Electron Microscopic Observations of Stromatolysis in Human Erythrocytes. (18124)

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(Introduced by O. H. Gaebler.)

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For an electron microscope study of *Treponema carateum,* preparations were made of tissue fluid from the skin lesions of a typical case of pinta. These preparations, not subjected to chemical fixation, showed a number of well-preserved erythrocytes and relatively abundant images which are thought to correspond to disintegrating, hemolyzed erythrocytes. The processes used to separate the treponemas from the other material present in the skin tissue fluid resulted in the hemolysis of most of the erythrocytes. The specimens were centrifuged in both normal saline and distilled water suspension for 30 minutes, respectively. A small portion of one tissue fluid batch was examined without centrifugal concentration and no distilled water was used in this case. The technic has been described in more detail elsewhere(1).

Observations. While no changes were observed in some cells, widely different degrees of hemolysis were observed in others, and some of the erythrocytes showed changes in cell surface configuration. A comparison of these latter images with those reported by several workers(2-10) using the optical microscope under controlled conditions of stromatolysis shows striking similarities. Further evidence of the stromatolytic nature of the observed changes is obtained by noticing appearances which are either identical or closely similar to the different stages of lysis of erythrocyte stroma reported by numerous workers. There is no basic difference in morphology, except for the superior image detail, between the electron micrographic appearances reported here and the photomicrographic ones depicted elsewhere. This is probably due in part to the absence of chemical fixation which is in accordance with previous findings(11).

Some images (Fig. 1 and 7) undoubtedly correspond to erythrocytes in relatively good condition, while others (Fig. 4, 14 and 15) are apparently erythrocytes which have dried flat to the supporting film in the usual processes of electron microscopy after suffering crenation and hemolysis. This interpretation also applies to other images such as those shown in Fig. 5, 11, 12 and 16, although they are often so distorted that they might be confused with dried liquid residues wherein the flow processes provoked by interfacial forces during drying might have given rise to filaments in the final image. However, their oval shape, their size (often decreased by osmosis, drying and electron bombardment), their similarity and incidence with the better characterized images from Fig. 6 and 8 to 10, as well as with unhemolyzed erythrocytes as in Fig. 1, the presence of filaments at the level of the upper portion of their seemingly collapsed envelopes and especially their marked similarity to known stromatolytic forms, all make their identification with stro-

