Application of the Immuno-peroxidase Method to the Study of Tumor Viruses

1. DNA Tumor Viruses

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I. INTRODUCTION

The immuno-peroxidase method, which was introduced by Nakane & Pierce in 1966 [1], appears to be increasingly available for studies of the intracellular or cell surface localization of various antigens. Utilizing this method the site of the production of various proteins, hormones, enzymes or gamma globulins has been decided within cells and tissues. Several reviews summarizing this technique point out the various advantages of this method, in comparison with immuno-fluorescein or immuno-ferritin methods used hitherto [2-8]. The principal advantages of the immuno-peroxidase method are characterized as follows: 1) Light microscopic staining sections are permanent and can be stored indefinitely. 2) The same preparation can be utilized both under light and electron microscopy. 3) Visualization by means of a cytochemical reaction increases the sensitivity of the probe through the amplifying effect of enzymatic activity. 4) Because of the small molecular size of peroxidase, the penetration of the labeled antibody into the cell or tissue is achieved more readily.

Since both immuno-fluorescein and immuno-ferritin methods have been widely utilized for studies of the development of various viruses or virus-related antigens in cells, the application of this new technique in the field of virology may be well expected. In 1969 this technique was applied for the first time for the detection of viral antigens, that is, the structural and T-antigens of both SV 40 and adenovirus type 12 by Wicker & Avrameas [9], and LCM virus by Abelson *et al.* [10]. Although they seem to have had considerable success, up to the present, practical applications of this method to problems in virology are surprisingly few. Using this technique, we have previously examined the localization of the structural antigen of polyoma virus in infected cells [11], which is one of the typical DNA tumor viruses. In the course of the experiment we encountered various problems that needed to be solved, since the immuno-peroxidase method had not yet been completely established. Although several studies are available in which this

method was used in the tumor virus, various problems appear to have been encountered. We have summarized and reviewed these problems here, paying particular attention to the immuno-peroxidase method itself. In this paper, studies on DNA tumor viruses are reviewed. The RNA tumor viruses, which are considered to have other problmes with regard to the immuno-peroxidase method, will be reviewed in another paper.

II. SV40

Wicker & Avrameas [9] used light microscopy with the immuno-peroxidase method to detect the virus antigens of SV 40 and compared the results with those obtained by the fluorescein labeling method. Infected MA 104 monkey kidney cells on coverships were fixed with acetone or 2% paraformaldehyde, and were followed by the indirect sandwich method for antigen detections. The cells were first incubated in rabbit or hamster antiserum against either the structural or T-antigens of SV 40. After being washed they were incubated in peroxidase-conjugated antibody using either sheep antirabbit immunoglobulins or rabbit antihamster immunoglobulins. In addition to peroxidase, alkaline phosphatase and glucose oxidase were successfully used as markers. Finally, the site of the enzyme label was revealed by classical histochemical ractions. Colored photographs of cells stained positively with those methods have revealed a remarkable similarity to the results of the immuno-fluorescein method [12]. Both the viral structural and T-antigens could be observed in infected cell nuclei. The T-antigen was in the form of intranuclear fibers and were consistent with the electron microscopic findings by the immuno-ferritin method [13, 14]. Both the structural and T-antigens could be simultaneously detected in the same preparation by using two enzymes as markers. Although the existence of a few non-specific stainings remained to be resolved, usefulness of the immuno-enzyme method for the study of tumor viruses was virtually proved by them.

In a subsequent study, the T-antigen of SV 40 was demonstrated at ultrastructural level by Leduc *et al.* [15]. Their work, as well as that of Abelson *et al.* [10], was the first application of the electron microscopic immuno-peroxidase method to problems of virology. The infected MA 104 cells were fixed in situ for 15 to 60 minutes in a freshly prepared buffered 1% paraformaldehyde solution. After being washed, the antigen was revealed by the indirect method described above. The cells were post-fixed in 2% OsO_4 and then gently scraped off the surface of culture tubes with a rubber policeman and centrifuged into a pellet. The pellet was dehydrated in alcohol and embedded in Epon. Ultrathin sections were examined without post-staining.

