INCLUSION BODIES IN EXPERIMENTAL HERPETIC INFECTION OF RABBITS.

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PLATES 41 AND 42.

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While few investigators claim to have demonstrated the actual infective agent in herpes,[1] certain peculiar nuclear and cytoplasmic inclusion bodies, which are said to be specific, continue to receive attention. Within the past 2 years it has been suggested, among other possibilities, that they are the infective agent or virus itself, "or particles of organic matter to which the virus tenaciously clings;" that they represent a combination between the virus and substances of cellular origin; that they are the expression of peculiar nucleolar changes caused by the virus; and lastly that they are merely products of cellular degeneration of dubious specificity.[2–5] The problem is more important than it might appear to be at first sight. It is not simply a question of the etiology of a relatively mild infection, with the appearance of which we are familiar, because information is accumulating to the effect that herpes may be but one of a group of diseases of unknown etiology. For instance, etiological relationship between herpes and lethargic encephalitis has been suggested.[4] Interesting inclusion bodies have likewise been described in vaccinia, chicken-pox, smallpox, and several other infections the nature of which is still under discussion.

[1] Kooy, J. M., *Klin. Monatsbl. Augenheilk.*, 1921, lxvi, 81.

[2] da Fano, C., *J. Path. and Bact.*, 1923, xxvi, 85.

[3] Lipschütz, B., *Arch. Dermatol. u. Syph.*, 1921, cxxxvi, 479.

[4] Levaditi, C., Harvier, P., and Nicolau, S., *Ann. Inst. Pasteur*, 1922, xxxvi, 77. Doerr, R., and Schnabel, A., *Z. Hyg. u. Infectionskrankh.*, 1921, xciv, 29. Levaditi, C., Ectodermoses neurotropes. Poliomyélite, encéphalite, herpès, Paris, 1922.

[5] Luger, A., and Lauda, E., *Wien. klin. Woch.*, 1921, xxxiv, 132.

As part of a wider study of the etiology of febrile herpes and of epidemic encephalitis that is being carried out by Dr. Flexner, Dr. Noguchi, and Dr. Amoss, we undertook the minute histological investigation of the inclusion bodies in question.

Infection by Intracerebral Injection of Virus.

Our first efforts were directed toward a repetition of da Fano's observations of the inclusion bodies of herpetic encephalitis in rabbits. To this end a total of eleven rabbits (Nos. 3, 5 to 9, 12, 13, 18, 21, and 30) were, in the course of our experiments, injected intracerebrally with about 0.2 cc. of 10 per cent H. F. strain of herpes virus. All showed typical symptoms at about the 4th day, when they were killed with chloroform.

Upon autopsy, sterility tests of the brain substance were made with plain broth, slant agar, and the Smith-Noguchi medium of all the rabbits except three (Nos. 3, 5, and 6). Upon incubation, the tubes remained sterile in all of these except two (Nos. 16 and 17, slant agar) in which a growth of a contaminating organism was obtained.

As a routine procedure, selected hemisections of the brain from the region of the anterior commissure, posterior commissure, and calamus scriptorius were preserved by the Orth-Müller method advised by da Fano,[6] but for some tissues the following supplementary fixatives were employed: 95 per cent alcohol, sublimate-acetic, alcoholic sublimate, 10 per cent formalin, and the mixtures of Flemming, Regaud, Carnoy, Marchi, Zenker (with and without acetic acid), and Pianese. Sections were colored by Giemsa's and Borrel's stains and, in special cases, with eosin-methylene blue, hematoxylin, fuchsin-methyl green, iron-hematoxylin, Sudan III, and other dyes.

When the plane of section passed through the lesion or in its vicinity (Nos. 3, 8, 9, 12, and 13) abundant granulations were observed resembling those described by da Fano. They were also seen in remote parts of the brain but not in such large numbers. For convenience, they may be considered under the three headings suggested by him.[7]

[6] The formula for Orth's fluid as given on page 91 of da Fano's paper is obviously a typographical error. We employed a mixture consisting of 9 parts of Müller's fluid and 1 part of commercial formalin.

[7] da Fano,[2] page 109.

