MICROKINETOSPHERES AND VP SATELLITES OF PINOCYTIC CELLS OBSERVED IN TISSUE CULTURES OF GEY'S STRAIN HELA WITH PHASE CONTRAST CINEMATOGRAPHIC TECHNIQUES*

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INTRODUCTION

In earlier reports (1, 2) which involved a study of Gey's HeLa strain of human cervical carcinoma (3–11), a variation of pinocytosis (cell drinking (5, 6, 12–22)) was described. This variation was manifested by the appearance of VP cells (variant pinocytic cells) at the periphery of HeLa cultures. This appearance was ascribed to the presence in the medium of human serum nutrients which in some manner altered the pinocytic mechanism as usually displayed by these cells when they are cultured in synthetic (medium Number 1066, Connaught Medical Research Laboratories (23)) and ascitic or pleural fluid nutrients (basic nutrients). In the "usual" form of pinocytosis, (Figs. 1 to 4) the fluid droplets rapidly reduced their size and rounded as they crossed the cytoplasm. In their minute and shrunken forms (1 to 3 μ) their index of refraction was altered so that with phase contrast optics (Bausch & Lomb) they changed from white to black. The droplet then entered the juxtanuclear aggregate of black and refractile granules. Pinocytosis in human serum nutrients varied from this "usual" form in that the fluid droplets of some or all of the pinocytes did not rapidly shrink but did, nevertheless, show a different index of refraction so that their phase display was reversed (white to black). The now black droplets or VP granules rounded and accumulated in the juxtanuclear zone. These resulting black spheres ranged from 4 to 25 μ and produced a cell of unusual appearance ((the VP cell) Figs. 5 to 8) which differed strikingly from the cells in cultures nourished only on the basic nutrients.

While attempting to elucidate this cellular activity, two new cell organoids

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were encountered (24, 25). The first, called the microkinetosphere, was originally recognized in the VP cells, but later observed in other pinocytically active cells as well. With time-lapse cinematographic techniques the active portions of these cells were repeatedly observed. It was soon apparent that small black granules (microkinetospheres) (Bausch & Lomb phase) which moved quite rapidly were always present in the cytoplasm where pinocytosis was active. Later, a certain affinity between the fluid droplets that were entering the pinocyte and the microkinetospheres was recognized. The second distinct organoid, which was observed only in the VP cells, appeared occasionally next to or on the surface of the droplets (VP granules) as a smaller black granule (Bausch & Lomb phase). If one VP granule in a cell showed this accessory granule so did most of the others in that cell. Similarly, if one VP cell of a culture showed this accessory body so did most of the other VP cells. Because the time-lapse cinematographic records revealed the close association of these two bodies, the name VP satellite was given this accessory granule. Originally, these VP satellites were observed as proportionately smaller than the VP granule and generally one-fourth to one third as large.

Because the microkinetospheres were apparently attracted to the incoming fluid droplets of pinocytosis and because the VP satellites were intimately related to the VP granules (a variant of fluid droplets), their relationship to each other was placed in question. The microkinetospheres were less than 1 μ in diameter and the VP satellites ranged up to 8 μ. This great discrepancy in size plus the fact that VP satellites had not always been a constant finding in VP cells disfavored a possible relationship. Further observations showed, however, that the microkinetospheres did become the VP satellites by a transformation described herein. It was also found that the VP satellites were present in all VP cells and had gone unrecognized because they were frequently minute and did not conform to the earlier concept of size.

