THE SUPRAVITAL STAINING OF VACCINE BODIES.

By EDMUND V. COWDRY, Ph.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 58 AND 59.

(Received for publication, June 26, 1922.)

INTRODUCTION.

To determine the nature of the conspicuous cell granulations found in the lesions of vaccinia is a problem in cytology of unusual interest which has continued to hold the attention of investigators.[1] They seem to represent the visible expression of a fundamental cellular reaction. From the historical point of view three lines of thought may be recognized in the development of our present knowledge of the subject, which, in its broader aspects, cannot well be considered apart from other diseases of unknown etiology.

1. On their discovery, the granulations were first named after their observers, Guarnieri's bodies in vaccinia and variola, Negri's bodies in hydrophobia, von Prowazek's bodies in trachoma, Mallory's bodies in scarlet fever, etc. Influenced, perhaps, by contemporaneous studies in malaria and other protozoal diseases, investigators have described complicated life histories based partly upon direct observations and partly upon analogy. The granules were hailed more or less tentatively as the actual parasitic causes of the diseases, were grouped among the protozoa, and were given generic and specific names, of which *Cytoryctes vacciniæ* in vaccinia, and *Neurocytes hydrophobiæ* in hydrophobia will serve as examples.

2. But closer and repeated scrutiny of the life histories did not turn out to be reassuring, and led investigators to question whether a real step had been made in advance. A reaction set in and several papers appeared showing most clearly that in some cases at least the granules are not cell parasites but deposits of pathologic nature. By common consent the protozoal terminology was abandoned and the names of the discoverers reinstated.

3. Faced by this association of more or less specific granulations with certain infectious diseases, the organisms of which have not been seen and remain almost

[1] The early literature is reviewed by Councilman, Magrath, and Brinckerhoff (1904), by Calkins (1904), by Paschen (1911), and by von Prowazek (1912).

wholly unknown, von Prowazek (1907) offered a compromise hypothesis according to which the cytoplasmic granules are of dual nature, consisting of microorganisms embedded in a substance of nucleolar origin produced by the cell in response to their action. Von Prowazek has proposed that these organisms be grouped in a new class which he has called Chlamydozoa—literally, mantle animals—to indicate this habit of being clothed in a mantle of cellular material. In his opinion they are characteristically intracellular parasites and differ from bacteria in their method of multiplication as well as in other respects. He conceived their life history to be somewhat as follows: In the stage of elementary corpuscles they are extracellular and filterable; soon after penetration through the cell membrane they may be detected in the form of tiny initial bodies. These become coated with cellular material, grow, mature, and break down with the liberation of large numbers of the original elementary corpuscles, which, in turn, enter and infect neighboring cells with a repetition of the process.

Lipschütz (1912) has devised the term Strongyloplasmata to indicate organisms endowed with much the same attributes. He groups (1921) under this heading the viruses of molluscum contagiosum, bird-pox, sheep-pox, vaccinia, scarlet fever, verruga peruviana, herpes (zoster, genitalis, and febrilis), varicella, variola, and paravaccinia, and is supported in his contention regarding vaccinia by Gins (1922) and other recent investigators.

It seems almost to have become the fashion to classify organisms about which very little is known in one or the other of these two groups. For example, during the war the ravages of typhus and trench fever demanded very close attention in both camps, which contributed directly to our knowledge of organisms which appear to be intimately concerned in the etiology not only of these two diseases but also of Rocky Mountain spotted fever and perhaps of tsutsugamushi disease, or Japanese river sickness. They have been called *Rickettsia* in honor of Ricketts who lost his life in Mexico in 1910 while investigating their behavior in typhus, but the term is unfortunate because it carries with it the suggestion that the organisms differ from ordinary bacteria and form a group exhibiting certain characteristics in common. Although it has not been possible to prove that during some phase of their life cycle they are of dual nature (consisting in part of cellular substance), they have been referred to the Chlamydozoa by da Rocha-Lima (1916), Jungmann (1919), and others.

Looking through the recent literature one finds the opinion repeatedly expressed that real advances in our knowledge of vaccinia will only be made with the help of new methods of technique.

Steinhardt and his associates (1913, 1914) and Harde (1921) have invoked the methods of tissue culture and have succeeded in obtaining a definite multiplication of the virus *in vitro* but have not been able to concentrate it greatly or to determine its relation to vaccine bodies.

What appears to be a new avenue of approach to the study of infectious diseases of unknown etiology is contributed by MacCallum and Oppenheimer (1922), who so concentrated the virus of vaccinia, through the method of differential centrifugation, that they have been able to observe directly certain granules, which may be the infective agents, under dark-field illumination and with ordinary powers of the microscope. "They occur singly or in small groups, or sometimes in tiny beaded chains. . . . . They stain faintly with Löffler's methylene blue, are gram negative, do not stain with neutral red or trypan blue, stain blue or red with Wright's stain, and take a fairly deep stain with carbolfuchsin." The authors believe that: "these granules are apparently identical with those seen in tissues and smears from vaccinia and smallpox by Prowazek, Paschen, Hallenberger, and others." A similar method is advised by Ségal (1922) for concentrating the virus of typhus fever.

A large supply[2] of tissues from vaccinated rabbits in various stages of the reaction has given me an opportunity to experiment widely in technique and thus to make a cytologic study of the cornea with three definite objects in view; namely, (1) to trace the origin of the material of which the vaccine bodies are constituted, (2) to ascertain whether it is in part of chlamydozoal nature, and (3) to determine how closely it is related to the granules described by MacCallum and Oppenheimer in vaccine lymph.

OBSERVATIONS.

If the edge of a sharp scalpel is passed over a typical corneal lesion and the cells thus obtained are immersed in physiological salt solution and examined unstained under direct illumination, they may be seen to contain a great variety of materials some of which are not visible in control preparations of normal corneas. These may be partly resolved into the following groups.