1. Intranuclear Particles.—Two types of nuclear inclusions were noted: (*a*) Basophilic bodies which are probably derived from nuclear chromatin as da Fano[2] has intimated. Reference to Figs. 1 to 7 will illustrate a close topographic association between the development of these bodies and changes in the nucleolus and nuclear chromatin. But the fact that these nucleolar changes do not exactly parallel other alterations in the cells is indicated by their frequent absence in highly vacuolated cells (Figs. 8 to 11). The inclusions represented in Fig. 3 should be compared with the *neurocorpuscles encéphalitiques* of Levaditi, Harvier, and Nicolau[4] as shown in their Plate I, Fig. 1. It is interesting to note that somewhat similar nuclear inclusions have been reported in nerve cells in the vicinity of a glioma by Lafora,[8] and others, though less pronounced, in normal nerve cells by Cajal.[9] (*b*) Irregular, amorphous masses of material colored red with Giemsa's stain and exhibiting a marked affinity for eosin. These were found to be particularly abundant in the hippocampus and seem to arise through a coagulation and alteration of the nucleoplasm (Figs. 3 and 6). Sometimes acidophilic material of this kind was observed to fill the nucleus almost completely, often giving it a swollen appearance so that the remaining chromatin was restricted to a thin layer just beneath the nuclear membrane (Fig. 27). These masses resemble nuclear inclusions previously described by many investigators including Lipschütz,[3] da Fano,[2] and Goodpasture and Teague.[10]

2. Relatively Large Extra- and Intracellular Formations.—Larger granulations corresponding to da Fano's descriptions were observed in abundance. Although a few were found within nerve and neuroglia cells, as well as in endothelial cells and polymorphonuclear leucocytes, the majority occurred in certain large mononuclear cells, which vital staining with trypan blue showed to be of the type of macrophages. The granules were always spherical and were optically of homogeneous consistency. They varied in diameter from about 0.6 to 5 microns (Figs. 22 to 31). The larger ones were very resistant

[8] Lafora, G. R., *Trab. lab. invest. biol. Univ. Madrid*, 1916, xiv, 103, Fig. 2.

[9] Cajal, S. R., *Trab. lab. invest. biol. Univ. Madrid*, 1910, viii, 52.

[10] Goodpasture, E. W., and Teague, O., *Proc. Soc. Exp. Biol. and Med.*, 1922–23, xx, 400.

to solvent action, appearing after all the fixatives enumerated on page 696. They stained blue with Giemsa, red with Borrel, and were strongly fuchsinophilic. They were blackened by the osmic acid contained in Marchi's fluid and Flemming's fluid and in a special mixture consisting of osmic, formic, and chromic acid, formalin, potassium bichromate, and chloride, and platinum chloride. They stained feebly with Sudan III, but gave a marked reaction when treated by Macallum's technique for the demonstration of masked iron. They resemble the inclusions which we have elsewhere described in vaccinia[11] in that they may be preserved by similar fixatives and that they exhibit an equally marked affinity for Borrel's stain which, we may note, was originally designed for the specific coloration of the corpuscles of Guarnieri; but they differ from the corpuscles of Guarnieri chiefly in being usually of smaller size, in their failure to indent conspicuously the nuclei of the cells containing them, and by the circumstance that they are not made up of several materials of different staining reaction but become colored uniformly throughout their substance.

That they are not readily autolyzed was shown by the following experiment. When Rabbit 3 showed typical symptoms of herpetic encephalitis, it was chloroformed and pieces of the brain were fixed in Orth's and Regaud's fluids. The remainder was placed in 50 per cent glycerol in the refrigerator at 4°C. Other pieces were preserved in the same way at 3 succeeding weekly intervals and sections were stained by the methods of Giemsa and Borrel. No reduction was noted in the large inclusion bodies, nor did they show a change in staining reaction. 9 weeks later the infectivity of the remaining material was proved (No. 30) and it is known that infectivity persists considerably longer.

Da Fano[2] has expressed the belief that these relatively large granules "originate from the progressive fragmentation of the nuclei of various elements, but chiefly of the polymorphonuclear leucocytes, under the influence of the virus." But the stains used by da Fano, *i.e.* toluidine blue, thionine, Azur II, methylene blue, and polychrome methylene blue, reveal only a fraction of the granules actually pres-

[11] Cowdry, E. V., *J. Exp. Med.*, 1922, xxxvi, 667.

