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A CINEMATOGRAPHIC STUDY OF THE PENETRATION OF CULTURED CELLS BY *TOXOPLASMA GONDII**

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INTRODUCTION

The cell penetration of *Toxoplasma gondii*, which has not been fully clarified, will only be solved conclusively by applying cinematography. In 3 preceeding cinematographic works, PULVERTAFT et al. (1954) and LUND et al. (1961) observed the propagation of toxoplasma inside cells. SCHMIDT-HOENSDORF & HOLZ (1953) reported on toxoplasmic penetration, in which penetration was complete within 30~60 minutes.

Active penetration of toxoplasma into cells was firstly reported by GÜIMARAES & MEYER (1942). PULVERTAFT et al. (1954) suggested two different ways in which the parasites gain entrance into host cells, active invasion or passive ingestion. Actively penetrating parasites did not multiply but were lethal to the cell; whereas the ingested ones were the proliferating forms.

In order to clarify the mode of the penetration of toxoplasma into cultured cells, a cinematographic work was done by the authors by applying phase contrast microscopy. Detail of the penetration which was particularly characterized by the perforating form of toxoplama is described in this paper.

MATERIALS AND METHODS

Toxoplasma gondii strain RH, originally isolated from a child by SABIN, was provided by Y. TSUNEMATSU, Institute for Infectious Diseases, University of Tokyo, and has been maintained in this department by mouse passage. In this study, 1770th to 1885th passages were used. The parasites were inoculated into mice and the peritoneal fluid of the infected mice were harvested on the 3rd day after inoculation. The fluid, containing numerous toxoplasma, was washed four times with the tissue culture medium described below, which was previously cooled. The number of toxoplasms was counted and adjusted by using hemocytometer to determine the multiplicity of infection.

PS cells (derived from pig kidney), supplied by M. ENDO, Institute for Infectious Diseases,

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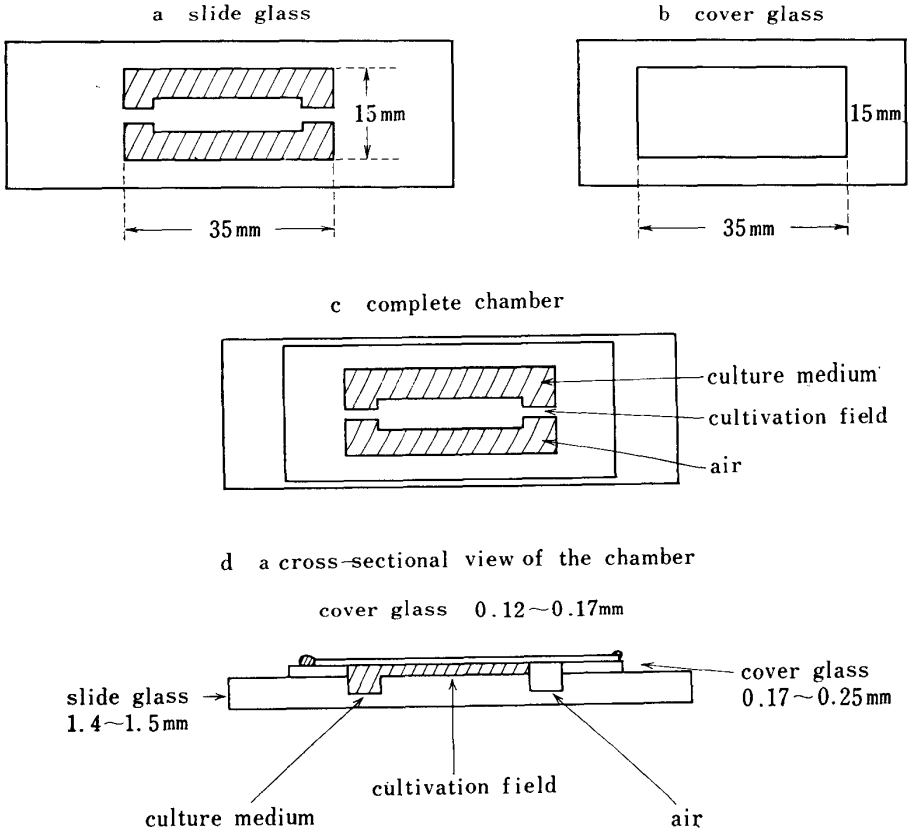
University of Tokyo, were cultivated at 37°C in a medium containing 10 per cent calf serum in EARLES solution with 0.5 per cent Lactalbumin hydrolysate (Difco) and 0.1 per cent Yeast extract (Difco). The calf serum showed no detectable cytoplasm-modifying antibody. pH of the medium was adjusted to 7.5 with a normal solution of bicarbonate. For the final concentration, 100 units of penicillin and 100 μg of streptomycin per ml was added to the medium.

Cultivation of PS cells was done in a specially devised chamber. The chamber was made from an ordinary microscopic slide (1.4~1.5 mm in thickness) and the cover slip. Both were partially dissolved by treating with hydrogen fluoride as shown in figures 1-a and 1-b. They were glued together with 0.5 per cent formval so as to make the chamber shown in figures 1-c and 1-d (a cross-sectional view).