1. Vaccine bodies, which are easily recognized by their relatively large size, low refractive index, and their tendency to be closely associated with the nuclei. Two of them are illustrated in Fig. 18, which

[2] This material was kindly placed at my disposal by Dr. Noguchi. It consisted of two rabbits 1 day after infection with vaccine prepared by a special method (Noguchi, H., Pure cultivation *in vivo* of vaccine virus free from bacteria, *J. Exp. Med.*, 1915, xxi, 539) by which it is freed from bacterial contamination, two 2 days after infection, three 3 days, two 4 days, one 5 days, three 6 days, one 7 days, one 8 days, one 9 days, and three controls, making nineteen in all.

should be compared with Fig. 1 representing a similar cell from an unvaccinated cornea.

2. Highly refractile spherical droplets which are usually gathered together in clumps and are probably of lipoidal nature.

3. A few scattered, rod-shaped mitochondria which may be definitely identified by the addition of a little Janus green, which colors them specifically when used in sufficient dilution (Fig. 20).

4. Large masses of coccal and bacillary shaped bodies of low refractive index, as represented in Fig. 19. Their occurrence is variable and does not seem to parallel the vaccine reaction. No independent motility could be established. They may be temporary phase granules owing their appearance to some alteration in osmotic conditions, or they may represent an accidental contamination with bacteria.

5. Large, highly refractile droplets of neutral fat which vary considerably in number, resist the solvent action of 1.5 per cent acetic acid drawn under the cover-glass, and are blackened by treatment with osmic acid.

Through the addition of brilliant cresyl blue 2 B[3] to the physiological saline solution in a concentration of about 1:25,000, the vaccine bodies are almost instantly stained a color varying from pink to dark blue (Figs. 3 to 17) and the stages in their formation may easily be studied. Why von Prowazek (1912) failed to obtain a definite color reaction with this dye I am unable to suggest, unless the sample used was of different constitution or employed in a different way.

As a control measure brilliant cresyl blue was applied to testicle lesions with the same result. It was found that the same material will stain a brick-red color with neutral red and that Janus green will color it when sufficiently concentrated. Unfortunately, staining with these dyes is largely a process of selective adsorption, so that it does not convey any information regarding the chemical constitution of the material stained. But it is, perhaps, not without significance, regarding the nature of vaccine bodies, that the dyes are true solutions commonly used for the study of cellular constituents, and that they have achieved no popularity as an aid to the study of microorganisms.

[3] For the manufacture of brilliant cresyl blue 2 B see Schultz (Schultz, G., Farbstofftabellen, Berlin, 1914).

Although they are relatively non-toxic (Hogue, 1922), these methods of supravital staining are open to serious question unless very carefully controlled. Since the cells are slowly dying, not through any toxic action of the stain but on account of traumatism and unfavorable environmental conditions, it is important not to prolong observations more than 30 minutes after the cells are removed from the cornea; certainly the moment that they show evidences of shrinkage, or of the adsorption of fluid with the release of molecular bombardment causing brownian movement, or of coloration of the nuclei, the preparations should be discarded.

The fragmentary appearance of the smaller vaccine bodies precludes any possibility of confusion with the segregation apparatus, originally described by Renaut, which is brilliantly colored by this technique in certain blood and connective tissue cells in which it takes the form of clusters of rounded droplets. Another point of distinction is that in smallpox similar bodies are found within the nuclei. A high development of this apparatus is suggested by Simpson (1922) as a criterion for the identification of monocytes.

With the fact in mind that in some respects the study of fixed tissues is less likely to be misleading because in them all vital activities are simultaneously arrested in some definite physiologic phase, every effort has been made to extend and to correlate both lines of inquiry. Many fixatives,[4] stains,[5] and microchemical methods[6] have been applied to infected and normal cornea and testicles.[7]

[4] Zenker's fluid with and without acetic acid, formalin, Regaud's fluid, Giemsa's sublimate mixture, acetic-sublimate, absolute alcohol, Altmann's fluid, Bensley's acetic-osmic-bichromate mixture, osmic acid in various concentrations, da Fano's modification of Cajal's silver method, etc., were used. In addition, "*Klatsch*" preparations, made as suggested by Ewing, were fixed by heat, by the vapor of formalin and osmium, by absolute alcohol, and in other ways.

[5] The stains used were Borrel's, Giemsa's, Löffler's methylene blue, Mallory's eosin and methylene blue, Wright's stain, iron-hematoxylin, Pappenheim's pyronine and methyl green, carbolfuchsin-acid violet, aniline fuchsin-methyl green, Herxheimer's Scharlach R, etc.

[6] Mayer's muchematein for mucus, the Macallum-Bensley iron reaction as suggested by Nicholson, Millon's reagent, and Schultze's oxidase reaction.

[7] I am also indebted to Dr. Noguchi for allowing me to study his extensive series of stained preparations of vaccinia in rabbits and in calves.

In corneal cells supravitally colored with brilliant cresyl blue, the irregularity in the form and distribution of the material from which the vaccine bodies are built is most noticeable. It varies from the very smallest aggregates, probably even beyond the limits of microscopic visibility, which do not concentrate the dye, and which cannot be resolved with the aid of the best lenses (Fig. 3), to roughly spherical masses upwards of 5 μ in diameter (Figs. 11, 12, and 15). This variability is exhibited by neighboring cells in almost any active stage of the process from 2 to about 9 days after vaccination. This is shown with equal clearness in fixed tissues (Figs. 21 to 45). After search through a number of preparations, or even in a single section, masses of material may be selected which correspond in size and shape to the elementary corpuscles, initial bodies, and reaction bodies described in the literature;[8] but considered all in all, so haphazard is their morphology that it is difficult to satisfy oneself of the existence of a progressive series of morphological changes indicative of independent growth other than a simple increase in size of the aggregates through the accretion of more and more material. It is equally difficult to make out a breaking down of the large masses with a liberation of the original tiny fragments. Fig. 35 may represent a dispersal of elementary corpuscles, or it may represent a stage in the condensation of small bodies into a larger mass, depending upon which way it is interpreted.

