Note on the Amoeboid Elements in the Blood of Helix aspersa.

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With Plates 9 and 10.

Introduction.

Recently a considerable amount of work has been carried out in this laboratory by Gatenby, Duthie, Hill, and Macdougald on invertebrate tissue culture (**4, 5, 6, 7, 8**). Bohuslav also figured growths from various organs of Helix (**1**), notably the atrium (**2**). As the work quoted above has been done with species of Helix only, it is desirable at this point to notice and compare the behaviour of the leucocytes in the blood of Helix aspersa. Kollmann (**12**), by his comparative studies, demonstrated the homology of the leucocytes in all groups; there is, therefore, no reason to suppose that the cell elements found in the blood are other than leucocytes.

I have to thank Dr. Gatenby for suggesting the problems that are discussed in this paper.

Previous Work.

In connexion with investigations on tissue culture of invertebrates it is proposed here to review the work of Bohuslav alone (**2**) since Gatenby, Hill, and Macdougald have mentioned previous work in a recent paper (**9**). Bohuslav in his second paper describes proliferation in the atrium after 12 hours, and compares the structure to that of the fibroblast network in the vertebrate cultures. The amoebocytes anastomose by hyaline bridges, and later clump in large or small groups (cp. Gatenby). Bohuslav compares the ease with which connective tissue elements are produced from vertebrate hearts, with the difficulty of producing them in Helix, and adds that it may be due to the post-embryonic nature of the latter.

Bohuslav's preparations were sterile, in a medium closely resembling Hédon-Fleig's fluid; networks remained alive for 22 days, and pulsation continued for 39.

Kollmann (**12**) made comparative studies of the leucocytes in the various groups of invertebrates and found similarity in histological evolution throughout. He also states that even in animals without definite lymph glands (Pulmonates, Lamellibranchs, Amphineura, Scaphopoda, &c.) there is no primitive character in the leucocytes to distinguish them from types possessing lymph glands.

Takatsuki (**14**) studied the amoebocytes in Ostrea edulis. He found granular and non-granular types. (The latter have not been found in Helix.) The granular leucocytes are actively amoeboid: 'After a lapse of about half an hour many become fully extended and may then measure 3 or 4 times their length in the contracted state. In this condition they are very thin, sometimes transparent. Some of them link up together to form an open meshwork of chains of cells.' When accumulated in masses, they do not fuse together, in fact those on the edge tend to creep away. In certain conditions amoebocytes form chains united by long protoplasmic threads; these strands may serve as entanglement for other corpuscles.

According to Goodrich (**11**) the pseudopodia of invertebrate leucocytes, described as freely projecting processes, are either figured from optical sections of folded membranes or due to changes taking place under abnormal conditions. He concluded that the leucocytes are provided with a more or less extensive membrane of cytoplasm, and that in most cases the membranous pseudopodia are normally expanded in the living animals, but that fine pseudopodia are absent. Schun-ichi Takatsuki's observations indicate that even the membranous expansion is less extended in the animal than when extracted in the blood.

Leo Loeb (**13**), working with Limulus amoebocytes, showed that the character of broad or fine pseudopodia (e.g. pseudopodia of Goodrich) depends on osmotic pressure and temperature. In contact with the glass the cells spread out, but the intensity of spreading out and number of spread-out cells is restricted.

Those cells not in contact with the glass remain contracted for a longer period.

Loeb's material is a blood-clot of Limulus, which is free from fibrin. He showed that under certain conditions (isotonic medium) structures are obtained which resemble connective tissue; hypotonic, slightly alkaline, resemble epithelium; hypertonic, resemble nerve- and glia-cells.

The clumping of the amoebocytes has long been known. It was described as plasmodium formation by Geddes (**10**), but they are actually not fused to that extent. No clotting occurs in lamellibranchs or gastropods.

G. H. Drew (**3**) describes in Cardium the agglutination of the corpuscles at the side of a wound when blood is escaping. Connecting strands of protoplasm stop the haemorrhage.

Personal Observations: A. Leucocytes.