FIGURE 1 *Procedure for preparing the cultivation chamber*



About 0.5 ml of the cells suspended in the culture medium was poured and spread on the cultivation field of the chamber. Without putting on a cover slip, the chamber was placed in a petri dish made air-tight by keeping the dish in a vinyl bag, and incubated at 37°C. Settled cells were found within 24 hours and could be maintained in good condition for 48 hours without changing the medium.

Twenty-four hours after the cultivation, when the cells were already settled, the medium was sucked up with a capillar pipette. The washed toxoplasms, suspended in 0.5 ml of the culture medium, were inoculated by pouring onto the culvation field of the chamber. The

number of toxoplasms was adjusted approximately to the proportion of one toxoplasma per cell. Finally, the chamber was covered with a cover slip as shown in figure 1-d, and sealed with vaseline mixed with equal volume of paraffin. Care was taken to keep the medium containing toxoplasms from overflowing into the space for air (fig. 1-d). Immediately after the inoculation and closure was completed, the chamber was placed in an incubator apparatus which maintained at 37°C temperature when the chamber was placed under the microscope.

A Beulieu microcinematocamera was set on Nikon phase contrast microscope magnification was ×400 (Nikon 40× objective in combination with Nikon p 10× ocular). Photographs were taken using a microscope lamp of 6.2 v, 5 amp., Fuji RP x-negative, and 16 mm film. Illustrations were made from the positive prints. A glass filter was used to avoid unnecessary heat from the light source.

RESULTS

1 Preliminary experiments

Investigation was initiated by preliminary experiments which might have influenced the toxoplasma infection. The experiments were done in a small glass bottle (3×5.5 cm) for tissue culture work. Intracellular parasites were observed 7 minutes after inoculation. The movement of the parasites toward the cells appeared to be more dependent upon gravity and other factors than upon parasitic motility. The time required for penetration was influenced by the thickness of toxoplasma medium which was overlayed onto the cultured cells. From the results of further preliminary works, the conditions for the main experiment were determined, which were summarized as follows.

a) Multiplicity of infectoin……5 toxoplasms per one cell,

b) Serum concentration of the culture medium……10%,

c) pH of the culture medium optimum for cell growth and for the invasion of toxoplasma into cells……7.5,

d) Elimination of toxoplasma-inhibitory effect of mouse ascitic fluid……This effect was paticularly shown by the mouse fluid obtained on the 4th and 5th days after infection. To avoid this inhibitory effect, ascitic fluid was obtained from the infected mice on the 3rd day after infection and toxoplasms in the ascites were washed 4 times with the culture medium before use.

e) Examination of the possibility of toxoplasma invasion by ingestion……For this purpose formalin killed toxoplasms were prepared. Penetration or ingestion of killed parasites into cells was never observed.

2 Main experiments

Twenty cases of the penetration of toxoplasma were filmed. It took about 5 minutes before the inoculated and sealed chamber could be photographed. This period of time was necessary for the selection of a suitable field for microscopy. Most films of the toxoplasmic penetration could be taken 15 to 30 minutes after the onset of the experiment.

a) Pattern of the movement of toxoplasms outside cells in the culture medium

Before coming into contact with cells toxoplasms usually showed various patterns of movement. The movement was characterized mostly by forward and backward movement,

somersaulting, and rotation along the long axis. Individual parasites did not show one particular type of movement but a combination of the above mentioned patterns was shown. The movement was usually vigorous the first 30 minutes but gradually became weak and usually stopped approximately 3 hours after the onset of the experiment.

b) Contact of toxoplasms to the cells

Toxoplasms came into contact with the membrane of the cells with either their acute or obtuse end. When attached to the cells, toxoplasms showed the following three types of movement, (1) turning the free end making a circular cone attaching one end to the cell, (2) rotate along the long axis. Sometimes, this movement was in combination with type (1) movement, (3) leave from and attach to the cell membrane repeatedly exhibiting forward and backward movement within a very small distance. The movement was vigorous in some parasites but not in others. Movement was of inconstant velocity in individual parasites. Some parasites stopped completely as time elapsed. They came alongside cell membrane and tended to become rounded themselves. These toxoplasms never penetrated into the cell.

c) Penetration of toxoplasms into cells

Free and attached toxoplasms could equally penetrate into cells. However, the mode of penetration was different between the two. 1) Toxoplasms already attached to cell membrane as described above, contrary to expectation, never penetrated through cell membrane. They moved to another part of the cell surface of either the same or different cells, and there they penetrated through. One particular movement or a characteristic preparatory motion before penetration was never noticed. 2) Toxoplasms moving freely in the culture medium suddenly took their course and penetrated directly into cells. No particular movement could be observed prior to the penetration. Only toxoplasms showing vigorous movement in the culture medium were able to penetrate the cells. Toxoplasms showing slow motion never invaded. In both cases, penetration was initiated at one end of the major axis of the parasite. It was not always easy to differentiate which end of the parasites penetrated first. Generally, toxoplasms were found to penetrate cell membrane with the obtuse end. However, we could not differentiate acute and obtuse ends with any degree of accuracy.