That we are in fact dealing with a tremendous development of material which does not owe its ultimate origin to an infective process is shown by the demonstration of traces of a similar substance when the same technique is applied to the unvaccinated cornea. Its substantial similarity, as observed in normal cells supravitally colored with brilliant cresyl blue and in normal cells after fixation in Zenker's fluid (less acetic acid) and staining with Giemsa's stain may be seen by comparing Figs. 2 and 22. There is also a close parallelism between its increase in amount, under the influence of vaccination, as one follows it in supravitally stained cells and in fixed tissues. When colored with a relatively strong solution of Janus green

[8] A close comparison may be drawn with Figs. 2 to 6 in the section on vaccine in von Prowazek's handbook to protozoa.

(1:10,000) the material changes to pink and bleaches with the formation of the color base and leuco base on reduction at the same time as do the other cytoplasmic constituents. After fixation in Altmann's fluid, and other mixtures containing osmium, it is, like the chromidial substance in gland cells, somewhat more diffuse. It would be unsafe to say that it occurs in definite aggregates in normal living cells *in vivo*, for the technique of its demonstration in living cells and in fixed tissues may easily, and probably does, produce artificial changes in the colloidal substratum of the cytoplasm. In other words, the material, as we see it, is the expression of the treatment we accord the cells; its occurrence in small quantities is not dependent upon vaccination.

Owing to their larger size, the vaccine bodies lend themselves more easily to microchemical analysis than does this primordial material, but beyond the statement that they are in part, at least, of protein nature, little may be said with certainty. Their resistance to tryptic and peptic digestion has been noted by von Prowazek (1912). Attempts to secure a definitely positive reaction to Millon's reagent and to the Bensley-Macallum test for iron have been unavailing, but my failure may be due to technical difficulties. Neither do they respond to Schultze's reaction for oxidase granules. They are almost immediately dissolved when a 1.5 per cent solution of acetic acid is drawn under the cover-glass. Since vaccine bodies which are apparently similar develop in the interstitial cells of the testis, and are recorded in the literature in many other locations, it follows that the original material is to be regarded as a fundamental constituent of protoplasm.

The phagocytosis of entire leucocytes and of other corneal cells is a phenomenon of common occurrence (Figs. 16, 39, and 40), but I think that the vaccine bodies do not arise through the digestion of red blood cells,—an old idea which Woodcock (1921) has recently reaffirmed,—because I have not been able to distinguish any traces of hemoglobin coloration in freshly teased corneal cells. The test for masked iron, on which he places so much reliance, can hardly be expected to differentiate between iron-containing compounds of hematogenous and nuclear origin. Moreover, the properties of the bodies, as detailed above, and the occurrence of minute deposits of similar material in unvaccinated corneas are not easily reconciled with this view. The

theory that they are formed through the phagocytosis of leucocytic fragments breaks down in the absence of data regarding consecutive stages in the process. In the normal cornea there is little or no breaking down of leucocytes, and after vaccination the leucocytic increase is somewhat variable and does not closely parallel the development of definitive vaccine bodies. Neither are vaccine bodies more abundant, or larger, near foci of leucocytic destruction.

It seems more likely that the material of which they are composed is endogenous. That it is not related to mitochondria is evident from the application of the Janus green reaction (Fig. 20) and from the general appearance of mitochondria in fixed preparations of the cornea (Fig. 45). Although I have not been successful in obtaining satisfactory slides of reticular material (Golgi apparatus) in the cornea, silver preparations of vaccinated testicles indicate that it does not constitute a source of confusion. In consideration of Ewing's (1904–05) studies with "*Klatsch*" preparations, a nuclear origin is strongly indicated, some investigators going so far as to suggest that in studying vaccine bodies we are dealing wholly or in part with extruded nucleoli. The avidity with which the vaccine bodies take up basic dyes and their topographic association with the nuclei are worthy of comment in this connection. The failure of brilliant cresyl blue to bring to light similar material within the nuclei (Figs. 2 to 17) does not militate against this conception because the dye is not able, in dilute solution and in so short a space of time, to penetrate through the nuclear membranes. At present it seems only safe to conclude that the material may be in part of nuclear origin. It would be very difficult to prove that it is wholly so.

When, under the stimulus of vaccination, the material is deposited in fairly large masses, it shows evidences of heterogeneity in both living cells and in fixed tissues. Supravital staining with brilliant cresyl blue reveals the presence of blue-staining droplets, or granules, within the larger pink-staining masses. The blue droplets vary in size, but only in rare instances are they as large as the pink-staining material (Fig. 17). They are also of less frequent occurrence. In some cases the vaccine bodies contain masses of high refractive index and irregular shape, which do not stain and which appear to be of less fluid consistency. Occasionally these occur free in the cytoplasm.