The shell was broken away and the mantle pricked a little behind the heart with a tapered needle. Samples of blood were taken at various intervals, from 15 minutes later, to alternate days for 5 days. Each successive sample showed an increase in the number of cell elements or leucocytes in the blood. The most remarkable increase was shown 15 minutes after the first wounding.

The normal number of cell elements in the blood of Helix is very small. They appear as transparent rounded cells, which shortly after extraction produce numerous fine pseudopodia. When a wound is made as described and subsequent samples taken in which there are large quantities of leucocytes they proceed to clump. The pseudopodia grow out, and coming in contact with the pseudopodia of other cells, join with them and slowly contract. This draws the cells together. Large clumps are formed in this way when the cells are crowded. Three separate cells were observed for twenty minutes. At first the shortest distance between them was about 2 diameters, but by the process described above, at the end of 20 minutes they were joined into a single clump.

If the drop of blood is placed on a slide and protected from evaporation (either by placing in a Petri dish, or, better, by

placing the drop on a coverslip and mounting on a vaselined slide), the clumps come in contact with the glass, begin to spread over it. The leucocytes radiate from the clumps with broad, flattened pseudopodia, and by anastomoses with other spreading groups create a network, recalling the appearance of the amoebocytes described in tissue cultures. The network is formed in between 30 minutes and an hour (cp. Takatsuki).

The phagocytic properties of these cells have been investigated. Owing to their transparent nature it is difficult to see whether or not particles have actually been ingested. But if a hanging drop preparation of blood is made, and fine particles of carmine added to the surface of the drop on the point of a needle, before the leucocytes have adhered to the glass, phagocytosis can be well seen. The carmine stays at the edge of the drop, but the leucocytes that reach the coverslip are clearly seen to be full of ingested particles. Although all cells do not ingest the carmine this cannot be taken as a distinction between two types of cells, because they do not all necessarily come in contact with the material.

Large, with scattered chromatin blocks, the nuclei are often bent and lobed in various ways. Although the clumps have the appearance of a plasmodium, the individual cells separate out and round off just before disintegration, which occurs after two or three days when kept in blood and protected from evaporation.

Fig. 1, Pl. 9, shows the clumped leucocytes in a sample of blood, taken 15 minutes after wounding. The spreading out and anastomosing into a network are well seen. This condition was reached about 40 minutes after extraction, the drop lying on a coverslip with a vaselined slide inverted over it. Four hours after extraction the slide, with the coverslip adhering to the vaseline, was turned upwards and the network photographed.

Fig. 3, Pl. 10, shows denser clumps, fixed in Carnoy's fluid, and stained in Mann's methyl-blue eosin. The subsequent spreading out in contact with the glass is well advanced except in the densest clumps.

The question whether two types of cells can be distinguished has been considered. No difference in type (e.g. lymphocyte and tissue cells) can be shown by ordinary staining methods

such as Leishmann's blood stain or Delafield's haematoxylin. The dense rounded cells found in fig. 4, Pl. 10, at xc and ec, seem at first sight to be different, in fact contrasted, in character to the more numerous, flattened cells. It will be shown later, however, why this conclusion is not admissible at present.

The appearance in figs. 1 and 3, Pls. 9 and 10, resembles the behaviour of the amoebocytes of Ostrea described (but not figured) by Takatsuki. The chains connected by long threads have also been seen, and they have rounded corpuscles trapped on them. But it should be noted that the chains are drawn out as such and do not have to settle on the slide. They must, in fact, have been formed already at the wound.

B. Heart Preparations.

The heart is dissected out and put in a drop of blood on a slide. Fragments of the atrium are cut with a fine sharp knife and mounted as hanging drop preparations in blood. Growths or migrations occur in less than 24 hours and produce a typical network. This remains alive from 3 to 6 days.