ent in the tissues. His fixation in Orth's mixture followed by Müller's fluid is an excellent preservative for mitochondria, which may be brought to light in addition to the other granulations, seen in Giemsa preparations, by staining in the usual way with fuchsin-methyl green. Specimens of this kind exhibit a bewildering array of granules of different sizes, colored all tints from red to green and purple. It is not a difficult task to establish a gradation in shape and size between typical mitochondria and some of the large droplets under consideration, but it is impossible without laborious solubility tests to ascertain whether a true chemical transition between the two actually exists. It is also doubtful whether some of these larger inclusions are not stages in the phagocytosis of injured red blood corpuscles, a process recently emphasized by Woodcock.[12]

For the present, therefore, we have to entertain the possibility that the granules in question are nuclear detritus, as suggested by da Fano, or that they are formed from mitochondria, or through the phagocytosis of erythrocytes, or by some method of which we have no knowledge whatsoever. More accurate analysis might even show that all of these "relatively large" granules do not belong to the same category. Nor can we evade the corollary that we have no good reason to suppose that they are all formed in one way, in spite of the fact that they may look superficially alike. Methods of chemical analysis are hazardous when projected into microscopic fields and are only helpful when we recognize the full extent of their fallibility.

Fortunately the exact nature of the granules is not of great moment in the etiology of herpes, because, although we have failed to find them in the brains of three supposedly normal rabbits (Nos. 28, 29, and 36) we have seen granules closely resembling them in the brain lesions of typhus fever in guinea pigs, and a survey of the literature reveals the fact that inclusions of similar morphology, though perhaps of different chemical constitution, have been reported in *moquillo*, a catarrhal infection of dogs.[13]

[12] Woodcock, H. M., *J. Roy. Army Med. Corps*, 1922, xxxix, 14, and earlier papers.

[13] del Rio Hortega, P., *Trab. lab. invest. biol. Univ. Madrid*, 1915, xii, 109, Fig. 7.

But in order to have a definite answer as to whether the granules are inseparable from the infection, three rabbits (Nos. 10, 11, and 14) were injected intracerebrally with a suspension of normal brain substance, instead of brain containing the virus, by means of exactly the same technique. They were chloroformed 7, 24, and 48 hours later and the tissues at the site of inoculation were, in each case, preserved in 10 per cent formalin and stained by the methods of Giemsa and Borrel. Sections from the first and the last mentioned animals contained a few relatively large inclusions (Fig. 31), apparently identical with those under consideration, but none were seen in the second rabbit.

An additional control is supplied by Merzbacher[14] who, some years ago, made a study of traumatic encephalitis in rabbits. He has reported and clearly illustrated (Plate II, Fig. 4) a series of granulations which blacken with osmic acid, like those under discussion, and which, in their morphology, suggest some of the inclusions described by da Fano and represented in his Figs. 20 and 21. The account given by Homén[15] of infective toxic encephalitis has also proved helpful.

3. Minute Bodies.—Very small bodies of rather irregular size were met with in larger or smaller numbers in all the brains infected by this intracerebral route (Figs. 11 to 20). Some of them closely resembled the "minute bodies" of da Fano as illustrated, for example, in his Fig. 21. In this drawing it will be noted that the bodies are subject to a relatively great variation in size, some being five times as large as others. This variability is even more marked in his Fig. 24. Smears from one brain (No. 18) were dried in air, stained with Giemsa, and carefully examined. They contained large numbers of very small bodies of coccal (0.34 micron), diplococcal, and bacillary form (0.34 by 0.5 micron) stained deep blue which were often surrounded by halos and were very much like the most uniform minute bodies mentioned by da Fano. These bodies (Figs. 11 to 20) were found to be Gram-negative and to occur chiefly in neuroglia cells, but also in nerve cells, in endothelial cells, in macrophages, and extracellularly. Some were intranuclear as illustrated in Figs. 15 and 17 to 19. They

[14] Merzbacher, L., *Histol. u. histopath. Arb.*, 1909–10, iii, 139.

[15] Homén, E. A., *Arb. path. Inst. Univ. Helsingfors*, 1919, ii, 1.

are somewhat smaller than the diplococci described by Rosenow and Jackson[16] in lethargic encephalitis and are present in much greater numbers, but resemble slightly some granules illustrated by Pollak[17] in nerve cells in puerperal eclampsia. They were not observed in tissues which had been allowed to undergo partial autolysis. In Marchi and Flemming preparations of No. 30 bodies of approximately the same size and distribution were blackened with osmic acid but they may or may not be the same as those with which we have to deal because the osmic reaction cannot be superposed upon Giemsa specimens.