Fixed preparations also furnish evidence of corresponding diversity in composition. Similar droplets are visible in the vaccine bodies after a variety of fixations, of which Zenker, acetic-sublimate, formalin, and Regaud may be mentioned. They remain uncolored after treatment with the Borrel combination (as suggested by Calkins) Mallory's eosin-methylene blue, carbolfuchsin-acid violet, and other dyes. The chromatophobe, refractile masses, above mentioned, have not been seen after fixation. With Wright's stain after Zenker fixation, a rough gradation may be established between small irregular vaccine bodies which are colored dark blue and certain larger ones in shades of gray which take a pink or a light robin's egg blue color, as represented in Figs. 27 and 36. Sometimes these larger masses are homogeneous, or they may be surrounded with a fringe of dark blue material, as indicated in Fig. 36. Staining with aniline methyl violet, treating with Gram's iodine solution, and differentiating in 95 per cent alcohol discloses a similar heterogeneity, as shown in Fig. 40. Giemsa's stain (Fig. 41) brings to light much the same effect, it being possible to modify at will the color values from dark blue to reddish blue by inserting traces of colophonium in the alcohol of differentiation, as suggested by Wolbach, Todd, and Palfrey (1922) for the demonstration of *Rickettsia*.

From these observations I infer that the material is labile, and that, as the larger vaccine bodies are formed, it undergoes some dissociation or takes up substances from the cytoplasm so that it loses its original optical homogeneity; but this change can hardly be regarded as suggestive of the presence in it of independent microorganisms in the hypothetical stage of initial bodies.

Neither is the physical behavior of the material suggestive of the presence in it of microorganisms. In cells which are still living, it has not been possible to detect evidence of independent motility. The smallest bodies show a tendency to be distributed fairly uniformly throughout the cytoplasm and the larger ones to be congregated near the nuclear membrane. In common with other cytoplasmic materials its form and position are determined by cellular stresses, by electrical charge, and by other physical and chemical forces. For example, in the depth of the lesion, where the cells are often pressed together laterally, the deposits of material fre-

quently assume an elongated shape and become disposed parallel to the long axis of the cell. When the shape of the cell is altered through manipulation, the shape assumed by the material also changes and in some cases it seems to flow upon the surface of the nucleus, as is shown in Figs. 5 and 9, but the movement exhibited is in no sense independent or ameboid. That the material is very fluid in consistency and exhibits but little internal organization requires no further proof. The larger masses are perhaps more fluid than the smaller, more angular fragments, and have rounded up into spheres by virtue of the law of least surfaces.

In this respect also the study of permanent preparations is confirmatory. The habit the material shows of conforming in shape to that of the cell under pressure is illustrated in Fig. 28—its flowing motion in Fig. 30. As in the living cells, so also here the larger masses are spherical, but they show another property in their tendency to lodge in infoldings of the nuclear membrane. Sometimes they appear to press into the nucleus from opposite sides (Fig. 33), while in other cases they are found in deep invaginations of its surface, which may be single or multiple, and which convey the impression, when cut in transverse section, that the vaccine bodies are actually intranuclear, as represented in Figs. 34, 38, and 40. But pictures of this kind are not so easily interpreted. Whether the vaccine bodies actively indent the nuclei by virtue of being of more solid consistency, or whether they flow into depressions produced in the yielding surfaces of the nuclei through shrinkage, it is difficult to say. It seems clear, however, that the indentations are artificial since they are not to be seen in living cells (compare Figs. 3 to 20).

Considerable attention has been devoted in the literature to the areas of clear cytoplasm, or halos, which surround the vaccine bodies (Figs. 23 to 35). Investigators have called to mind approximately similar halos as accompaniments of bacteria in tissues. The fact that they are not visible in the case of vaccine bodies supravitally stained with brilliant cresyl blue is not very helpful, because neither do I find the bacterial halos in insect material. Nevertheless, it can hardly be denied that their appearance is strongly suggestive of shrinkage at interfaces between fluids of different consistency and composition. That their extent is in some measure proportional to the size of the

vaccine bodies will be seen by comparing Figs. 23 and 27 with Fig. 33. The presence of slight halos about the nuclei (Figs. 25 and 26), though probably produced in the same way would hardly be taken to mean that the nuclei behave like independent microorganisms.

In discussing the significance of cell inclusions in scarlet fever, Mallory (1903–04) expressed the opinion that:

> "In order to prove that a series of bodies are stages in the developmental cycle of a protozoon we are dependent on three things, namely, ameboid motion, definite and characteristic morphology of the various bodies found, and the demonstration that the bodies go through a progressive series of changes or developmental cycle resulting in increase in size followed by division and the re-formation of the small bodies from which the series started."

From the foregoing account it is apparent that I have not been able to fulfill any of these conditions.

The problem is complicated by the fact that well formed vaccine bodies have been repeatedly recorded outside the cells. In some of my preparations certain bodies, bearing a resemblance, perhaps entirely superficial, to intracellular vaccine bodies, are to be seen in the fibrous tissue beneath the epithelial layer of the cornea. Whether they have been formed *in loco* or have escaped from degenerating epithelial cells or leucocytes cannot be definitely stated in the absence of specific criteria for their identification. The chlamydozoal hypothesis does not provide for their development apart from the cells. Attention has already been directed to the fact that according to this theory they are said to be of dual nature, consisting of intracellular parasites in the form of initial bodies cloaked in a mantle of material elaborated by the cell. This assertion belongs to the category of statements which are easy to make and exceedingly difficult either to prove or to disprove. If the granules described by MacCallum and Oppenheimer are in fact the infective agents, and, if they maintain throughout their life cycle the morphological and tinctorial properties ascribed to them, then it appears to be unlikely that the virus is contained within the vaccine bodies. But, should the virus be beyond the limits of visibility with the best lenses, then its relation to the vaccine bodies is not easily determined.

While I have not made a cytological study of other diseases attributed to chlamydozoal organisms, a careful review of the literature does indicate, that, as far as the nature of the visible cytoplasmic granulations is concerned, their inclusion in a single category is scarcely warranted. Particularly does this appear to be the case in trachoma (Solovief, 1921).

