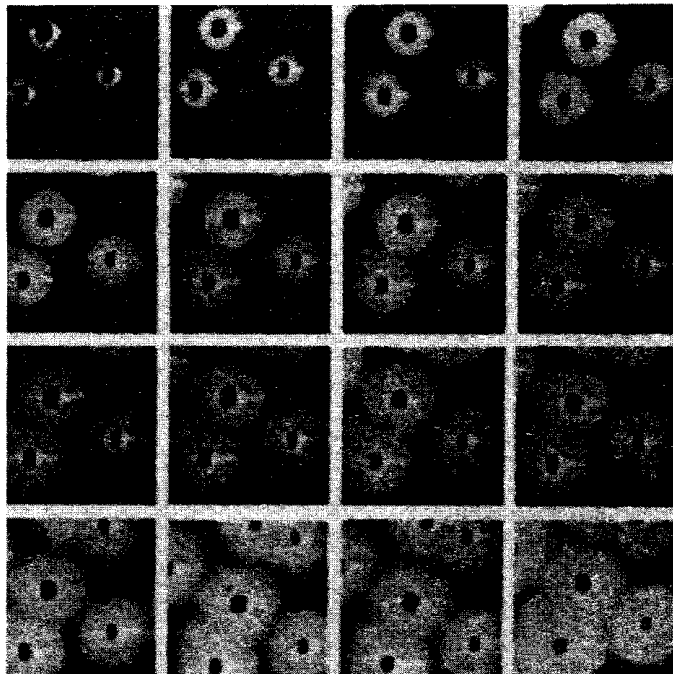


Fig. 8. Unceasing growth of fibrin clots initiated by adding glass beads to the plasma placed in a closed Petri dish. The photographs correspond to the following moments of time from the start of incubation (frame sequence from left to right, from top down): 1, 10; 2, 16 min, interval between the following frames 3 min.

of the system [8] (in this case the distance over which the concentration thrombin wave can travel).

As shown by experiments with fluorescence, the leading edge of the moving concentration thrombin profile is steep (Fig. 5, 1–4). Only after arrest of growth of the clot does one observe the usual diffusional blurring of the resulting region of raised concentration of AMC (Fig. 5, 5–9). This indicates that after arrest of clot growth the formation of new fluorescent products ceases, and those appearing earlier diffuse into a zone where there is no longer any clot.

The structures formed in the course of clotting grow at rates far exceeding the characteristic diffusional rates. The practical independence of the growth rate right up to the zone of arrest from the type of activating surface and also “steep” sharply delineated spatial boundaries of the clot formed (thrombus) are not at all characteristic of passive, that is purely diffusional, distribution of matter in space. These observations agree with the notions that growth of the thrombus at the early stage is determined by the spread of a quite steep thrombin autowave [7, 8].

The interaction of two thrombi growing towards each other (Figs 4 and 9) agrees well with the predictions of the hypothesis of [7]. However, at present there is not enough evidence to allow one to state with confidence that the zones of non-coagulation are actually associated with inhibition of the action of thrombin and we would say not with the depletion of fibrinogen. The published findings on molecular mechanisms of clotting [1–3] appear insufficient for understanding the nature of the processes of arrest of thrombus growth.

Comparison with the phenomenological model [8] shows that experimentally the same regimes are observed qualitatively as predicted by the model. In experiments with the lid open, growth of the thrombus is observed with sharp arrest and formation of distinct linear zones of

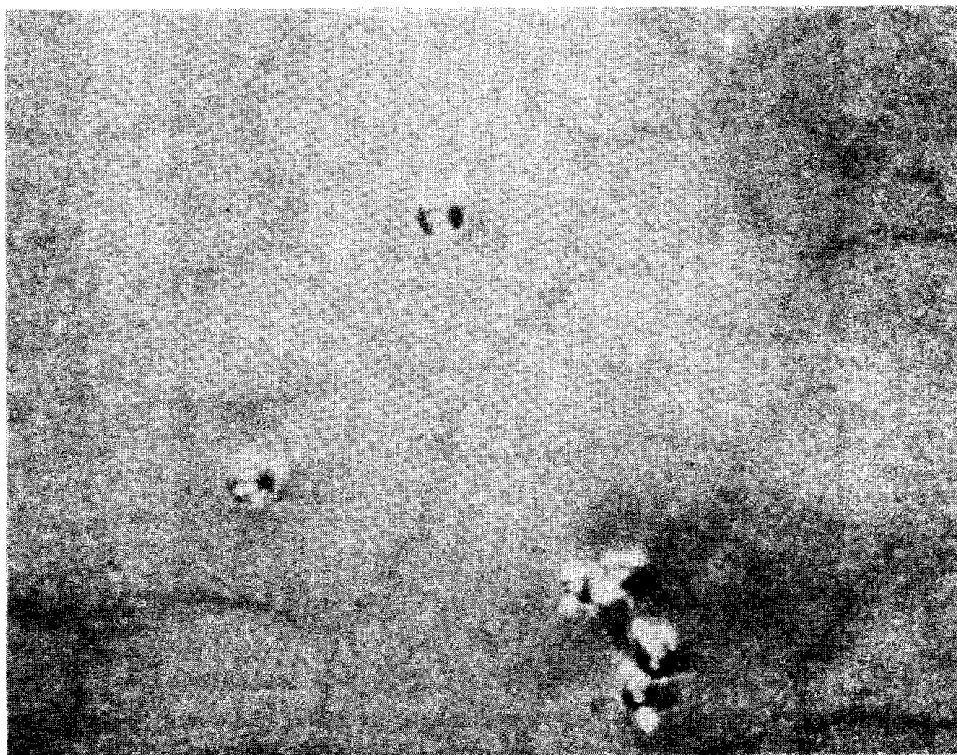


Fig. 9. Spatial structure appearing in the Petri dish after fusion of the clots in the plasma where the thrombi grew unceasingly. The photograph corresponds to 40-min incubation.

inhibition (Fig. 4). According to the model, regimes of such a kind must operate for a high clotting activational threshold. With fall in the activational threshold, according to the model, ring structures must form in a narrow zone of values. Such structures could sometimes be actually observed in experiments, although the concrete conditions of their realization are still not entirely clear. A further fall in the activational threshold in line with the model must lead to replacement of the fast by the slow growth phase. Indeed, this happens in the experiment. Continuous slow growth of the clot after formation of the main core is particularly clearly observed in experiments with the lid closed (Figs 7 and 8).

In conclusion, we would note that although the series of experiments run by us actually revealed the predicted phenomena of formation of localized and spatially regular structures in the blood plasma, which is a cogent argument in favour of the previously advanced two-wave hypothesis, it seems to us premature to consider the hypothesis as proved. Thus, the differences in the character of clot growth in the situation with open and closed lids indicate that a major role in the phenomena recorded may be played by convection. Only further experiments to elucidate not only the qualitative but also the quantitative characteristics of thrombus growth will finally shed light on the mechanism responsible for the spatial dynamics of thrombus formation.

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