With a view to investigating further the significance of these bodies under more favorable conditions, we directed our attention to a close examination of the brains of rabbits infected by other routes than intracerebral injection, thus obviating all mechanical injury. In this connection it is to be recalled that da Fano made a similar control by the examination of one rabbit inoculated intranasally in which he succeeded in finding the same minute bodies which he observed following intracerebral inoculation.

Infection by Intraperitoneal and Subcutaneous Injection, by Skin Scarification, by Nasal Implantation, and by Corneal Inoculation.

The animals employed for study exhibited typical symptoms with considerable regularity in about 10 days time. The same bacteriological tests were made and turned out to be negative. Twenty-two rabbits were thus employed, as follows:

4 infected by intraperitoneal injection (Nos. 20, 23, 37, and 38).
7 infected by subcutaneous injection (Nos. 15, 19, 22, 31 to 33, and 40).
4 infected by skin scarification (Nos. 24, 26, 27, and 39).
3 infected by nasal implantation (Nos. 16, 17, and 25).
4 infected by corneal inoculation (Nos. 1, 2, 4, and 41).

In several brains the same intranuclear and relatively large granules were observed, which, as we have shown, cannot be considered to play the part of causative agents, so that henceforth it was the

[16] Rosenow, E. C., and Jackson, G. H., Jr., *J. Infect. Dis.*, 1923, xxxii, 144.
[17] Pollak, E., *Arb. neurol. Inst. Wien. Univ.*, 1906, xiii, 1, Fig. 15.

minute bodies which engaged all our attention. These were found in rapid succession in smears of six brains (Nos. 16 to 21) as well as in sections of two others (Nos. 23 and 27). They were not observed in similar brain smears of three guinea pigs employed in typhus fever experiments but they were detected in two of the three normal control rabbits (Nos. 28, 29, and 36) and in one rabbit which succumbed to septicemia but never showed symptoms suggestive of herpetic encephalitis (No. 27), but they were not seen in six other experimental septicemia rabbits. Not only were these minute bodies intensely stained in Giemsa preparations but they looked almost black and gave a peculiar impression of hardness. It was not found possible to identify them, with certainty, in living tissues stained supravitally with methyl green (W. T. M.), brilliant cresyl blue 2 B, Janus green (M. L. B), neutral red, and Nile blue sulfate or to ascertain their solubility in 0.1 per cent solutions of HCl and KOH. Some of their reactions were tested by the method of superposition, as follows: Certain of the minute bodies were identified in Giemsa preparations by a camera lucida drawing and mechanical stage readings. The preparation was then decolorized and restained by the Borrel combination and it was found that the same granules exhibit a strong affinity for magenta. Bodies similarly identified in a Giemsa preparation were decolorized and tested by the Macallum hematoxylin method for masked iron. Their reaction was negative. Proceeding in this way it was discovered that they resist solution when treated for 5 minutes with concentrated HCl, from which it may be inferred that they are not composed of chromatin. Our next task was to determine whether these minute granules have any natural coloration. Granules identified in Giemsa preparations were destained with 1 per cent potassium permanganate followed by 5 per cent oxalic acid, dehydrated, mounted, and reexamined and found to be colorless. On the chance that, in this case, the granules might have been altered through oxidation, another clump of granules was decolorized in 50 per cent alcohol which removed all the blue stain and left them a light brown color. From this we concluded that some of the minute bodies are pigments, perhaps of the type described by del Rio Hortega[18] in neuroglia cells

[18] del Rio Hortega, P., *Trab. lab. invest. biol. Univ. Madrid*, 1916, xiv, 19, Fig. 8.

using the method of Achúcarro, notwithstanding the fact that they are to be found in albinos (No. 27). But obviously it would be unsafe to state that all of the minute bodies are pigments. Some of them have certain features in common with the "methylene blue granules" illustrated by Alzheimer,[19] particularly in Plate XXVIII, which should be compared with some of da Fano's drawings and with our Figs. 11 to 13 and 20. In further effort to check our results by reference to minute bodies which may be of similar nature in conditions other than herpes, mention should also be made of the tiny and remarkably uniform granules discovered by del Rio Hortega[18] in a case of softening (of the brain) by the use of special silver methods, which, in turn, closely approximate to others described by Havet.[20] While we feel that many of the minute bodies which have been reported in the central nervous system in herpetic encephalitis are of neuroglial origin, a similar interpretation is less plausible for inclusions in other tissues devoid of neuroglia, although every organ contains fixed and wandering connective tissue elements possessing granulations which may not be so very different from those to be found in neuroglia cells.