In comparing the properties of the granules described by MacCallum and Oppenheimer in vaccine lymph with those of typical intracellular vaccine bodies, several points of similarity and of dissimilarity may be noted. (*a*) Like the granules of vaccine lymph the vaccine bodies are not specifically colored with trypan blue, and are colored various shades of blue and red with Wright's stain. They apparently surpass the granules of vaccine lymph in the intensity of their coloration with Löffler's methylene blue and especially with carbolfuchsin. (*b*) Unlike them, they are Gram-positive in the sense that they resist decoloration with alcohol after staining with aniline methyl violet and treatment with Gram's iodine. They are, moreover, intensely stained with neutral red. But still more striking is the difference in morphology. Instead of being characterized by their uniformity, the vaccine bodies are extremely variable. They bear no resemblance to tiny beaded chains of streptococci, as do the granules in vaccine lymph described by MacCallum and Oppenheimer.

To the claim that these reactions are not specific and mean but little one way or the other,—which is freely admitted,—and, further, that one would not expect the granules in vaccine lymph to maintain their original uniformity of morphology after penetration into the cell where they find themselves under entirely different surroundings, it is but natural to advance the observation of the existence of traces of material similar to the vaccine bodies in unvaccinated corneal cells. If the granules which these investigators have described are in truth the infective agents, they cannot be taken to correspond with material seen in uninfected cells, however imperfect our knowledge of its chemical constitution may be.

Since, however, the report of MacCallum and Oppenheimer is of a preliminary nature, many important details being reserved for a subsequent contribution, it seemed desirable to obtain lymph from the

same source and to repeat their work in order to make a more direct comparison with intracellular vaccine bodies.[9]

Bearing in mind the results obtained by MacCallum and Oppenheimer, I took about 5 cc. of this lymph, centrifuged it at high speed for 1 hour, drew off the turbid supernatant fluid,—which in their experiments contained the virus,—and divided it into six portions. Through the addition of varying amounts of glycerol to samples of Locke's solution, solutions were made up to the following specific gravities, determined by hydrometer: 1.11, 1.12, 1.13, 1.14, 1.15, and 1.16. One of these fluids was added to each of the six portions and centrifuged. Although the fresh supernatant fluid was examined in each case, no aggregates suggestive of organisms were found. In attempting to prepare permanent specimens, the relatively large amount of glycerol constituted a considerable handicap. Fixation in formalin vapor and absolute alcohol proved unsuccessful, but after prolonged treatment of smears with the vapor of a slightly warmed 2 per cent solution of osmic acid followed by desiccation, it was possible to color certain granulations intensely blue with Giemsa's stain in the lymph which had been centrifuged in a fluid of 1.14 specific gravity. It will be recalled that MacCallum and Oppenheimer believe the specific gravity of the virus to be about 1.12 or 1.13.

On close examination these granules were seen to be roughly spherical and to vary in diameter from 0.2 to 0.4 μ. Sometimes the larger ones are slightly angular. They occur free or embedded in a loose coagulum which stains a light lilac color. Although they are quite frequently grouped end to end in series, the individual segments are not always of the same size and rarely exceed four or five in number. The same granules are tinted gray, not black, with osmic acid. Judging from the brief description by MacCallum and Oppenheimer their appearance is only remotely suggestive of the more uniform bodies which they found.

[9] I am again indebted to Dr. Noguchi for his kindness in giving me a large supply of vaccine lymph which he obtained from the H. K. Mulford Laboratories through the courtesy of Dr. W. F. Elgin.

CONCLUSIONS.

Vaccine bodies in living corneal cells may be specifically stained by the addition of a small quantity of brilliant cresyl blue 2 B to the physiological salt solution in which they are being observed. Their appearance by this method (Figs. 3 to 17) corresponds with that seen in fixed preparations (Figs. 22 to 42). Both lines of study reveal the existence of traces of similar material in unvaccinated corneal cells. As this increases in amount during the reaction, it behaves like an integral, cytoplasmic constituent of fluid consistency and shows no evidence of being endowed with any measure of independent vitality. The low grade of structural differentiation which it does exhibit, in living cells as well as in fixed tissues, is not suggestive of the presence within it of independent microorganisms. The material differs radically in its morphology and microchemical reactions from the granules observed by MacCallum and Oppenheimer in vaccine lymph.

BIBLIOGRAPHY.

Calkins, G. N., The life history of *Cytoryctes variolæ*, Guarnieri, *J. Med. Research*, 1904, xi, 136.

Councilman, W. T., Magrath, G. B., and Brinckerhoff, W. R., I. The pathological anatomy and histology of variola, *J. Med. Research*, 1904, xi, 12.

Ewing, J., The structure of vaccine bodies in isolated cells, *J. Med. Research*, 1904–05, xiii, 233.

Gins, H. A., Untersuchungen über die für Variola und Vaccine spezifischen Zellveränderungen, *Z. Hyg. u. Infectionskrankh.*, 1922, xcv, 255.

Harde, E. S., Some observations on the virus of vaccinia, in Mémoires publiés à l'occasion du Jubilé de Élie Metchnikoff (16 Mai 1915), Paris, 1921, xii, 107.

Hogue, M. J., A comparison of an amœba, *Vahlkampfia patuxent*, with tissue culture cells, *J. Exp. Zool.*, 1922, xxxv, 1.

Jungmann, P., Das wolhynische Fieber, Berlin, 1919.

Lipschütz, B., Die ätiologische Erforschung der "Einschlusskrankheiten" der Haut, *Zentr. Haut- u. Geschlechtskrankh.*, 1921–22, iii, 3.

MacCallum, W. G., and Oppenheimer, E. H., Differential centrifugalization: A method for the study of filterable viruses, as applied to vaccinia, *J. Am. Med. Assn.*, 1922, lxxviii, 410.

Mallory, F. B., Scarlet fever. Protozoon-like bodies found in four cases, *J. Med. Research*, 1903–04, x, 483.