Materials and Methods

Stock cultures of strain HeLa were maintained in roller tubes at incubator temperatures (37 $\pm$ 1.5° C.) in 2.5 to 3.0 cc. of the basic nutrient. This basic nutrient was composed of a 50-50 mixture of an ascitic or pleural fluid and medium Number 1066. This nutrient also contained 10-day chick embryo extract (10 per cent), phenol red (0.0015 per cent), penicillin G (1,000 units/cc.) and occasionally human serum (5 per cent). HeLa cells were cultivated in colonies on the glass walls of the tubes and harvested by gently scraping them into the nutrient with a long spatula. The cultures were transferred to multipurpose culture chambers (26, 27) by a technique described earlier (24). HeLa cells cultivated by this method were available for phase contrast observations without special preparation. When the cultures had attained a diameter of 4 to 5 mm., the basic nutrient was withdrawn and replaced by 100 per cent serum nutrients which produced the VP cells.

Weak Janus green solutions (1:10,000) were prepared in Earle's balanced salt solution and slowly injected into the culture chambers containing either the basic or serum (100 per cent) nutrients. Neutral red solutions (0.05 per cent) were also prepared in Earle's balanced salt

solution and injected into chambers which had had their nutrient removed. After 1 minute the neutral red solution was removed and the nutrient replaced.

RESULTS

Microkinetospheres (MKS)—These tiny cytoplasmic bodies (Figs. 1 to 14) appeared to be a most active component of pinocytic cells. As originally observed, they were spherical to oval in shape and measured 0.3 to 0.6 μ. These facts contributed to the derivation of their name. With phase contrast optics (Bausch & Lomb) they appeared black. Under brightfield illumination they were yellow green as were the VP granules, but at other focal levels appeared blue green. After vital staining with Janus green the microkinetospheres did not show a color change as did the filamentous mitochondria. Stained mitochondria appeared a blue green with the brightfield optics and, as observed with phase contrast, they were swollen and less active. The microkinetosphere activity did not appear to be suppressed by Janus green staining. Their refractive and reflective qualities made the staining reaction with neutral red equivocal.

Functionally they demonstrated their peculiar role in the pinocytic process. They seemed to be attracted to the incoming fluid droplets and to make contact with them. Microkinetosphere-fluid-droplet combinations occurred from the peripheral and turbulent zone of membrane ruffling to the more centrally located nucleus. Microkinetospheres of cells bathed in serum nutrients adhered and remained attached to the fluid droplets as they entered the cell. In basic nutrients the microkinetosphere contacts with fluid droplets were of short duration (few minutes only), after which the microkinetosphere moved on to contact other droplets. Although no visible means of locomotion was detected, the microkinetospheres in their apparent attempt to contact the incoming fluid droplets were often observed to move in a direction opposite to the motion of the entering droplets. Fluid droplets of pinocytes in basic nutrients apparently responded to the microkinetosphere contacts by (1) shrinking, (2) rounding, and (3) shifting their index of refraction as indicated by the change in phase (white to black). Fluid droplets of pinocytes in serum nutrients responded only by the rounding and phase shifting to black. In the former situation, as mentioned above, the fluid droplets disappeared into the juxtanuclear aggregate, and in the latter this phase shift produced the large VP granules. With brightfield optics the entering fluid droplets of the cells of both pinocytic types were clear. When neutral red was added, these droplets remained clear but as they advanced across the cytoplasm they assumed a red hue which closely coincided with the change in refractile properties and with the phase reversal.

VP Satellites—These accessory bodies were seen only in the VP cells. They occurred as bodies compressed against the VP granules (Figs. 5 to 16), appeared black with phase contrast optics (Bausch & Lomb), and stained vitally with neutral red. With Janus green both the VP granule and the VP satellite stained a

blue green. They were first visualized only in some of the cells cultivated in serum nutrients.

In reviewing many forming VP cells at high magnification (43× and 97× objectives), it became evident that although the VP satellites were not noted when the VP cells were first observed, they were perhaps there. Actually, some satellites were flatter than others and assumed larger sizes. Others were less adherent to the related VP granule and were interpreted initially as only an adjacent small granule. It was this situation which led to the former belief that only some VP cell cultures contained VP satellites. The fact was, however, that all VP granules had these accessory bodies and they were simply more obvious in some cultures than in others. VP satellites which appeared conspicuously with the 10× and 21× objectives were large and have been termed macrosatellites. Those, that were noticeable only through the higher power objectives (43× and 97×), were termed microsatellites. Generally speaking, VP cells of a culture possessed one size-type of satellite. The macrosatellites measured 1 to 8 μ and were proportionately smaller than the corresponding VP granule by about one-third to one-fourth. The microsatellites measured 0.5 to 1.0 μ and were only slightly larger than the microkinetospheres.