The Cornea, Skin, Liver, Suprarenal, and Pancreas of Herpetic Rabbits.

1. Corneæ.—We examined the corneæ of the four rabbits (Nos. 1, 2, 4, and 41) mentioned on page 701, and of two others (Nos. 16 and 17) which developed corneal vesicles following nasal implantation of the virus. For comparison with the normal, we used corneal preparations made in connection with our study of vaccinia. Granules like those described by da Fano were readily found (Fig. 21). They did not differ noticeably from those which we have described in the central nervous system. In their morphology some of them call to mind the so called Russell bodies of plasma cells recently described by Dubreuil and Favre.[21] Minute bodies of pigment were

[19] Alzheimer, A., *Histol. u. histopath. Arb.*, 1909–10, iii, 555, Plate XXVIII, particularly Figs. 9 to 14.

[20] Havet, J., *Trab. lab. invest. biol. Univ. Madrid*, 1916, xiv, 74, Fig. 24; 76, Fig. 27.

[21] Dubreuil, G., and Favre, M., *Compt. rend. Soc. biol.*, 1914, lxxvii, 372.

not observed in the corneæ, but were found to be present in the neighboring skin of the eyelid (No. 16).

2. Liver, Suprarenal, and Pancreas.—About 0.3 cc. of 10 per cent virus was injected into the liver, suprarenal, and pancreas of two etherized rabbits. The inoculated areas were excised in one rabbit killed with chloroform (No. 35) 7 hours later, since, according to Lipschütz[3] the intranuclear inclusions in the cornea are best developed at this time, and, in the other (No. 34), after 24 hours as suggested by Goodpasture and Teague.[10] The tissues were fixed in Zenker, with and without acetic acid, and in Carnoy's fluid. Similar tissues from a control rabbit (No. 36) were preserved in 10 per cent formalin. Sections were colored with Giemsa's stain and hematoxylin and eosin. The only change consisted of a slight accumulation of leucocytes and the presence of a few granules of different sizes in the blood cells and connective tissue elements. Careful search revealed the presence of a few intranuclear inclusions which, however, did not seem to us to be specific to the herpetic infection.

CONCLUSIONS.

We have thus far failed to observe any inclusion bodies in herpetic lesions which in our opinion may properly be interpreted as microorganisms. Concerning the exact nature of the granules so fully reported by others before us, we hesitate to commit ourselves other than to indicate certain points of resemblance to nuclear débris, red blood cells in the process of phagocytosis, mitochondria, pigment, the "methylene blue granules" of neuroglia cells, and the Russell bodies of plasma cells. Obviously, the fact that the individual granules in a section stain in the same way by Giemsa's method, or the technique of Borrel, and look alike is no good reason to suppose that they are of the same composition. We suspect that several of our methods of staining depend more upon physical forces than upon the actual chemical constitution of the substances which become colored. So that granules which stain alike may not only differ in composition but may also have been recruited from different sources. This fact makes inferential chemical analysis extremely difficult since it is next to impossible to superpose all our reactions upon a single granule of microscopic size. Consequently, if we apply one microchemical

test to a group of granules, another test to another group, and so on, we cannot be sure that we have been dealing throughout with the same substance or substances. It is our belief that the inclusions which are so abundant in herpetic lesions do not represent a concrete class of granulations *sui generis* but that they are of variable composition and are derived from several sources.

EXPLANATION OF PLATES.

All the drawings were made with Zeiss apochromatic objective 1.5 mm., compensating ocular 6, and camera lucida at a magnification of 1,500 diameters.

PLATE 41.

FIGS. 1 to 10. Nerve cells from the nucleus caudatus of a rabbit (No. 3) injected intracerebrally with 0.2 cc. of 10 per cent H. F. virus which showed typical symptoms 4 days later. The tissue was fixed in Regaud's fluid and stained with fuchsin-methyl green, illustrating intranuclear inclusions.

FIGS. 11 to 19. Cells and isolated nuclei observed in air-dried smears stained by Giemsa's method from the brain of Rabbit 18, which received 0.2 cc. of 10 per cent suspension of the brain of No. 3 intracerebrally and showed characteristic symptoms 4 days later. The cells and some of the nuclei contain numbers of minute inclusions, colored deep blue, which subsequent staining proved to be Gram-negative.