Details of the penetration were photographed and are shown in figure 2, which was compiled from successively taken films. A parasite, 64 minutes after inoculation, came into contact with a cell membrane with one end (figs. 2-a~2-e). Five seconds later, the parasite formed a fine papilla at the attached end (fig. 2-f). Soon, the papilla became larger (fig. 2-g). In the next stage, the parasite was a diplococcus or gourd-shape (fig. 2-h). Then, the constriction of the parasite moved backward as if it had a papilla at the another end (fig. 2-i). Finally, the parasite recovered its original shape and advanced a limited distance in the cytoplasm (fig. 2-j). Whole the procedure described above indicated the parasite passed a very narrow hole (about 1.5 μ diameter), which was smaller than that of the parasite. After finishing the above process the movement of the parasite ceased at once. It took usually several minutes before the parasite initiated the next much slower movement toward nucleus. Accordingly, the above process was considered the penetration of toxoplasms through cell membrane.

It took only about 25 seconds from the first appearance of the papilla at the attached end to the disappearance of the parasitic constriction at the rear end.

The parasite which penetrated through and advanced a little further stopped once. The time required for the above penetrating procedure, from the formation of a small papilla to the first stop of the parasite in the cytoplasm after penetration, was 25～75 seconds. The average penetration time of the 20 cases was 40 seconds.

A bright halo was always found around each parasite free in the liquid medium (fig. 2-a). The halo could still be seen when the parasite was located below the cell membrane after penetration (fig. 2-j). The brightness of the halo disappeared gradually and became very weak with further movement of the parasite in the cytoplasm, and disappeared when the parasite finally stopped near the nucleus.

d) Behavior of toxoplasms after penetrated into cytoplasm

After penetration, the toxoplasma stopped and then began to move toward the nucleus (figs. 3-a～3-d and 3′-a～3′-d). This time the speed of the intracytoplasmic movement was much slower. The toxoplasma finally stopped near the nuclear membrane but never attached to nor invaded the nuclear membrane. The time needed to finish the above intracytoplasmic movement varied from 10 to 90 minutes. The variation was due to the distance from the parasite to the nuclear membrane. In addition, toxoplasms did not always take the shortest route toward the nucleus.

e) Cellular response to the toxoplasmic invasion

In normal cells, intracytoplasmic granules were generally distributed densely around the nucleus but rather sparse near the cell membrane. Sometimes the granules gathered making several groups in cytoplasm. The granules showed an active motion similar to Brownian movement. The motion was partially active where the granules were crowded.

No common response of granules was observed in 20 cells after invasion of toxoplasms. In a few cases, however, many granules gathered around the invading parasite. Several minutes later, the granules dispersed as before and those remaining around the invading toxoplasma gradually became slow in movement. We could observe one cell in which all the granules suddenly became sluggish when the toxoplasma invaded. It was found, while the invading toxoplasma was moving toward the nucleus, that granules around the toxoplasma became more sluggish than those near the nucleus. Generally, granules of the cells infected with toxoplasma were more slack than those of normal cells.

f) Escape of toxoplasma from the cell

One case of toxoplasma departing a cell was observed which gave us additional data. The detail was photographed in figure 4.

A cell was found, 4 min and 30 sec after infection, already invaded by two toxoplasms. One toxoplasma began to move in the cytoplasm, and left the cell (figs. 4-a～4-m). The brightness of the halo was recovered gradually while the intracytoplasmic parasite was moving toward the cell membrane. And the halo was completely regained when the parasite was escaped from the cell (figs. 4-n～4-p). The host cell was not damaged by the escape.

Another toxoplasma in the same cell changed its position in the cytoplasm, coincidentally (figs. 4-f～4-j).

The former location of these toxoplasms was noticeable as toxoplasma-shaped vacuoles remained (fig. 4-p).

The constriction of the parasite always found at the time of invasion was not clear.

The cells from which toxoplasma escaped were found to be rather inactive, because the granules in the cytoplasm were sluggish.

DISCUSSION

Whole the procedure of toxoplasma penetration into cells, described in this study, was as if the parasite passed a narrow hole, whose diameter was smaller than that of the parasite. This is one of the original findings of this report.

A fine papilla formed prior to the penetration seems to bore a narrow hole through the cell membrane or perforate an invagination of the cell membrane. The fact that toxoplasms were constricted when passing through cell membrane indicates the flexibility of the parasites. Recovery of cell membrane, if this happens, at the penetration point seems to occur rapidly. Invagination of cell surface such as described only once by MANWELL & DROBECK (1953) was not observed.

In most of the 20 penetration cases, a tendency of the parasites to penetrate by the obtuse end of the major axis was observed. BRINGMANN & HOLZ (1953), GUSTAFSON et al. (1954) described, by using electromicrographs of thin sections of toxoplasma, the acute end of the parasite as showing a very distinct organelle, designated "conoid", which was a hollow, truncated cone 0.15～0.25 mμ in diameter and 0.2～0.3 mμ large. However, we could not exactly differentiate the acute end under phase contrast microscope. Therefore, the question of whether the parasite penetrated at the acute end or at the obtuse end was not answered in this study.