VP cells which contained macrosatellites and which were actively engaged in pinocytosis were selected for time-lapse studies, and although the satellites had been observed to develop during periodic visual observations the true mechanism was not revealed until the films were viewed. Fluid droplets entered the cells and were contacted by the microkinetospheres. This contact was a permanent union. As the phase reversal of the contacted fluid droplet progressed, additional microkinetospheres moved about the surface of the droplet. (There may have been some shrinkage of the droplet during the intermediate graying phase, but, after the droplet had become the intensely black VP granule, there was a long period (several hours to a day or more) during which it retained without too much variation a relatively large size). The number of microkinetospheres revolving around one droplet varied. Later two microkinetospheres cohered and eventually coalesced (Figs. 9 to 16). This happened several times until the satellite was fully developed by this process, which required approximately 1 to 4 hours. As the VP granule blackened, the satellite formation and orbital motion diminished. Eventually, the fully formed satellite appeared firmly anchored to the surface of the VP granule.

Observations recorded here were typical for the conditions used. Usually after one or two microkinetospheres had touched it, the pinocytic vacuole of a cell in the basic nutrient contracted and changed its refractive index. It was assumed that the contact with the microkinetosphere played a role in this process because uncontacted vacuoles remained unchanged. Some vacuoles seemed, however, to be refractory because they failed to contract after numerous contacts.

DISCUSSION

The question of whether the action of the microkinetospheres contributed to the constituents of the fluid droplets or removed one or more of its components cannot be decided on the basis of these observations. From the data collected, one or the other and possibly both occurred inasmuch as their relationship appeared to be a constant one and certain definite changes followed their union. It was noted, for example, that fluid droplets commence to become red in neutral red solutions after the microkinetospheres have made contacts with them. The microkinetospheres transform into VP satellites which, in turn, attach to the VP granules. The microkinetospheres do not stain vitally with Janus green as do the filamentous mitochondria and were, therefore, not considered a part of the chondriome. However, the VP satellites and the VP granules both stain with Janus green, and since the satellites are aggregates of microkinetospheres, a chondriomal relationship may truly exist.

Gey (6, 21) has pointed out a definite relationship between the fluid droplets of pinocytosis and the filamentous mitochondria. It was not the intent of this report to refute his observations but merely to illustrate another relationship between the fluid droplets and a component of the cytoplasm, the microkinetospheres. Although mitochondria are often abundant in the pinocytic areas screened, they are not of constant occurrence. Indeed areas of pinocytosis have been observed which were devoid of mitochondria, but such areas were never devoid of microkinetospheres. Mitochondrial contacts with the fluid droplets appeared to be either accidental (Gey showed droplets tearing through mitochondria) or of a very short duration and have never been seen to unite with the fluid droplets, as in the formation of the VP granule-satellite complex.

The idea was advanced earlier (24) that the microkinetospheres may be a cellular source of enzymes and, as such, carry these enzymes to the fluid droplets. This may account for the droplets displaying neutral red coloring after the microkinetosphere contacts. That droplets in VP cells contain serum components and human sera contain enzyme inhibitors (28) may be factors influential in the formation of the VP granules. It might be assumed that the enzyme(s) delivered to the serum fluid droplets of the VP cell by the microkinetospheres are neutralized by the serum enzyme inhibitor(s) also in the fluid droplets. The droplet is, therefore, not digested until sometime later and consequently does not shrink but, because of the enzymatic addition, it does change its index of refraction, as indicated by the phase reversal. Presumably the enzyme is continually carried to the droplet as more microkinetospheres become involved and the neutral red coloring and phase reversal become stronger. The differences in the size of the satellites among the cultures stimulated by various sera could then have been directly proportional to the enzyme inhibiting power of the serum. In other words, more microkinetospheres might be required to neutralize the serum droplet when it contains greater