Paschen, E., Über den Erreger der Variolavaccine. Immunitätsverhältnisse bei Variolavaccine, in Kraus, R., and Levaditi, C., Handbuch der Technik und Methodik der Immunitätsforschung, Jena, 1911, suppl. 1, 465.

von Prowazek, S., Chlamydozoa. I. Zusammenfassende übersicht, *Arch. Protistenk.*, 1907, x, 336.

von Prowazek, S., Vaccine, in von Prowazek, S., Handbuch der pathogenen Protozoen, Leipsic, 1912, i, 122.

von Prowazek, S., and Lipschütz, B., Chlamydozoen. Allgemeines, in von Prowazek, S., Handbuch der pathogenen Protozoen, Leipsic, 1912, i, 119.

da Rocha-Lima, H., Beobachtungen bei Flecktyphusläusen, *Arch. Schiffs- u. Tropen-Hyg.*, 1916, xx, 17.

Ségal, J., The association of the virus of typhus fever with the various blood elements, *Brit. J. Exp. Path.*, 1922, iii, 95.

Simpson, M. E., Vital staining of human and mammalian blood, with special reference to the separation of the monocytes, *Anat. Rec.*, 1922, xxiii, 37.

Solovief, P., Examen histologique de quelques pièces de trachome expérimental du lapin et du magot, *Arch. Inst. Pasteur*, 1921, i, 388.

Steinhardt, E., Israeli, C., and Lambert, R. A., Studies on the cultivation of the virus of vaccinia, *J. Infect. Dis.*, 1913, xiii, 294.

Steinhardt, E., and Lambert, R. A., Studies on the cultivation of the virus of vaccinia. II, *J. Infect. Dis.*, 1914, xiv, 87.

Wolbach, S. B., Todd, J. L., and Palfrey, F. W., The etiology and pathology of typhus, Cambridge, 1922.

Woodcock, H. M., An introduction to the study of hæmatophagy, *J. Roy. Army Med. Corps*, 1921, xxxvii, 418.

EXPLANATION OF PLATES.

PLATE 58.

The figures have been drawn with a Zeiss apochromatic objective of 1.5 mm., compensating ocular 6, and camera lucida at the table level, giving a magnification of 1,500 diameters. All represent living corneal cells in physiological saline solution viewed by direct illumination. Figs. 1, 18, and 19 are unstained cells. Fig. 20 is a cell supravitally stained with Janus green B and the remainder are of cells stained with brilliant cresyl blue 2 B. The corpuscles of Guarnieri, or vaccine bodies, were colored pink and blue, while the nuclei remained quite unstained.

FIG. 1. Living, unvaccinated, and unstained corneal cell in which rod-like mitochondria and other granular material may be distinguished.

FIG. 2. Cell from the same animal, supravitally stained with brilliant cresyl blue, showing traces of the material of which the vaccine bodies are built.

FIG. 3. Cell 4 days after vaccination, supravitally stained in the same way, showing a larger amount of the same material.

FIG. 4. Cell 5 days after vaccination similarly treated. The material shows signs of heterogeneity in the form of clear unstained vacuoles.

FIG. 5. Cell 4 days after vaccination, similarly stained, showing the spread along the surface of the nucleus of a vaccine body, in which it is possible to distinguish materials of different staining reaction.

FIG. 6. Cell from the same cornea, stained in the same way, containing a larger vaccine body, exhibiting both unstained vacuoles and blue-stained granules.

FIG. 7. Cell 2 days after vaccination similarly stained.

FIG. 8. Cell 5 days after vaccination, stained in the same way and containing a heavy deposit of material.

FIG. 9. Cell 2 days after vaccination stained in the same way.

FIG. 10. Cell 1 day after vaccination, containing several small heterogeneous vaccine bodies.

FIG. 11. Cell 5 days after vaccination, with a very heavy deposit of material.

FIG. 12. Cell 5 days after vaccination, with a large solitary vaccine body.

FIG. 13. Cell 2 days after vaccination, containing a solitary vaccine body in which large and small droplets may be distinguished, which took the blue stain.

FIG. 14. Cell 5 days after vaccination, containing several vaccine bodies with droplets (or granules) which took a blue coloration.

FIG. 15. Cell 5 days after vaccination, containing a very large solitary and apparently homogeneous vaccine body.

FIG. 16. One corneal cell phagocytosed by another in a 5 day lesion.

FIG. 17. Cell from the same cornea, containing a vaccine body in two parts staining differently.

FIG. 18. Unstained corneal cell 7 days after vaccination, containing two large and slightly refractile vaccine bodies closely applied to the nuclear membrane. The finer granulations may be resolved into indistinct rod-like mitochondria and a few highly refractile droplets of lipoid-like material.

FIG. 19. Corneal cell from the same lesion, also unstained. It contains a mass of coccal and bacillary bodies which is only very occasionally met with.

FIG. 20. Cell from a 7 day vaccination, supravitally stained with Janus green to demonstrate more sharply the mitochondria. It contains a vaccine body as large as the clear unstained nucleus.

PLATE 59.

These figures have been drawn under the same magnification. All of them represent fixed and stained preparations. At least two factors are concerned in their small size, as compared with Figs. 1 to 20: first, the shrinkage resulting from the technique employed, and second, the tendency of the living cells illustrated in Figs. 1 to 20 to become slightly flattened, thus increasing their apparent size, but not their actual volume.

FIG. 21. Normal corneal cell from a control animal fixed in Zenker's fluid and colored with Giemsa's stain. The irregular deposits of material in the cytoplasm are colored dark blue in the original preparation. On infection this gives rise to typical vaccine bodies.