Less than 50 per cent. of the preparations grew at first, when they were taken at random from unwounded snails. It was later found that 'growth' or migration was almost certain if the hearts were taken from individuals wounded with a needle 4 or 5 hours before. The cut edges of the fragments in this case, if observed immediately, were found to be a mass of rounded cells. A few minutes later the outermost cells had produced short pseudopodia. The edge was in fact bristling with leucocytes. Next morning they had spread out along the coverslip and produced the characteristic network. It is to be noted that although the migration and network formation are most remarkable in contact with the coverslip, migration occurs at all layers through the drop and also at its lower surface. The cells are, however, more rounded and less inclined to form networks in these positions.

The phagocytic properties of these cells are also shown by introducing finely-ground carmine. As they are already in close contact with the glass, the carmine was introduced this time suspended in Ringer solution. After about an hour the carmine

was washed off in a stream of Ringer solution, but a certain amount remained adhering to the cells and some was unmistakably ingested.

Fixing in Carnoy's fluid, and staining by Hansen's method, did not remove the carmine, so a permanent preparation could be made by blotting the coverslip instead of dehydrating in alcohol.

The staining properties of these cells in Leishmann's stain, and also haematoxylin, are in no way different from those of the leucocytes already described. Neither do they show a distinction into two types of cells, although the same difference in shape occurs. The lobed and deformed nuclei found in the leucocytes are found here also. The resistance of these cells to bacteria is considerable. One preparation remained sterile for 6 days. (No aseptic precautions are taken beyond flaming the coverslips and slides.) Clumping, as described by Gatenby, occurs here also; rounding off and numerous large vacuoles precede degeneration.

Similar migrations were produced in preparations mounted in Hédon-Fleig medium, pH 7·2. This is a lower pH than the one used by the workers quoted, but Bohuslav states the optimum to be 6·8 for Helix pomatia. A sample of blood taken at the same time as making the preparation was 7·8. pH 7·2 then came between these two and gave good results.

The migration in blood is shown in fig. 2, Pl. 9. The preparation was fixed and stained on the fourth day. Swarms of bacteria are visible and many have been ingested by the amoebocytes. The latter are rounding off in many cases, preliminary to disintegration. The gap between the ex-plant and the migrated cells was produced during fixation.

Discussion.

In Vertebrata increase in the number of leucocytes, following wounding, has long been known; it is in fact an inflammation. But the source of the increase in Invertebrata is doubtful, especially in the case of Helix, in which no lymph gland is recorded, and although serial sections were made no lymphogenic tissue was observed. The first cases observed were from individuals which had been wounded several days before, so that it was suggested that the cells which came out in the blood might be

the connective tissue elements repairing the wound. When the number was found, however, to increase within 15 minutes of wounding this explanation was no longer possible.

Kollmann states that all multiplication takes place by mitosis in the circulating cells; but 15 minutes is too short a time for mitosis to take place. Also, in the extracted cells, although they were left in blood no indications of mitosis were seen.

Kollmann denies the occurrence of amitosis in these cells, and compares the lobed and deformed nuclei to the polymorph nuclei so familiar in Vertebrate leucocytes. If this be true the multiplication cannot be explained by amitosis either.

The cells of the connective tissue in the mantle cavity-wall stain very similarly to those in the blood. The only remaining explanation seems to lie in diapedesis, or migration through the blood-vessel walls from the connective tissue. Sections show indications of this.

It is to be noted that the increased number of leucocytes after 15 minutes is not merely due to a loss of liquid; because there is an increase also from day to day when the amount of liquid has been replaced, and samples taken then may be quite opaque with leucocytes.

The histology of the mantle cavity-wall is described by Gatenby and Duthie (1932). The connective tissue is very loose, and narrowly separated from the blood-vessels. It is possible that the cells in the connective tissue are identifiable with the leucocytes, and also that in culture they are the 'amoebocytes' which wander out. The same authors have described reparation of a wound, which begins with a plug of these cells. Figures suggestive of amitosis were found in this case. Gatenby and Hill (1934) describe the absence of the Golgi apparatus in some amoebocytes, in which it is usually present. This they conclude indicates rapid amitotic division in which the Golgi material remains entire in one daughter-cell. The possibility of amitosis is not therefore excluded.