amounts of enzyme inhibitor, and the satellites of this situation might be larger and proportionate in size to their parent VP droplets. By this same reasoning, after the inhibitor has been neutralized, the digestive function proceeds and the VP granule dissolves slowly (1 to 3 days) as revealed by the time-lapse movies.

Only recently Novikoff *et al.* (29, 30) have described "dense bodies" in liver fractions and in parenchymal sections of rat liver observed with the electron microscope. These "dense bodies" measured 0.25 to 0.50 μ and were rich in acid phosphatase and the other enzymes of de Duve' "lysosome fraction" (31, 32). Similar structures were described as "microbodies" by Rouiller and Bernard (33) and as "peribiliary bodies" by Palade and Siekevitz (34), Watson and Siekevitz (35), and Palade (36). Straus (37) isolated droplets from rat kidneys after intraperitoneal injection with egg white. These were characterized by their high acid phosphatase activity and stained supravitally with neutral red and Janus green. Later, Straus (38) isolated droplets from normal rat kidneys which contained the same enzymatic spectrum as de Duve's "lysosomes" and Novikoff's "dense bodies." He presented three fractions of droplets which ranged from 1 to 5 μ (fraction I) to 0.1 to 1.0 μ (fraction III). The enzyme activity of these granules was 10 to 15 times higher than the mitochondrial fraction. Chicken monocytes in tissue culture were observed by Weiss and Fawcett (39) to show acid phosphatase cytochemically in an area comparable to the Golgi zone only after their transformation to macrophages. This appeared to be related to their enhanced phagocytic activity. HeLa cells were examined for their acid phosphatase content by Green and Verney (40), using the methods of Rutenberg and Seligman (41). They found the cytoplasm of young HeLa cells to be diffusely sprinkled with discrete coarse and fine granules which resembled in size and distribution those bodies which normally stained with Janus green B supravitally. This short review of work with the electron microscope, and biochemical and cytochemical techniques, seems to relate the structures known as "lysosomes," "dense bodies," "microbodies," Straus's droplets, "peribiliary bodies," the acid phosphatase positive areas of macrophages, and HeLa cells to the microkinetospheres and VP satellites of HeLa cells presented herein. This relationship, though not proven, does support the hypothetical reasoning on VP satellite size. For example, the size of the "dense bodies" (0.25 to 0.50 μ) very closely approaches the size of the microkinetospheres (0.3 to 0.6 μ), and fraction I (1 to 5 μ) of Straus's droplets closely approaches the size of the VP satellites (1 to 8 μ).

The immediate importance of the microkinetospheres seems to lie in the fact that they are integral components of the cell, specifically involved in the entrance of the fluid environment into the cell (by pinocytosis). Their role in this activity is yet to be defined, but should prove of considerable interest.

SUMMARY

By tissue culture methods and with the use of phase contrast, interference color contrast, and time-lapse cinematographic equipment, the activity of a cytoplasmic organoid, termed the microkinetosphere, has been followed and correlated with pinocytosis. A transformation of several microkinetospheres by coherence and coalescence into the solitary VP satellite was observed in cells undergoing pinocytosis in serum nutrients. A correlation of both of these structures to cytoplasmic organoids described by others, notably with the electron microscope, and a hypothesis on the nature of the microkinetosphere and its transformation was presented.