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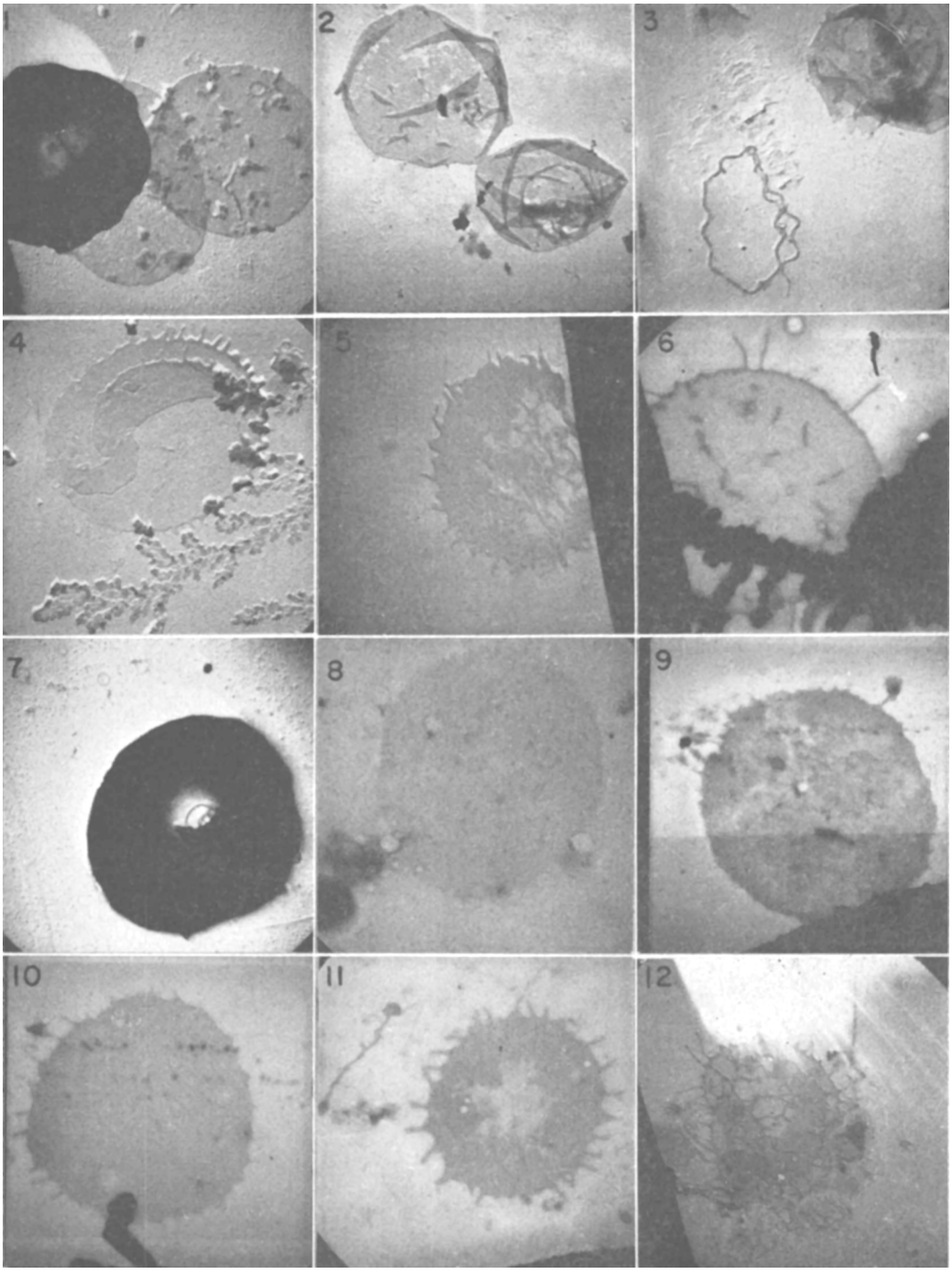

Fig. 1 to 5 and 7 and 12 shadow-cast with chromium at angle of about 12°. Magnification × 4,000.

matolyzing erythrocytes highly probable. This contention receives added support from the formation of similar filamentous processes in a large number of materials also rich in phospholipids (1,10).