To determine whether toxoplasma had already invaded the cytoplasma or still remain outside cells, attention was given to the existence of a bright halo around toxoplasma. A bright halo was always found around each extracellular parasite, brightness of the halo gradually decreased after the perforating form was recognized and completely disappeared when the invading parasite finally stopped (near the nucleus).

The halo has been already observed by MUHLPFORDT (1952), GUSTAFSON et al. (1954) and SOURANDER et al. (1960). But they thought the halo might be an artifact and did not mention the possible relation of the halo to the intra- or extracellular localization of toxoplasma.

GÜIMARAES & MEYER (1942) were the first to report active penetration of toxoplasma into cells. According to SCHMIDT-HOENSDORF & HOLZ (1952) the time required for the penetration was 30～60 minutes. PULVERTAFT et al. (1954) suggested 2 different ways in which parasites gain entrance into cells, active invasion or passive ingestion. Actively penetrating parasites did not multiply but were lethal to the cells whereas the ingested ones were the proliferating forms. Although their findings have not yet been adequately discussed, it should be

pointed out that the cells they used were a mixed population of fibroblasts, lymphocytes and macrophages. In addition, these cells were grown on a serum agar medium.

Only 40 seconds were necessary for complete penetration, as clearly shown in this study. ROBINEUX (1956) reported that the time required for phagocytosis of staphylococci by leukocytes and macrophages was 20 minutes. MIYAMOTO & YAMAMOTO (1958) described that tubercle bacilli were not phagocytized by cultured macrophages before 15 minutes. Some period of time is thus necessary for complete phagocytosis. It is obvious that the time spent for toxoplasma invasion is considerably shorter. This could be explained by the fact that toxoplasma actively penetrated and was not phagocytized.

A characteristic constricted shape of toxoplasma, revealed only during penetration, should be emphasized. This is satisfactory support of active penetration.

Penetration of toxoplasma always occurred by either end of the major axis. This happened in all 20 cases of penetration without exception. If toxoplasms were phagocytized, their intake by cells must occur in every direction.

Less active toxoplasms which were left at 37°C for a considerable period of time and formalin killed toxoplasma did not invade. Neither they developed the characteristic perforating form.

All the above findings support the theory that toxoplasma infection is due to the active penetration of the parasite.

The knowledge of the penetration or invasion of toxoplasma shown in the present tissue culture system would be very helpful in investigating toxoplasma infection particularly the fundamental ones such as host to host transmission, mechanisms of neutralization test, etc.

The escape of toxoplasma from an infected cell was extremely interesting. The escape was due to the movement of toxoplasma itself. Since the cell from which toxoplasma escaped was found rather inactive, it is probable that toxoplasma is highly sensitive to the nutritional condition of host cell.

SUMMARY

Cinematographic observation with phase contrast microscopy was undertaken to elucidate a course of penetration of cultured cells by *Toxoplasma gondii* (RH strain). The results are summarized as follows:

1) The parasites which moved toward the cells showed active, irregular movements. No penetration was observed by less active or completely inactived parasites.

2) In the course of penetration of the cell membrane, the parasite attached itself to the cell membrane at a pole of its major axis. The attached portion of

the parasite formed a rostrum with which it seemed to bore a small hole in the cell membrane through which it entered the cytoplasm. The hole or invagination must be smaller than the parasite because when passing through, the parasite was constricted. The time required for penetration of the cell averaged about 40 seconds.

3) Following the penetration, the parasites moved slowly in direction of the cell nucleus, but none were observed to penetrate the nuclei.

4) No common, definite cellular response was observed but, in general, the movement of the cytoplasmic granules surrounding the parasites decreased with time.

5) Escape of toxoplasma from the infected cell was observed, which was due to the movement of toxoplasma itself. Since the cell from which the toxoplasma escaped was found rather inactive, it is probable toxoplasma is highly sensitive to the nutritional condition of host cell.

ACKNOWLEDGEMENT

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EXPLANATION OF PLATES

Photographs were taken at 5 seconds intervals, ×750.

PLATE I The process of penetration of toxoplasma

Fig. 2-a 64 min*
Fig. 2-b " and 5 sec
Fig. 2-c " 10 "
Fig. 2-d " 15 "
} Show rotating movement of the extracellular parasite.