As for the 'growths' or migrations that occurred in the heart preparations, these are almost undoubtedly migrations of leucocytes which had collected in the heart. In most cases the individual had been previously wounded and the leucocytes

were seen as soon as the preparations were made. The staining and phagocytic properties of the cells produced from the heart are the same as those of the leucocytes. Bohuslav states that if blood is used as a medium it produces toxins which cause the cells to degenerate. This is certainly not always the case, because the pieces of heart live up to 6 days in nothing but blood, pulsating for at least the first three. It is true that networks are not formed if the drop of blood contains much debris, but the toxins are more likely due to the latter than to the blood itself. It might be argued that two types of cells are here concerned: those that can resist the toxin (i.e. leucocytes), and those that cannot. But the similar behaviour found in the cells in Hédon-Fleig solution makes this doubtful. Here again, therefore, the amoebocytes described by Bohuslav from heart cultures are probably identical with the leucocytes.

Bohuslav's early pictures recall the appearance both of the leucocyte clumps and of the heart preparations described above.

Another significant fact is that the atrium only is found to grow; as Bohuslav says, only a few odd amoebocytes are produced from the ventricle. This difference is accounted for by the smaller lumen of the ventricle, which does not allow many leucocytes to collect.

The growth described from the heart in this and other cases is probably the same phenomenon as the migration from the 'cellfibrin' tissues described by Loeb.

With regard to the question of two types of cells being found either in the blood after wounding or in the heart migrations, these facts are against it: as Loeb remarks in Limulus amoebocytes, the number of spread-out cells is restricted, and those cells which are not in contact with the glass remain contracted for a longer period; also, if the cells are fixed immediately the blood is drawn, before they have time to settle, all the cells are dense and rounded. These observations may explain the presence of the cells in fig. 4, Pl. 10, at XC and EC. Some of them may of course be necrotic as they are often enclosed in flattened cells (EC), and the dying or dead cells described by Gatenby and Hill are similar in appearance.

Conclusions.

1. Clumps of leucocytes of Helix aspersa may simulate the networks described in tissue cultures.

2. Less than 50 per cent. of the heart preparations grew at first, when they were taken at random from unwounded snails. It was later found that 'growth' or migration was almost certain if the hearts were taken from individuals wounded with a needle 4 or 5 hours before.

3. The preparations remain sterile up to 6 days (in blood), without special aseptic precautions, probably owing to the leucocytic nature of the cells.

4. The 'outgrowths' produced from mantle-wall and other tissues, in Helix, are migrations of leucocytes.

5. At the present moment the conclusion seems forced upon one that the various cell elements in blood, in tissue culture, and in such connective tissue networks as are found in the mantle cavity-wall, cannot be differentiated one from another by current fixation and staining technique.

DESCRIPTION OF PLATES 9 AND 10.

(Microphotographs by Mr. Douglas Glen.)

Plate 9.

Fig. 1.—Leucocytes in blood, extracted 15 minutes after wounding, living condition. The network was formed 40 minutes after extraction and photographed 4 hours after. (×386 circa.)

Fig. 2.—Atrium, 4 days in blood. Fixed in Bouin's fluid, stained Delafield's haematoxylin and eosine. Swarms of bacteria (B) are seen, some of which are ingested by the amoebocytes. Many of the amoebocytes are rounded off preparatory to disintegration. (The large gap between the atrium ex-plant and the amoebocytes at one point is a shrinkage space caused during fixation.) (×147 circa.)

Plate 10.

Fig. 3.—Clumps of leucocytes from blood of an individual wounded 15 minutes previously. Clumping occurred first, the clumps subsequently spreading in contact with the glass. Fixed Carnoy's fluid, stained methyl-blue-eosine. (×147 circa.)

Fig. 4.—Blood from individual wounded several days previously. Clumps spreading, fixed 2 hours after withdrawal in Bouin's fluid and

stained in Delafield's haematoxylin and eosine. The rounded type of cell is seen at XC and EC. The irregular shapes of the nuclei are also apparent. (×386 circa.)