Unhemolyzed erythrocytes are found in

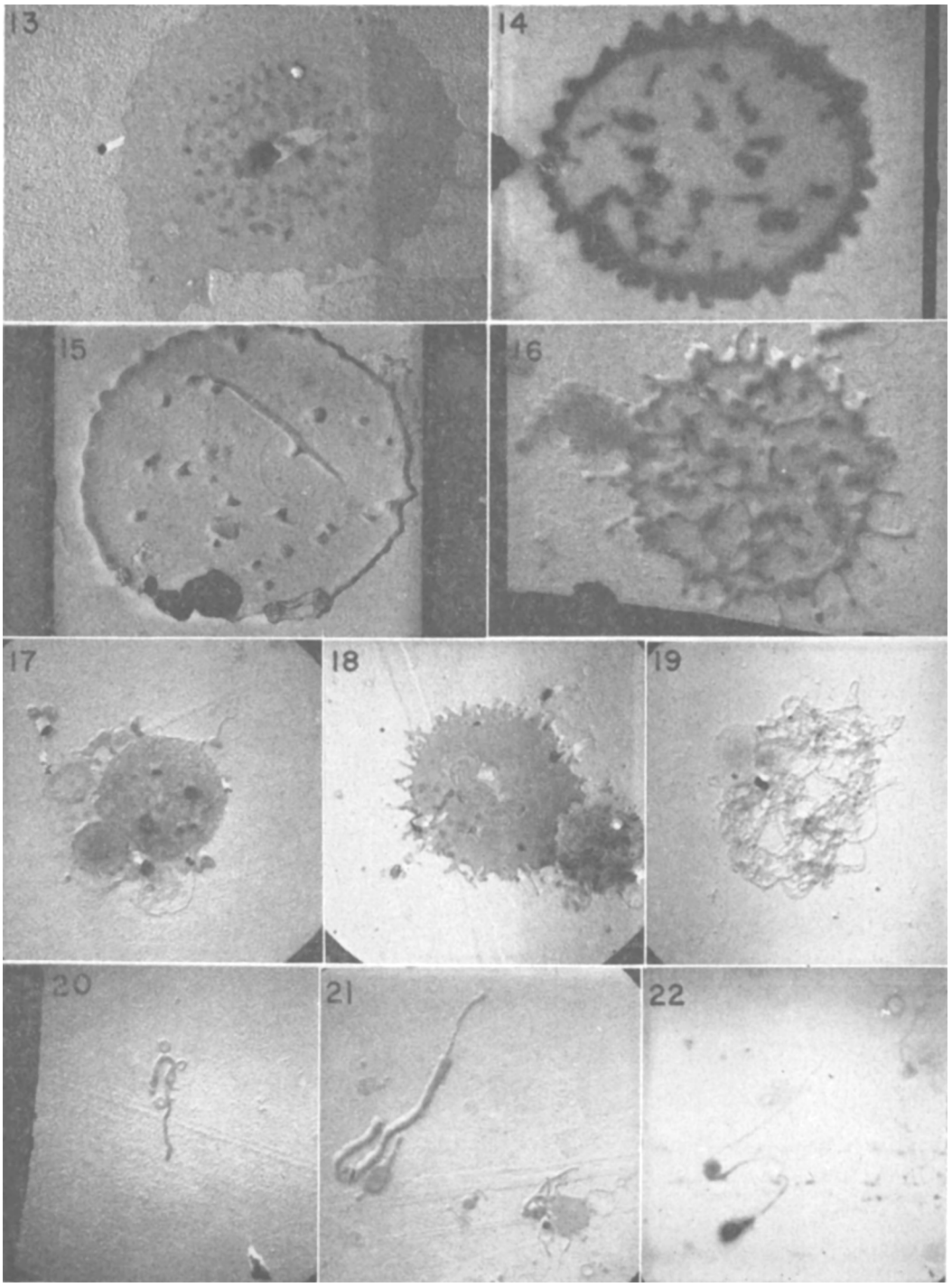

Fig. 13, and 15 to 21 shadow-cast with chromium at angle of about 12°. Magnification: Fig. 13, ×6,000; Fig. 14, ×4,800; Fig. 15, ×5,600; Fig. 16, ×4,600; Fig. 17 to 22, ×4,000.

Fig. 1 and 7. Hemolyzed erythrocytes with no apparent change in configuration are illustrated in Fig. 1. No processes are found in the erythrocytes from Fig. 2 except folds

in the erythrocyte envelope. According to de Robertis *et al.*(12), this envelope appears to correspond to the plasma membrane plus some external layers of cytoplasm. On the other hand, folds were observed in fixed chick embryo erythrocytes(11), but no folds have been seen with the electron microscope in chick embryo erythrocytes which were not subjected to chemical fixation. Probably the preparation of the specimens which was unlike that of the present case accounts for the difference observed. Folds are seen in torn erythrocytes (Fig. 3) and they can be seen also in erythrocytes showing short, relatively broad processes (Fig. 4).

The density of the periphery and of the processes themselves which arise from the periphery of the erythrocytes (Fig. 6 and 14) may be explained by persistence of hemoglobin at these levels. This is supported by the finding of partially hemolyzed (Fig. 9) and unhemolyzed (Fig. 1 and 7) erythrocytes in the same preparation. Unhemolyzed, nonnucleated erythrocytes often show a less dense center in electron micrographs which indicates less hemoglobin in this area (Fig. 1 and 7). This may support the contention that hemoglobin tends to persist at the cell periphery even in partially hemolyzed erythrocytes.

Seifriz(7) in his work on living human and amphibian erythrocytes remarks on how easily the cytoplasm flows into the processes formed on the cell surface. This fact apparently has some bearing upon the explanation for the images in Fig. 14, 16 and others. In these figures while much of the corresponding image is flattened as the envelope collapses under hemolysis, some hemoglobin has apparently been left at the level of the processes which consequently do not flatten to the same extent. The density of the processes in the unshadowed images (Fig. 6 and 14) and the raised appearance of these processes in shadowed fields, depicted in Fig. 4, 5 and 16, also support the same argument.