Fig. 2-e " 20 " ……Contact with cell membrane with one end
Fig. 2-f " 25 " ……Forming a fine papilla at the point of attachment
Fig. 2-g " 30 " ……Papilla becoming larger
Fig. 2-h " 35 " ……Diplococcus-like or gourd-shaped form

* Indicates time after infection

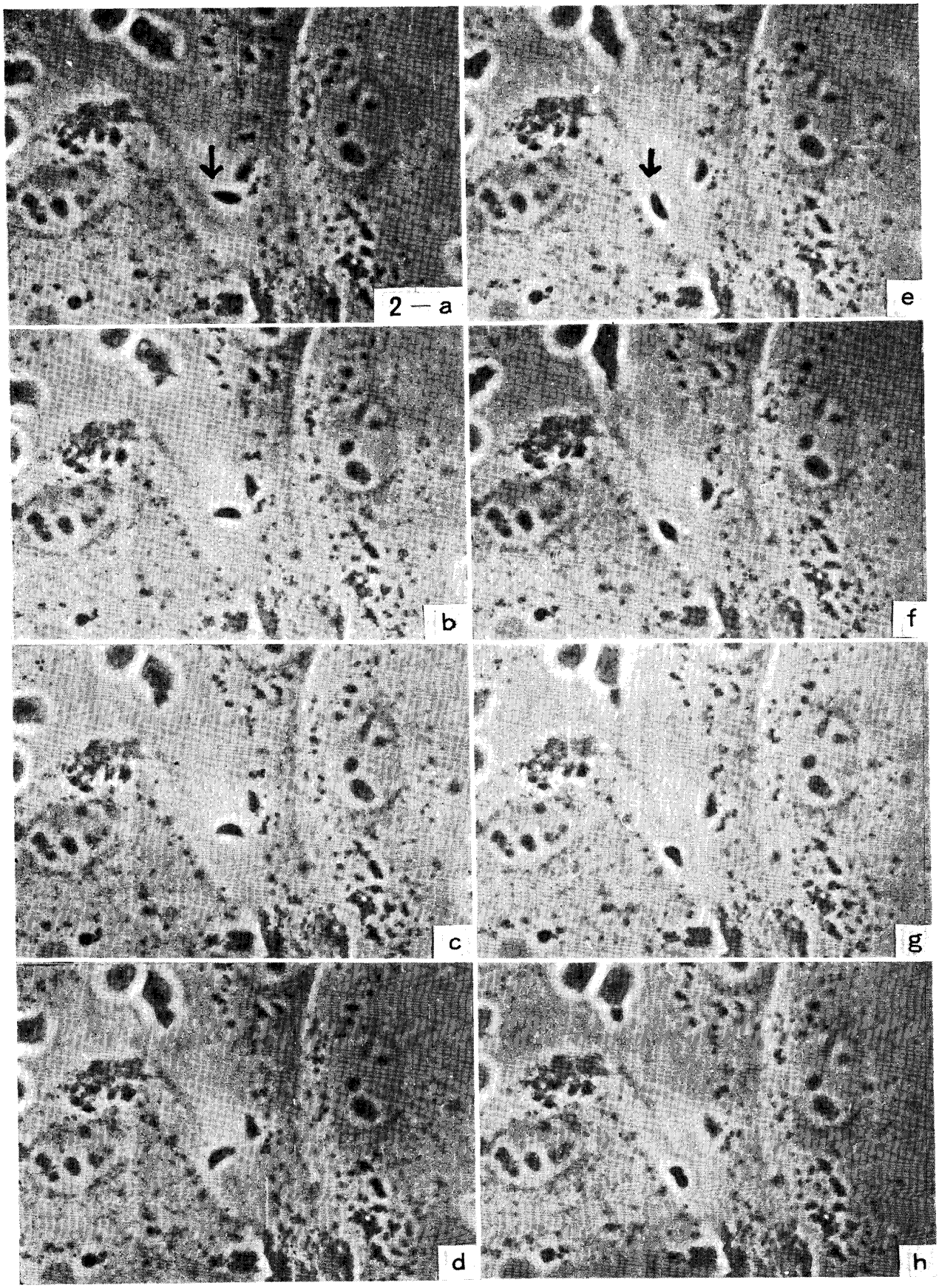
2 — a
e
b
f
c
g
d
h

PLATE II

Fig. 2-i 64 min and 40 sec……Constriction moved to the rear end.

Fig. 2-j " 45 " ……Penetrated parasite recovered the shape.

Fig. 2-k 73 min and 5 sec
Fig. 2-l 89 min and 45 sec } Disappearance of the bright halo