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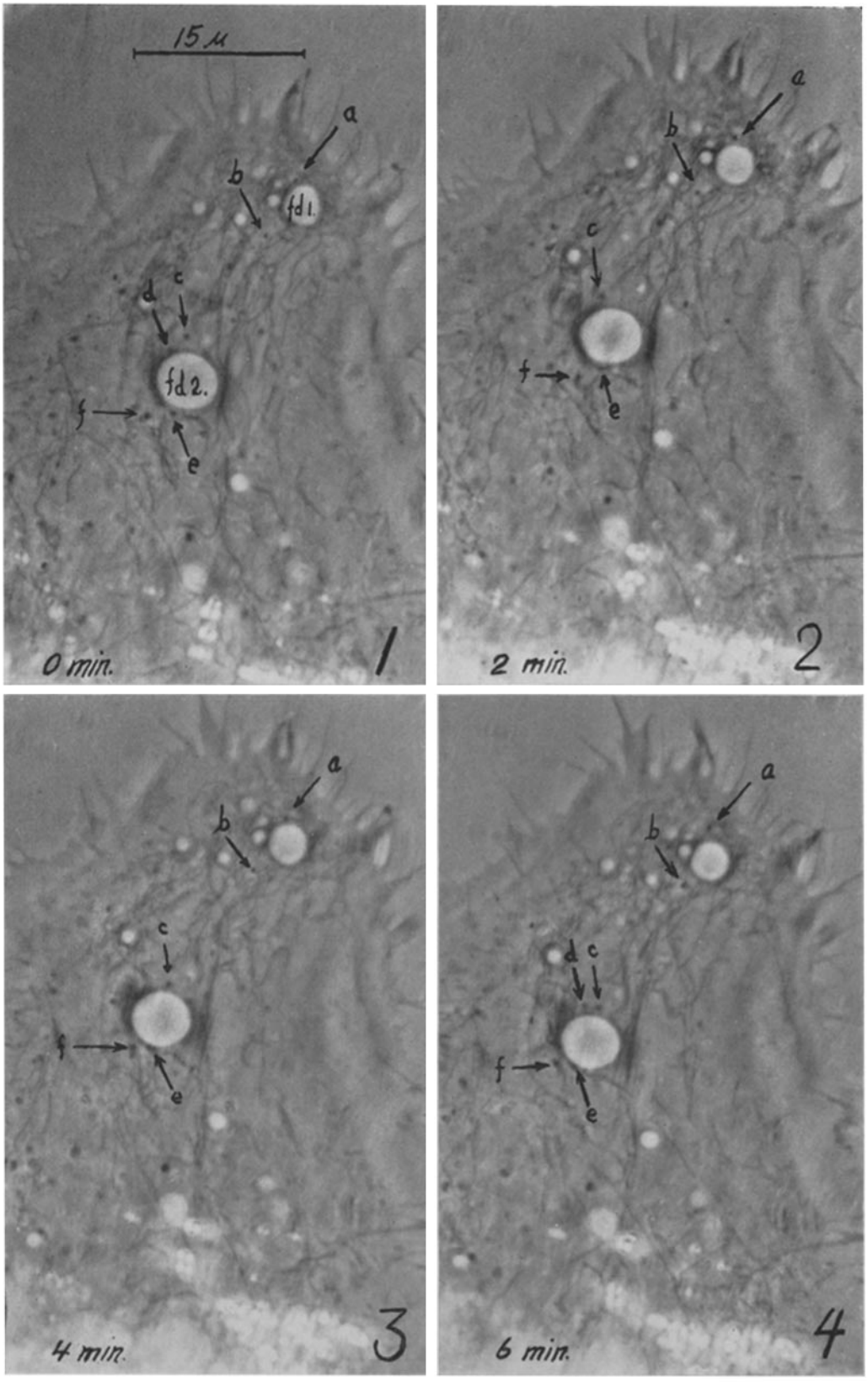
EXPLANATION OF PLATES