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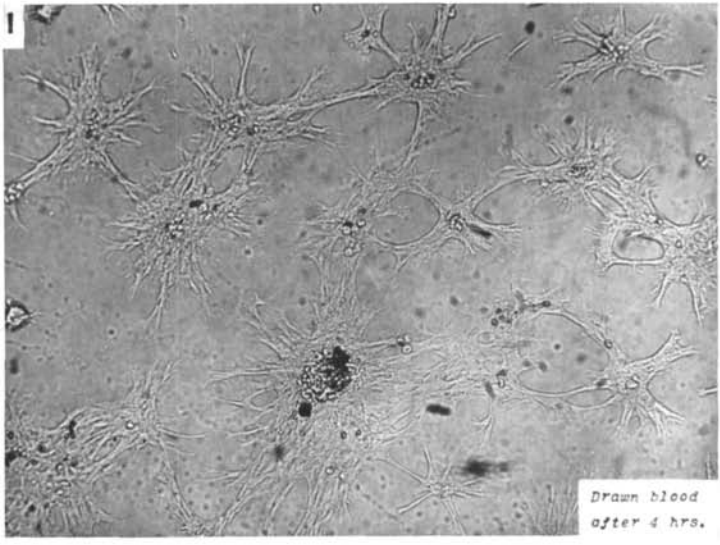
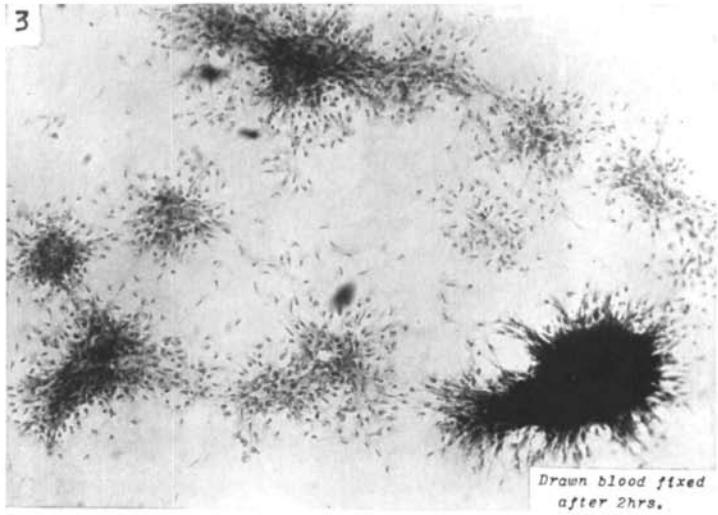

1
Drawn blood
after 4 hrs.


2
Atrium Ex-plant.
B
After four days
in blood.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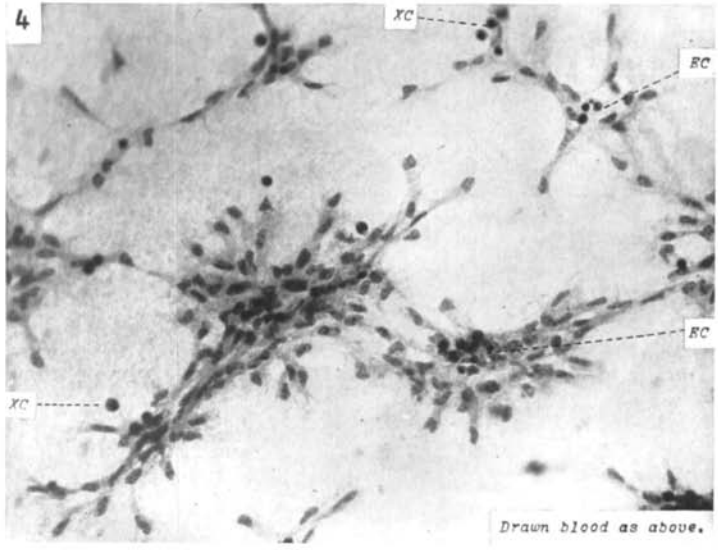
4
XC
EC
EC
XC
Drawn blood as above.