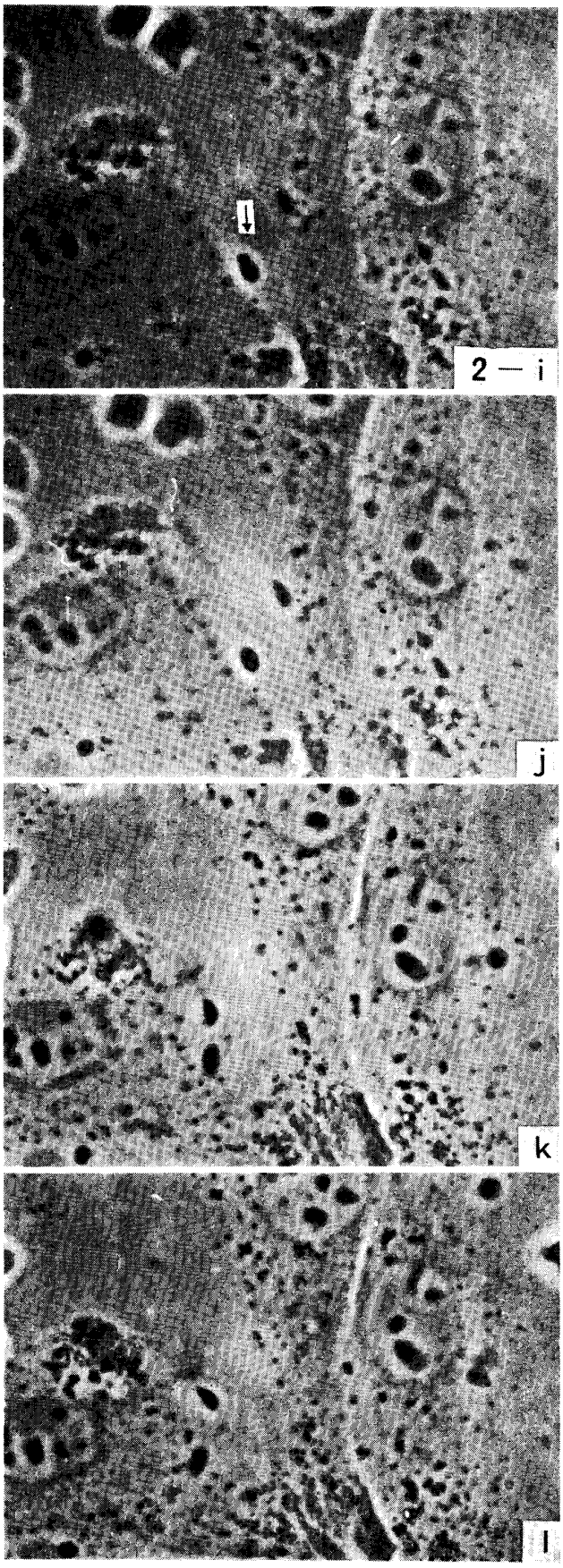
2 — i
j
k
l

PLATE III Intracytoplasmic moving toward the cell nucleus after the penetration

A

Fig. 3-a 5 min

Fig. 3-b 64 min and 10 sec

Fig. 3-c 95 min and 50 sec

Fig. 3-d 120 min

B

Fig. 3'-a 16 min

Fig. 3'-b 27 min and 20 sec

Fig. 3'-c 44 min

Fig. 3'-d 101 min and 15 sec

} The parasites were moving slowly in the direction of the cell nucleus.