PLATE 42.

FIG. 20. Small capillary with endothelial and adjacent neuroglial cells in the fissura hippocampi of a rabbit (No. 23) injected intracerebrally with 0.2 cc. of a 10 per cent suspension of the brain of No. 18. The tissue was fixed in 10 per cent formalin and treated with Giemsa's stain. This colored many minute inclusions deep blue, some of which were observed free within the lumen.

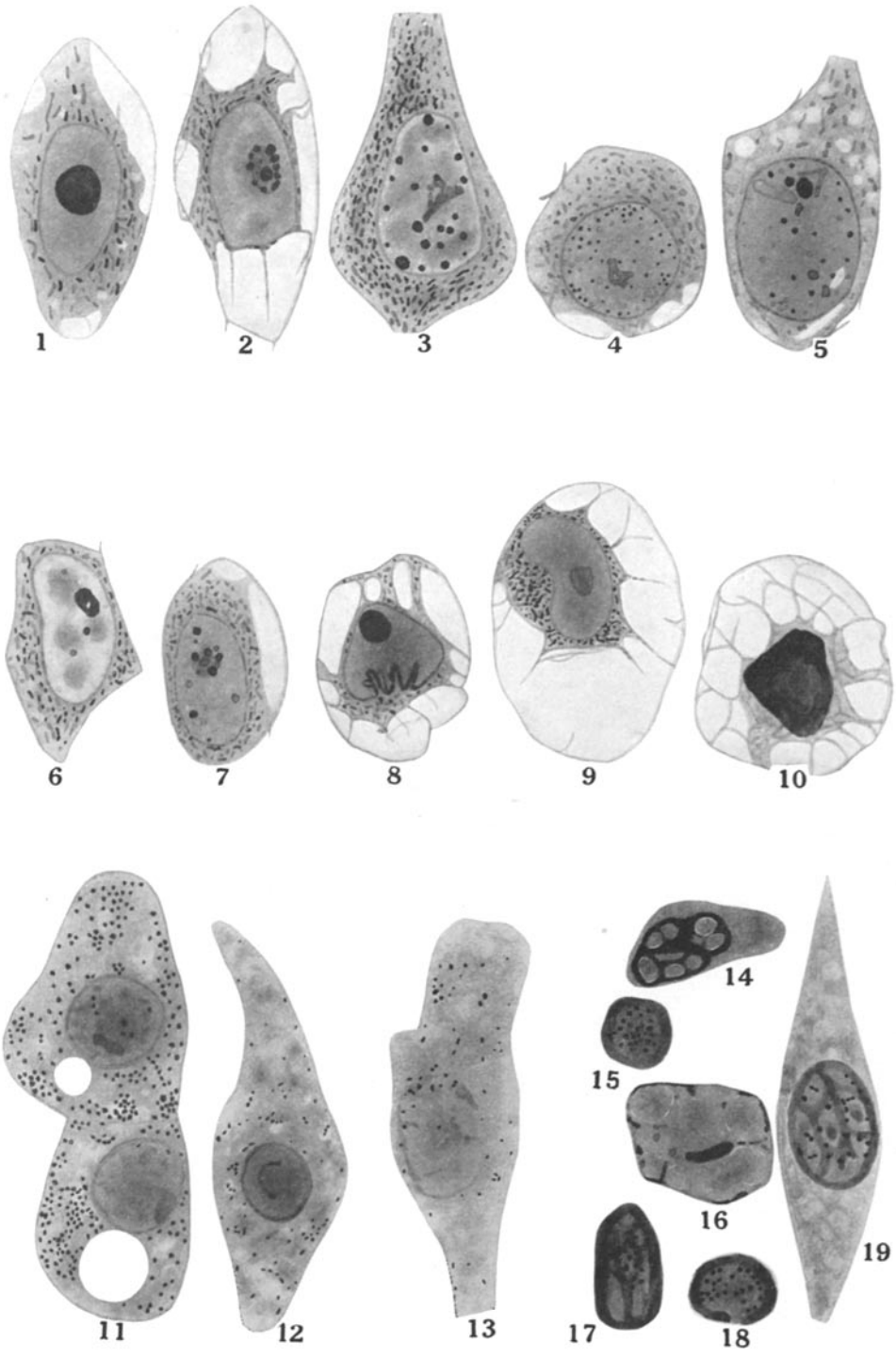
FIG. 21. A clump of phagocytes in the cornea of a rabbit (No. 1) infected by direct implantation with the virus, fixed in Zenker's fluid and colored by Giemsa's method. They contain somewhat similar "minute bodies" colored dark blue and sometimes surrounded by halos.