Positively and heavily stained nuclei exhibited a fairly uniform distribution of the T-antigen throughout the nucleoplasm except in the nucleoli. There was another pattern of the antigen distribution in the form of a reticulum network, which was interpreted to represent an association of the antigen with the nuclear chromatin. It was suggested by them that the loose network pattern of the antigen might indicate an earlier stage of antigen formation, although further experiments with the time lapse after infection would be necessary for clarification. It is strange that the cytoplasm of the cells with a heavily stained nucleus was usually strongly damaged and was stained non-specifically (Plates 2 b, 3 a and 3 b in Ref. 15). Even in the control cells either uninfected or infected, marked non-specific cytoplasmic staining was observed. This might attribute to the fact that whole serum was used rather than purified antibody. Since the fine structure of cells was also not satisfactorily preserved, an improvement of the whole mehtod, especially for cell fixation, seems to have been essential for their work.

Recently, Baba *et al.* [16] examined the ultrastructural localization of the T-antigen in SV 40-transformed kidney cells. They successfully stained the T-antigen by applying the immuno-peroxidase method directly on ultra-thin sections of the cells, which had been fixed with paraformaldehyde and then embedded in water-soluble plastic (glycol methacrylate). They explained that a diffusion method was abandoned because of the difficulty in achieving penetration of the peroxidase-conjugated antibody into cells. In the diffusion method, tagged antibodies are allowed to penetrate into fixed cells and to interact with antigen sites prior to embedding and thin sectioning. In the surface-localizing method used by Baba *et al.* [16], on the other hand, thin or semithin sections of fixed and embedded tissues were prepared prior to the detection of antigenic sites with immunotracers. In addition to glycol methacrylate, polyethylene glycol, Epon and albumin have been employed as the embedding materials for this purpose. A detailed explanation and a comparison of diffusion and surface-localizing methods was summarized recently [8]. Some investigators have suggested that aldehyde fixation of cells might not be suitable for the immuno-peroxidase technique in the diffusion method, because the peroxidase-conjugated antibody could not penetrate into cells through the fixed-cell membrane [8].

Baba *et al.* confirmed the intimate relationship between the T-antigen and the nuclear chromatin in SV 40-transformed cells. The nucleolus and the cytoplasm were usually negatively stained. Although they could detect the T-antigen by a skillful surface-localizing technique, the fine structures of cells illustrated by them leaves something to be desired. When we used the methacrylate as the embedding material, extraction of cell organells could

not be avoided during the embedding procedure. Since previous immuno-ferritin studies suggested a specified filamentous structure as the T-antigen of SV 40 [13, 14], precise localization of the T-antigen may require further clarification.

Localization of the structural antigen of SV 40 in infected cells was examined by Shabo *et al.* [17] by using the ultrastructural immuno-peroxidase method. The infected cells fixed in situ for 1 hr in cold 4% paraformaldehyde were directly reacted with the peroxidase-conjugated rabbit immunoglobulin against the SV 40 structural antigen. Numerous intranuclear SV 40 virions of heavily infected cells were hardly stained in spite of distinct breaks in the nuclear membranes, whereas several clumps of intracytoplasmic virions were densely stained (Fig. 6 in ref. 17). Although they have insisted that the result was in agreement with the previous immuno-ferritin studies [14, 18], there seems to be a clear contradiction between them. Immuno-ferritin studies [14, 18] showed that in addition to the intracytoplasmic virions the amorphous nuclear material around the intranuclear virions was tagged strongly by the immuno-ferritin, whereas no intranuclear virions were tagged. Moreover, it should be recalled that their work did not agree with the former fluorescein microscopic [19] and immuno-peroxidase studies [9], since they illustrated clearly the existence of SV 40 structural antigen throughout the nuclei of the infected cells. There seem to be some points for further improvement in the study by Shabo *et al.* [17], for example, the simultaneous experiments at light microscopic level, and the use of further purified preparation of the peroxidase-conjugated antibody with application of a column fractionation or absorption techniques.