In Fig. 15 there are rounded, rectangular areas of high contrast which are very likely images of sodium chloride crystals. Sodium chloride residues are seen also in several other fields, for example in Fig. 4, 17 and 18. Since the shadows cast by the processes are in general of a rather constant length, it is likely that they lie mostly in the object plane. The image from Fig. 13 shows dense areas of irregular contour and marked density, which throw no shadows and are likely the manifestation of dense areas within the envelope. The large particle which throws a shadow is a crystal.

Discussion. Many workers have reported appearances identical or similar to the crenated aspect shown by a number of the erythrocytes presented here. Seifriz(7) obtained evidence which supported the suggestion of Brinkman and Dam(13) that crenation of erythrocytes was not an osmotic phenomenon. Bechhold(4), Salén(5), and Rockwood(6) observed these stromatolytic forms in unhemolyzed erythrocytes from preparations where hemolysis was occurring. Kite(2), Oliver(3), Takeuchi(8) and Auer (9) did likewise in unhemolyzed erythrocytes which were standing under various other conditions. In a few cases these forms were found attached to other erythrocytes(8,9). Seifriz(7), in a study of living human and amphibian erythrocytes pointed out that changes in the physical properties of the erythrocyte envelope resulted in changes in the cell surface configuration. These changes could be provoked by microsurgical manipulation or could appear spontaneously. They consisted of pseudopod-like processes, globular extrusions and conical processes, all at the erythrocyte envelope, and papillae or filaments at the cytoplasm only. These papillae closely resemble many of the filaments depicted here, especially in the rather frequent terminal swelling (Fig. 1, 4, 5, 11 and 12). In some instances, the papillae observed by Seifriz left vacuolar depressions in the cell below the envelope, which are apparently identical with the depressions seen here in Fig. 5 and 11. A pseudopod-like process appears to have occurred in Fig. 13, while

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globular and conical processes may be seen in Fig. 14 and 15, respectively. With the optical, dark field microscope, Furchgott(10) followed the process of disintegration of hemolyzed, human, beef, and calf erythrocytes. As the initial stage under the action of lyotropic salts, ghosts showed small buds at the cell periphery. Then, these buds progressed away from the ghost periphery, thus constituting filaments which in a further stage separated from the ghost either intact or broken into smaller threads. Finally, these smaller filaments disintegrated into small particles unresolvable in the ordinary, bright field microscope and whose diameter was estimated as about 100 millimicrons was the dark field microscope. It was technically impossible to follow the whole process of stromatolysis in the electron microscope, but isolated appearances descriptive of all the stages reported by Furchgott are represented and depicted in this report. Furchgott observed stromatolysis under certain conditions even by the action of normal saline solution and of distilled water.

If Fig. 14, 16 and 12 are examined consecutively, the order for the progress of filament formation is noticed, which follows Furchgott's observations(10) that the first production of globular processes subsequently narrows until definite filaments are formed. To a less extent, this is true of Seifriz's findings(7) too, since he stated that while changes at the erythrocyte envelope progressed from pseudopod-like processes, to globular, to conical extrusions, filaments were cytoplasmic processes protruding through the envelope. On the other hand, observing Fig. 7 to 12, a quite gradual development of the progressive stages of filament formation is noticed while globular or conical processes are apparently missing. That this developmental order may actually occur seems to be supported by the appearance from Fig. 4 as well as by Seifriz's remarks(7) on the cytoplasmic origin of filaments in contrast to the origin of the globular and conical processes he observed. However, it should be pointed out that both definite filaments and blunt and conical processes may be seen in the same erythrocyte (Fig. 5 and 16).