PLATE 223

FIGS. 1 through 4. Margin of pinocytic cell of strain HeLa photographed with phase contrast objectives (Bausch & Lomb). The interval from time zero for each photograph is indicated in their lower left hand corners and the magnification scale is shown in Fig. 1. Microkinetospheres (MKS) that made contacts with two entering fluid droplets (*fd 1* and *fd 2*) are indicated by arrows and the letters *a* through *f*. Note MKS_a contacted *fd 1* in Fig. 1, moved away from *fd 1* in Fig. 2, returned to *fd 1* in Fig. 3, and again moved away in Fig. 4. Note that MKS_b progressively moved toward *fd 1* in all four figures. MKS_c moved toward and MKS_d moved away from *fd 2* in all four figures. MKS_e and MKS_f moved toward *fd 2* in all four figures. Other microkinetospheres and large numbers of filamentous mitochondria are also evident in the photographs. The highly refractile area at the bottom of each photograph is referred to in the text as the juxtanuclear aggregate. × 1800.



(Rose: Microkinetospheres of pinocytic cells)

PLATE 224

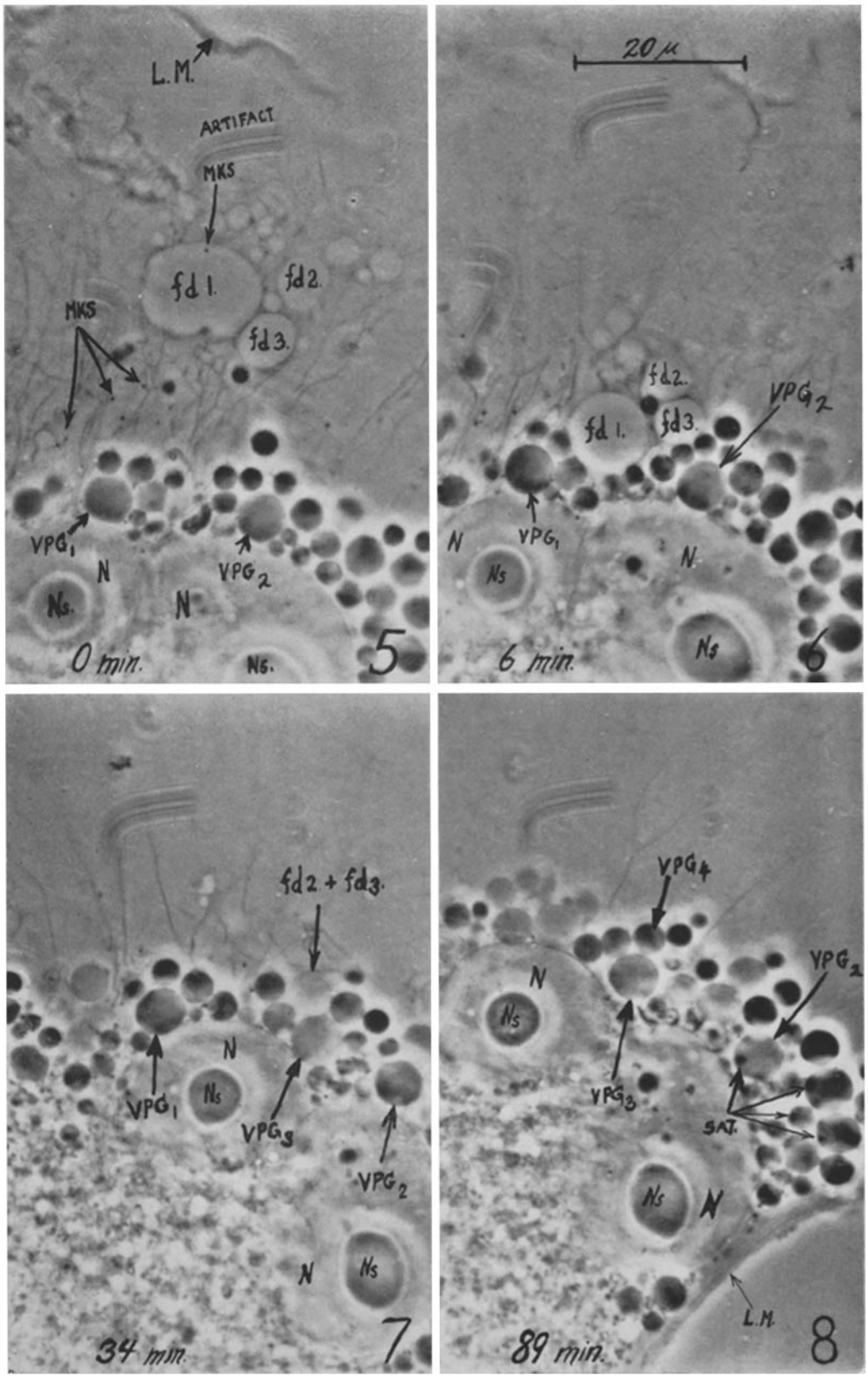
FIGS. 5 through 8. Portion of variant pinocytic cell (VP cell) of strain HeLa in which is displayed the transformation of fluid droplets to VP granules (phase contrast, Bausch & Lomb). The interval from time zero for each photograph is indicated in the left hand corners and the magnification scale for each photograph is shown in Fig. 6. × 1350.