FIG. 22. Cell about 2 mm. from the lesion in a cornea 1 day after vaccination, fixed in Zenker's fluid without acetic acid and colored with Giemsa's stain. The same irregular material is again colored dark blue.

FIGS. 23 and 24. Cells 5 days after vaccination, fixed and stained in the same way, showing an increase in the material giving rise to vaccine bodies.

FIG. 25. Cell 6 days after vaccination, fixed and stained in the same way, representing very high development of the small bodies.

FIG. 26. Cell 5 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain, illustrating the formation of larger vaccine bodies.

FIG. 27. Cell from a lesion of 6 days, fixed in Zenker's fluid and colored with Wright's stain, containing a large vaccine body stained light blue (robin's egg color), partly surrounded by a mantle of dark blue material, and other vaccine bodies disposed just beneath the cell membrane.

FIG. 28. Cell 4 days after vaccination, fixed in Zenker's fluid and stained with carbolfuchsin and acid violet which shows up the vaccine bodies in crimson against a light green background. It indicates an accommodation in the shape and position of the vaccine bodies to that of the cell.

FIG. 29. Cell from a 4 day lesion, fixed in acetic-sublimate and colored with Giemsa's stain, containing a very deeply colored and homogeneous nucleus.

FIG. 30. Cell from a 5 day lesion, fixed in Zenker's fluid and colored with Giemsa's stain, showing a large vaccine body containing clear droplets of uncolored material, closely applied to the nuclear membrane.

FIG. 31. Cell 1 day after vaccination, fixed in Zenker's fluid without acetic acid and colored with Giemsa's stain, containing a solitary spherical vaccine body.

FIG. 32. Cell from the same preparation possessing a smaller single vaccine body.

FIG. 33. Cell 6 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain, showing the hour-glass-like indentation of the nucleus by the vaccine bodies.

FIG. 34. Cell 6 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain, showing vaccine bodies occupying depressions in the nuclear membrane.

FIG. 35. Cell 2 days after vaccination, fixed in Giemsa's sublimate mixture and colored with Giemsa's stain, showing solitary and accessory vaccine bodies.

FIG. 36. Cell from a lesion of 6 days, fixed in Zenker's fluid and colored with Wright's stain, containing a large vaccine body stained light blue (robin's egg tint), with an irregular envelope of very dark purplish blue material.

FIG. 37. Cell 5 days after vaccination, representing the pocket-like indentation of the nucleus.

FIG. 38. Cell from the same preparation as Fig. 34.

FIG. 39. One corneal cell phagocytosed by another containing well developed vaccine bodies, 5 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain.

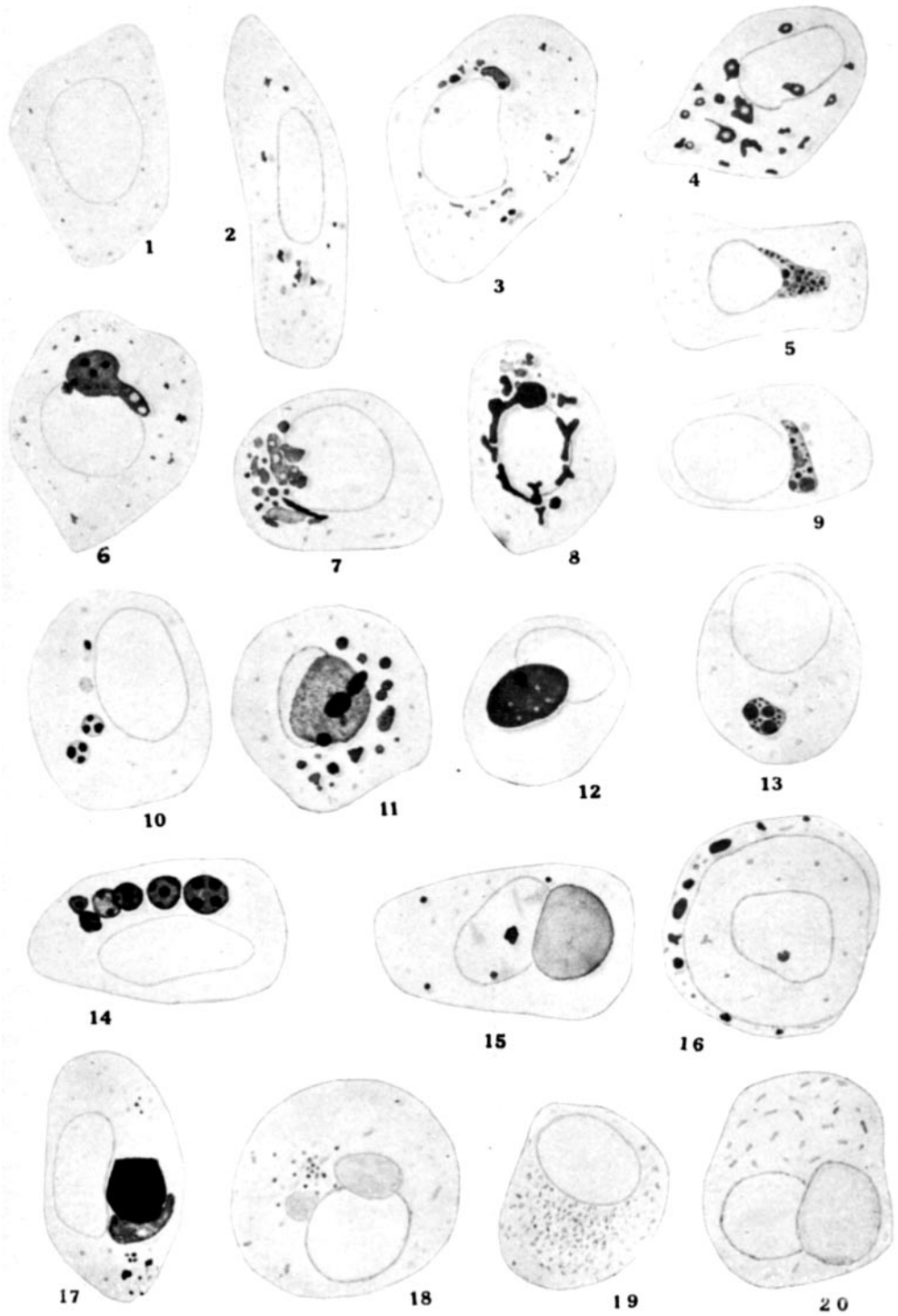
FIG. 40. A similar act in phagocytosis, in which both cells contain vaccine bodies, from a cornea 6 days after vaccination, fixed in Zenker's fluid, stained with methyl violet, treated with Gram's solution, and decolorized in 95 per cent alcohol. The vaccine bodies retain the stain much more strongly than do the nuclei. One of the bodies in the phagocytosed cell is made up of both Gram-positive and Gram-negative material.