A B

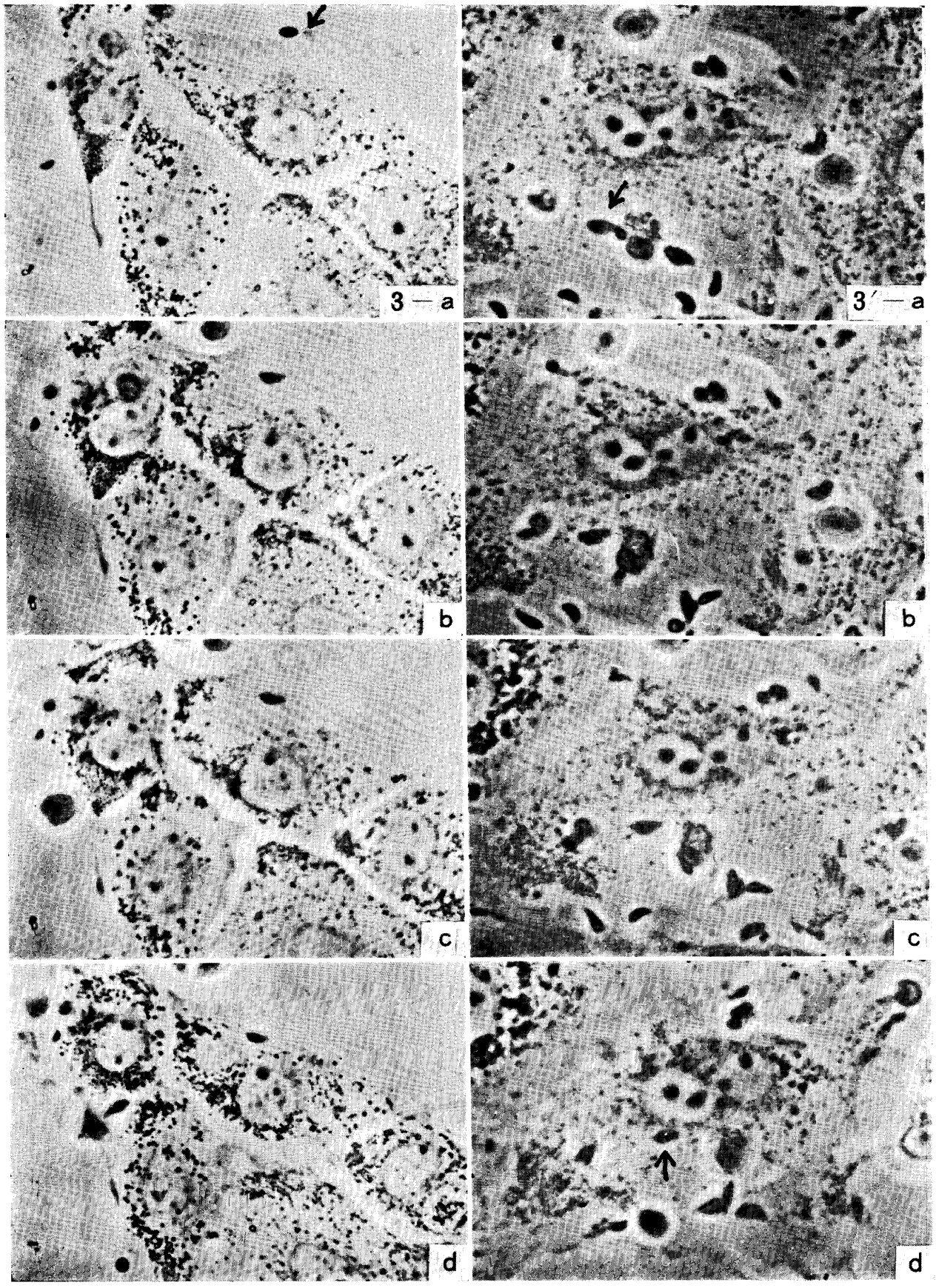

PLATE IV Escape of the parasite from the infected cell

Fig. 4-a 4 min and 30 sec……The parasite immobile in the cell

Fig. 4-b 〃 35 〃

Fig. 4-c 〃 40 〃

Fig. 4-d 〃 45 〃

Fig. 4-e 5 min and 50 sec

Fig. 4-f 〃 55 〃

Fig. 4-g 6 min

Fig. 4-h 〃 and 5 sec

} Intracellular movement toward cell membrane

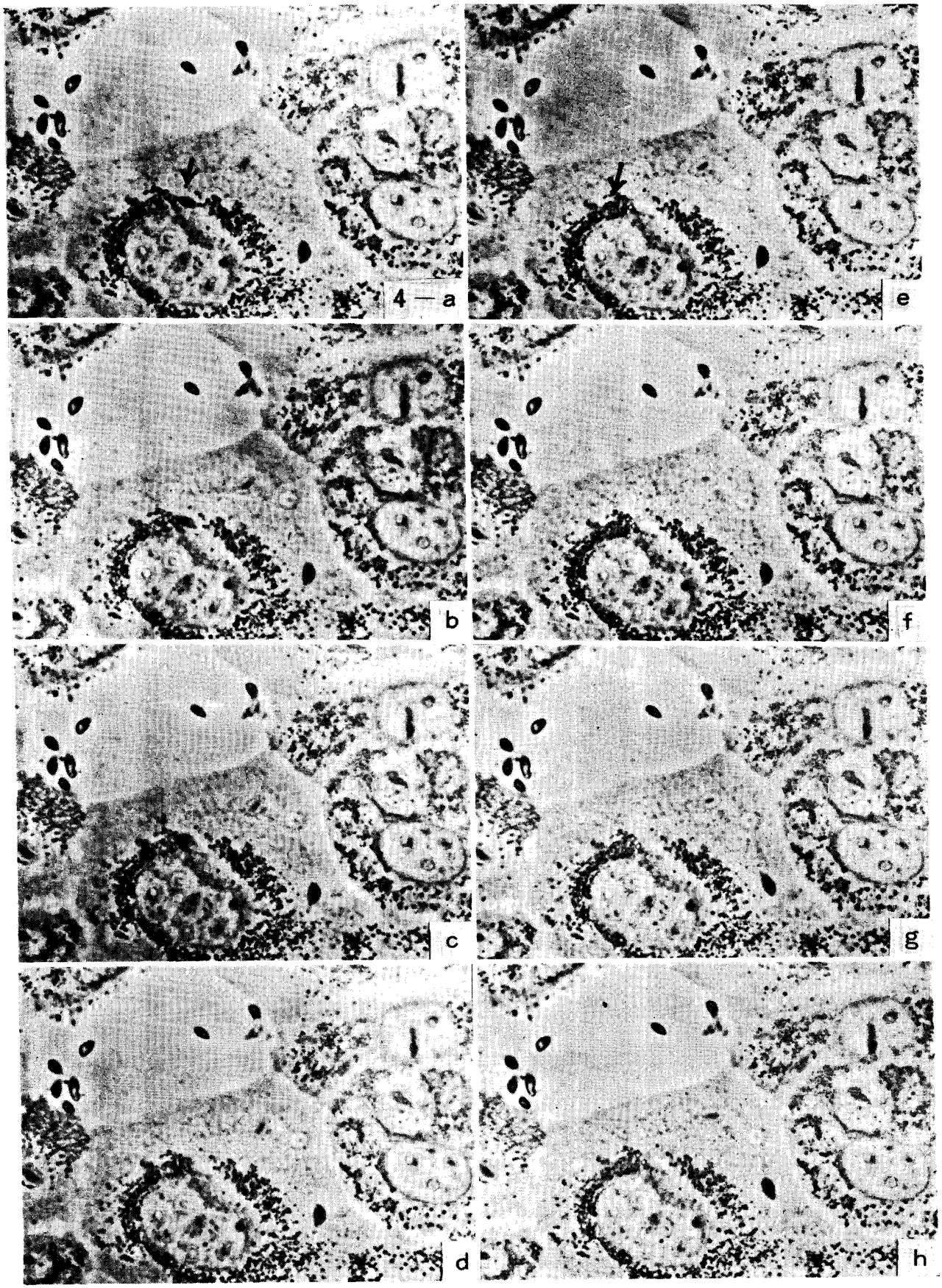
4 — a
e
b
f
c