FIG. 22. Leucocytes and large extracellular bodies in the fascia dentata of Rabbit 3.

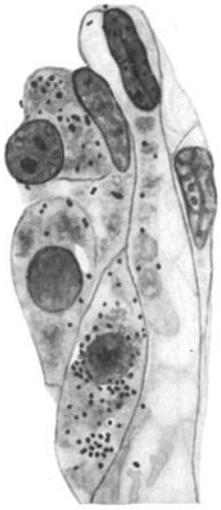
FIGS. 23 to 26. Selected macrophages from the meninges of Rabbit 30 which was injected intracerebrally with the brain suspension of No. 3, fixed in Flemming's fluid and stained by Giemsa's method. They contain a red blood cell and pigment (Fig. 23), leucocytes (Fig. 24), fat droplets (Fig. 25), and amorphous protein (Fig. 26).

FIG. 27. The nuclei of two nerve cells, and two leucocytes, with many inclusions in the stratum radiatum of the hippocampus of Rabbit 3 after Regaud fixation and Giemsa staining.

FIGS. 28 to 31. Selected cortical cells of a rabbit injected intracerebrally with a suspension of normal brain substance by means of exactly the same technique. Tissues taken 7 hours later were fixed in 10 per cent formalin and stained by the method of Giemsa. A variety of inclusions was observed in nerve cells (Fig. 28), in neuroglia (Figs. 29 and 30), and in certain phagocytes (Fig. 31).



(Cowdry and Nicholson: Experimental herpetic infection.)



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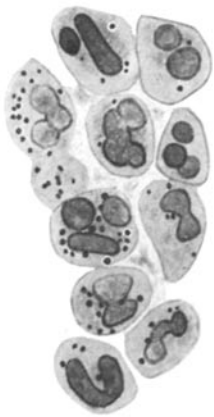

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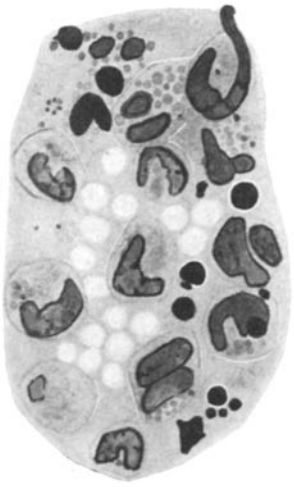

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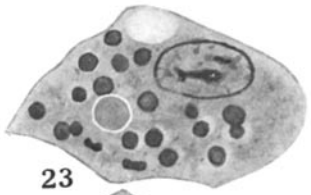

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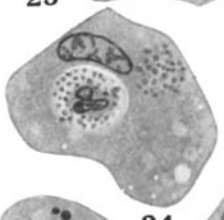

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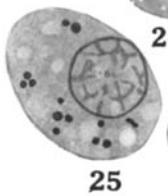

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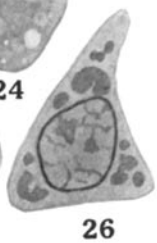

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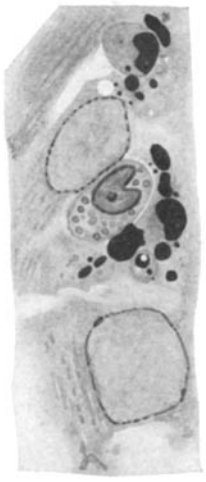

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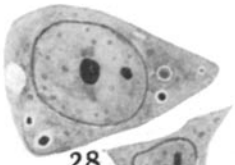

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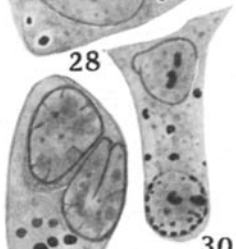

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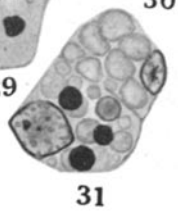

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(Cowdry and Nicholson: Experimental herpetic fnfection.)