FIG. 41. Cell from a cornea 5 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain, containing a large vaccine body, the center of which was colored dark blue and the periphery light blue.

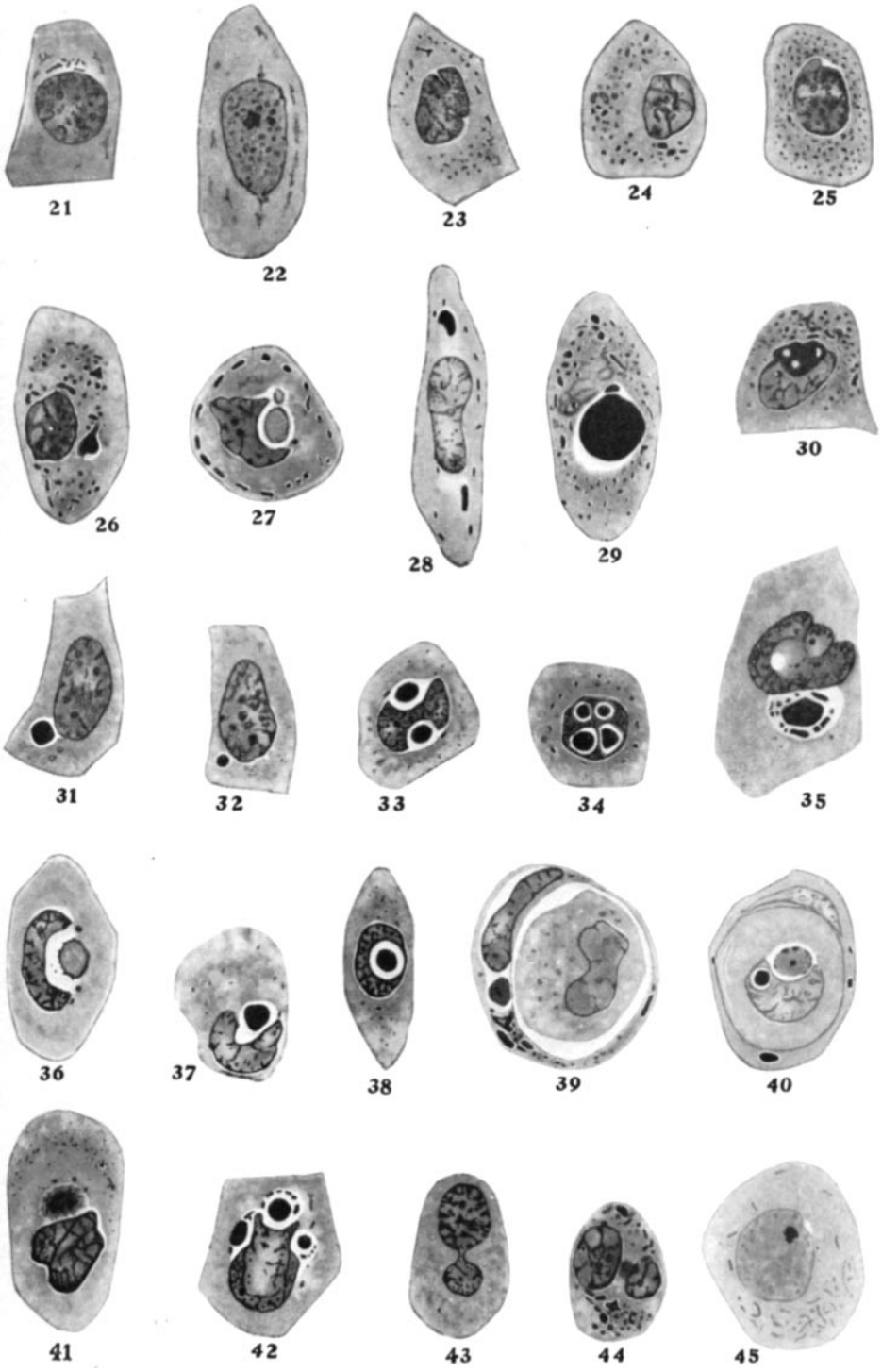
FIG. 42. Cell from the same preparation as Fig. 35, illustrating also nuclear infoldings and halos.

FIGS. 43 and 44. Constricted and binucleated cells from the same specimen as Fig. 41.

FIG. 45. Cell distant about 4 mm. from the lesion in a cornea 3 days after vaccination, fixed in Altmann's fluid and stained with fuchsin and methyl green. The mitochondria are rod-like and filamentous. They stained bright red in sharp contrast to the homogeneous green-colored cytoplasm in which the material destined to form the vaccine bodies is not visible.



(Cowdry: Supravital staining of vaccine bodies.)



(Cowdry: Supravital staining of vaccine bodies.)