The identification of the appearances in these micrographs with the different stages of stromatolysis is supported further by the very close morphologic similarity between the images in Fig. 17 to 22 (the bundles of threads in Fig. 19, the isolated filaments in Fig. 20 to 22 and the apparent cellular debris in Fig. 17 to 22) and the late stages of stromatolysis as reported by Furchgott(10). Furchgott noticed the resemblance to the so-called myelin forms of many of his stromatolytic structures. The former arise from the myelin sheath of nerve fibers as well as from other biological structures and even from simple chemical compounds of phospholipid nature. In this regard he stressed the high phospholipid content of erythrocyte ghosts. From shadow-casting considerations, only those erythrocytes which are relatively unchanged by the handling (Fig. 1 and 7) have any residual thickness. All of the others have flattened to the surface of the supporting film in such a way as to throw very little shadow except at the periphery. No changes were observed in the specimens during electron bombardment, and if specimen changes did occur they must have been due to drying alone. Some of these effects have been discussed earlier(11).

Although it is not possible to follow erythrocyte stromatolysis directly with the electron microscope, this instrument does offer increased resolution over the light microscope, and with the aid of shadow-casting an additional advantage in contrast, especially over the cell surface. Increased detail of the stromalytic processes is then to be expected. The correlation of light and electron microscopic information is very valuable when interpretations are being made in such a study.

Summary. Widely different degrees of stromatolysis were observed electron microscopically in erythrocytes which occurred in preparations of *Treponema carateum* taken from tissue fluid of skin lesions. A comparison of electron images with light microscopic images of cells under controlled conditions of stromatolysis showed striking similarities. Isolated appearances in the electron micro-

graphs similar to all the stages of disintegration of hemolyzed erythrocytes reported by Furchgott from light microscopy are represented and observed.

Received July 5, 1950. P.S.E.B.M., 1950, v75.

Action of Vitamin B_{12} in Counteracting Glycine Toxicity in the Chick.* (18125)

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Cary *et al.*(1) and Rubin and Bird(2), working with rats and chicks, respectively, have observed growth-inhibitory effects from high levels of protein in diets now known to be low in vitamin B_{12}. McGinnis *et al.*(3) reported that the nonprotein nitrogen content of the blood in chicks fed diets high in protein and deficient in the "animal protein factor" was higher than in chicks fed the same diets supplemented with this factor. Zucker and Zucker(4) observed a similar condition in rats. Since that time, vitamin B_{12} has been shown by Ott *et al.*(5), Lillie *et al.*(6), and others, to be an important part of the animal protein factor. Recently, Charkey *et al.*(7) have demonstrated that the levels of nonprotein nitrogen and amino acids in the blood were higher in vitamin B_{12}-deficient chicks than in chicks fed vitamin B_{12} (Merck & Co., APF Supplement No. 3). These workers concluded that vitamin B_{12} appears to function in metabolism by enhancing the utilization of circulating amino acids for building fixed tissues. These observations are in agreement with those noted in this laboratory (unpublished data).

In view of these findings, the present study was conducted to determine the effect of feeding different levels of glycine to chicks receiving various amounts of vitamin B_{12}. Evidence is presented to indicate that vitamin B_{12} is concerned in the metabolism of glycine in the chick.

Experimental. New Hampshire chicks of mixed sexes, obtained from dams kept on raised wire platforms and fed a ration low in vitamin B_{12}, were used in these experiments. The chicks were maintained in electrically heated batteries, and feed and water were supplied *ad libitum*. All glycine supplements were made at the expense of cerelose (glucose). Merck & Co. APF Supplement No. 3 was used as the source of vitamin B_{12}.

In Exp. 1, day-old chicks first were fed basal diet 122 (developed in this laboratory, Table I) during an 18-day preliminary period to further deplete them of vitamin B_{12}. This diet was deficient in vitamin B_{12} and contained 35% protein. At the end of the depletion period, six comparable groups of 15 chicks each were selected on the basis of body weight. Groups 1, 3, and 5 were fed the basal diet plus 0, 3, and 30 μg of vitamin B_{12} per kilo, respectively. The other 3 groups were fed these same diets except that 1%

* Scientific paper No. A287. Contribution No. 2233 of the Maryland Agricultural Experiment Station (Department of Poultry Husbandry). This work was supported in part by a grant from the Research Grants Division of the National Institutes of Health, United States Public Health Service.

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