III. POLYOMA VIRUS

First, it should be realized that polyoma virus as well as SV 40 belong to the PAPOVA group and the developmental processes or sites of the specific viral proteins in infected cells can be assumed to be the same in each. This supposition seems to be proved by the previous morphological or biochemical studies reported on both viruses [20].

An immuno-peroxidase study of the polyoma T-antigen has not yet been reported, probably because of the difficulty in obtaining an antiserum with a sufficiently high titer against the polyoma T-antigen. An immuno-ferritin study has not been published as yet, either. As to the site of the structural antigen of polyoma virus in infected cells, we previously presented a preliminary report regarding the use of the immuno-peroxidase method [11]. We describe briefly the methods and results throughout the experiment here, and discuss the problems encountered.

An indirect method was used. Rabbit antiserum against the polyoma structural antigen (virions), which was purified by sucrose density gradient centrifugation, and sheep antiserum against rabbit gamma globulin were prepared. Polyoma virus used throughout the experiment was a small plaque strain derived from SE-4B5 and orginally obtained from Dr. B. Eddy, National Cancer Institute, Bethesda, Maryland, U.S.A. Peroxidase (Sigma, type VI) was conjugated with the purified sheep gamma globulin by Avrameas' method using glutaraldehyde [22]. The peroxidase-conjugated gamma globulin was purified on a Sephadex G-200 column and then concentrated. The kidney cells from suckling Swiss mice cultured in petri dishes were inoculated with polyoma virus at a high multiplicity of infection (moi 10).

Prior to the fixation of the cells, three fixatives; 2% glutaraldehyde, 2% paraformaldehyde and acetone, were checked against the possibility of preserving the polyoma antigenecity. Polyoma virions were incubated for a definite time in a cold fixative and then dispersed into a large volume of PBS. After centrifugation, the remaining antigeneity of the virus was examined by HA titer (Fig. 1). Consequently, the polyoma HA antigen was proved to be rapidly affected by the incubation in glutaraldehyde, and hence both paraformaldehyde and acetone were exclusively used as the fixatives. The cells at 38 hr after the infection were fixed in situ either for 60 min in cold 2% paraformaldehyde buffered with PBS, or for 10 min in acetone. After washing for 2 or 3 hr in PBS, the cells were incubated for 12 hr in the rabbit antiserum against the polyoma capaid antigen at 4°C. After washing again they were further incubated for 12 hr in the peroxidase-conjugated goat antibody against normal rabbit gamma globulin at 4°C. Washing with PBS for 12 hr followed. Benzidine reactions were carried out for 10 min with Karnovsky's solution [22]. After the cells were postfixed for 1 hr in 1% osmic acid, they were scraped off the surface of the petri dishes,

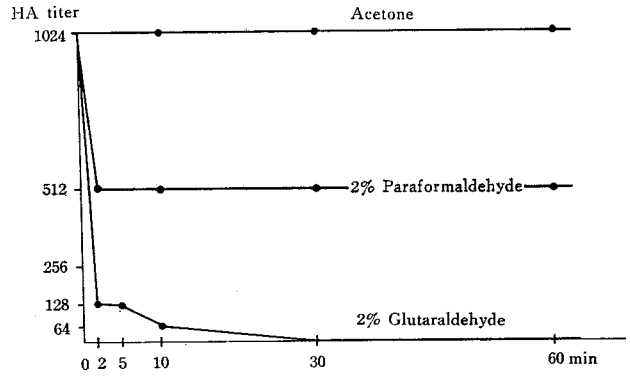

Fig. 1. Effect of fixatives on polyoma virus HA titer.

suspended in PBS and centrifuged into pellets. The pellets were rapidly dehydrated in ethanol and embedded in Epon 812. Thin sections were cut and examined under a Hitachi HS-7 electron microscope without post staining. Semi-thin sections were also cut and stained lightly with toluidine blue for light microscopic observations. Cells left on the surface of the petri dishes without scraping were also dehydrated and mounted for light microscopic examination.