FIG. 5. This photograph shows an active portion of the cytoplasm in which several fluid droplets have been ingested. Three large ones are labelled *fd 1*, *fd 2*, and *fd 3*. Microkinetospheres (*MKS*), filamentous mitochondria, and VP granules (*VPG*) are shown in the photograph. Other symbols mark the nucleus (*N*), nucleolus (*Ns*), and cell limiting membrane (*LM*).

FIG. 6. Note that the fluid droplets *fd 1*, *fd 2*, and *fd 3* have moved into the mass of VP granules between VPG_1 and VPG_2 and that they have shrunken and rounded.

FIG. 7. Note here that *fd 1* shows a reversed phase display, appears black, and now is represented VPG_3. *Fd 2* and *fd 3* have coalesced and appear as one round droplet (*fd 2* + *fd 3*).

FIG. 8. VPG_4 now appears as the replacing element for the coalescing droplets *fd 2* and *fd 3*. VPG_2 shows a very prominent satellite (*SAT*), as do several of the other VP granules in the lower right hand field. Note that there is no shrinkage of VPG_2 in any of the photographs.



(Rose: Microkinetospheres of pinocytic cells)

PLATE 225

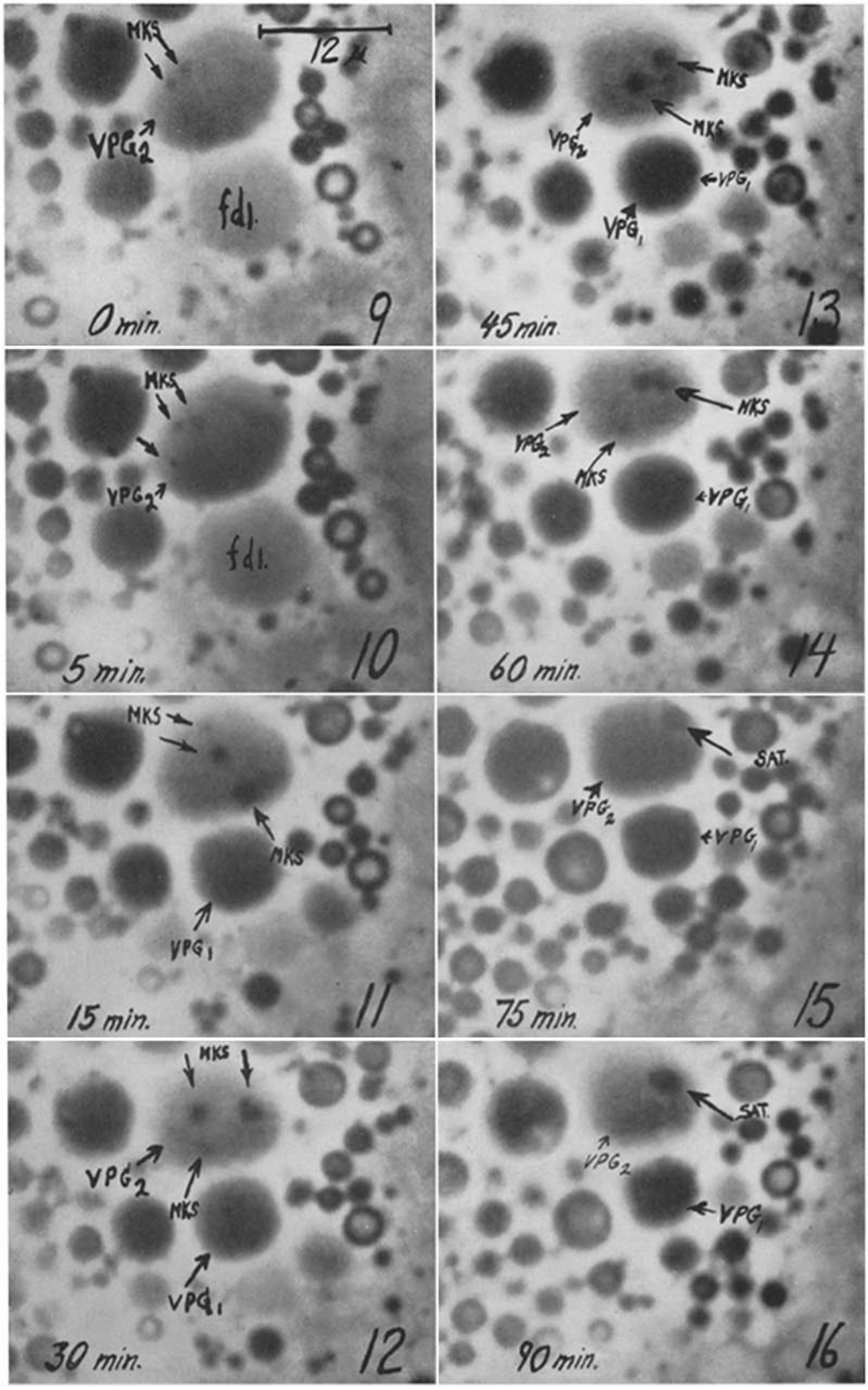
FIGS. 9 through 16. These phase contrast photographs were prepared from time-lapse 16 mm. motion picture film and demonstrate the transformation of several microkinetospheres (*MKS*) into a solitary VP satellite on one VP granule. The time intervals are given in the lower left hand corner of each photograph and the magnification scale is shown in Fig. 9.

FIGS. 9 through 11. Fluid droplet *fd 1* is shown undergoing a reversal of its phase display to become VPG_1. VPG_2 shows a significant transformation. Note that there are two microkinetospheres (*MKS*) shown on VPG_2 in Fig. 9, three in Fig. 10, and that the three in Fig. 11 are considerably enlarged.

FIGS. 12 through 14. Microkinetospheres (*MKS*), that were whirling about the surface of VPG_2, show progressive enlargement and in Fig. 14 a coalescence of two of the larger bodies is apparent.

FIGS. 15 through 16. The final fusion of the microkinetospheres (*MKS*) has occurred and the resulting solitary VP satellite (*SAT*) now shows less motion about the surface of the VP granule. Note that VPG_1 has undergone very little shrinkage since its phase display reversal.

These photographs represent only isolated events and many more coalescing phenomena were observed than are described here. For instance, the three *MKS* bodies of Fig. 10 are not necessarily the same three evident in Fig. 11. The lower *MKS* in Fig. 11 is probably a product of the coalescence of three or four smaller microkinetospheres. × 1750.



(Rose: Microkinetospheres of pinocytic cells)