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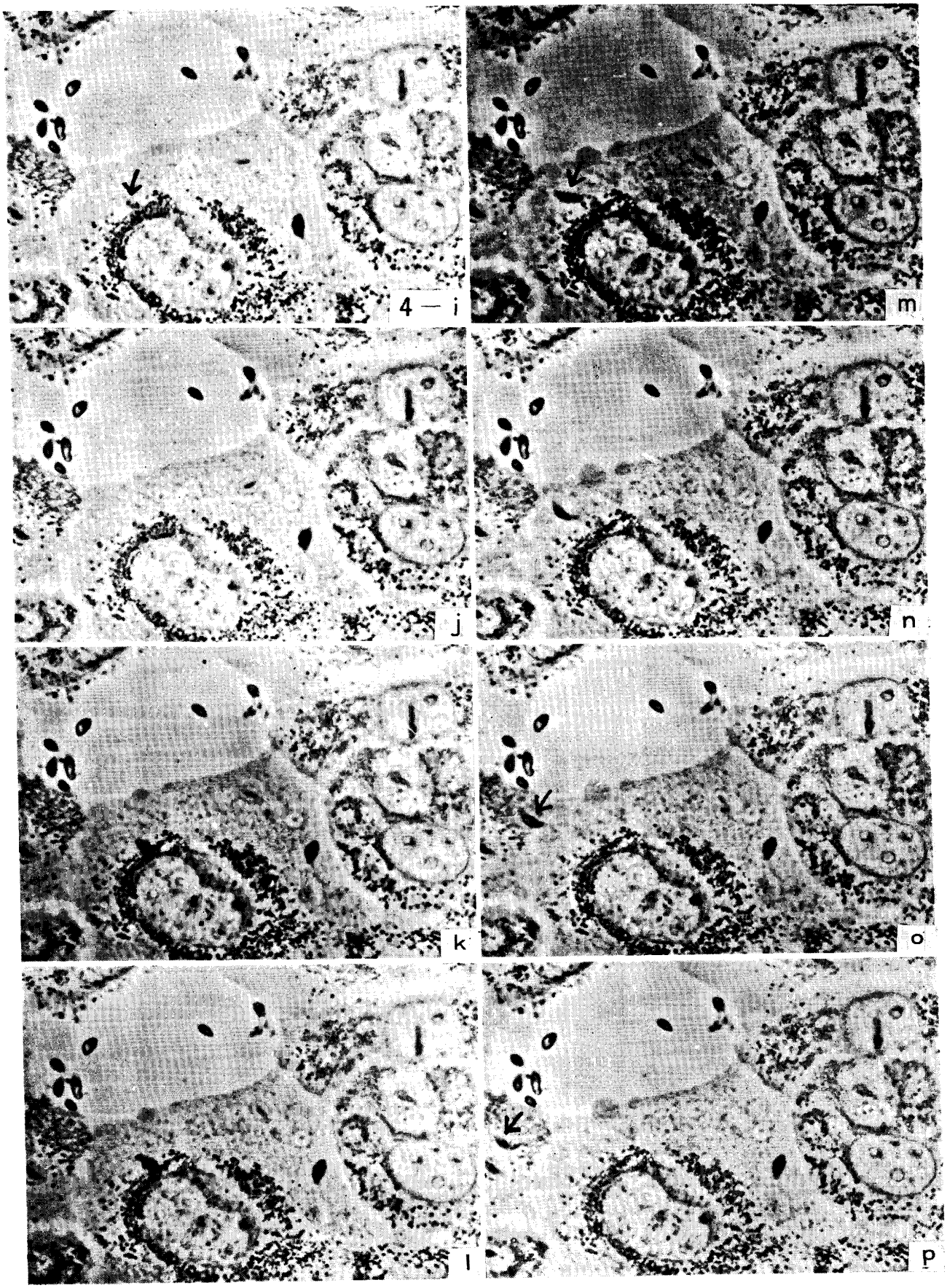
PLATE V

Fig. 4-i	6 min and 10 sec	Intracellular movement toward cell membrane
Fig. 4-j	" 15 "	
Fig. 4-k	" 25 "	Escape from the cell, leaving cell membrane
Fig. 4-l	" 40 "	
Fig. 4-m	" 55 "	
Fig. 4-n	7 min and 15 sec	Parasite free outside cell
Fig. 4-o	" 20 "	
Fig. 4-p	" 25 "	


4 — i
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