Two kinds of control were employed. In one, uninfected cells were processed exactly as above. In the other, infected cells were treated with normal rabbit serum or PBS instead of the serum containing anti-polyoma capsid antibody. Another type of control study was carried out by fluorescein microscopic studies, namely, the infected cells were incubated in the FITC-labeled antibody instead of in the peroxidase-conjugated antibody.

By use of the light microscopic immuno-peroxidase method, localization of the polyoma capsid antigen was shown exclusively in the nuclei of infected cells (Figs. 2, 3). Figure 2 represents a classical picture obtained by immunofluorescence and Figure 3 gives the peroxidase-immunohistochemistry. Acetone was used as the fixative for both cases. Although the latter seems to present a somewhat clearer picture of the details of cell structures, the general findings are of the same type. Many nuclei were strongly positive and others were weakly positive or completly negative. Nucleoli were usually eliminated from the staining. As such results were completely consistent with those in studies on SV 40 and polyoma virus previously reported by others [9, 12, 23], the immuno-peroxidase method at the light microscopic level seems to have been thoroughly established, in other words polyoma virus and SV 40 belong to the same group of viruses and the site or appearance of the virus capsid antigen in infected cells should be considered as the same. Certainly, this seems to be true regarding the acetone-fixed cells because of no background staining in any of the control experiment was shown. However, many problems remained to be solved in the case of paraformaldehyde-fixed cells. Even in controls, the weak nuclear and/or cytoplasmic stainings were often encountered either in uninfected cells or the infected cells followed by normal rabbit serum incubation. Figures 4 and 5 show semi-thin sections of Epon-embedded cells fixed with acetone and paraformaldehyde, respectively. Although the cell structures are ill-preserved and disappearances of some nuclear materials are obvious in acetone-fixed cells (Fig. 4), the specific staining against the polyoma capsid antigen is much clearer and stronger, and no background staining is shown. On the other hand, the nuclear staining in paraformaldehyde-fixed cells was faint and infrequent (Fig. 5). Furthermore, the distinct non-specific staining in the cyto-

plasm or on the cell membranes could not be removed despite the frequent washing of cells. It was noticeable that a similar staining as shown here is often encountered, even in the negative control preparations (not presented here).

Figures 6, 7 and 8 illustrate electron micrographs of the polyoma virus-infected cells fixed in paraformaldehyde and followed by the specific staining procedure described above. The fine structure of cells seems to be preserved very well except for some disappearance of nuclear materials. Certainly, several nuclei could be positively stained without any counterstaining with lead or uranyl acetate, although debris of reaction products was also observed near the cell membranes (Fig. 6). Both a stained and a non-stained nucleus were shown in marked contrast in a picture (Fig. 7). There were a marked increase of density of the nuclear inner membrane and diffuse nuclear materials in the positively stained nucleus. But, contrary to our expectation, the polyoma virions scattered within the nucleus were scarcely increased in density. A higher magnified photograph from the same nucleus shown in Figure 7 indicates the evidence clearly (Fig. 8). Such findings seem to coincide completely with those of the previous reports on SV 40 by the immuno-ferritin method [14, 18].

In the acetone-fixed cells, on the other hand, the numerous polyoma virions within the nuclei were strongly stained with the reaction products (Figs. 9, 10). Although the acetone fixation certainly destroyed the fine structure of cells and generally tended to increase their electron density, the nuclear morphology was preserved relatively well and the reaction products against the corresponding antigen could be clearly identified by its intensive density. As a natural consequence the boundary of the virions became unclear and their diameter increased (Fig. 10). The cytoplasm, nucleolus and the nuclear amorphous materials were usually eliminated from the specific staining. Thus, these results seem to be inconsistent with the previous reports on SV 40 [14, 18] and with our present study on the paraformaldehyde-fixed cells. We believe the findings seen in the acetone-fixed preparations to be valid, because non-specific staining was definitely not seen in either normal or infected cells. Moreover, the increase of the diameter and the lack of clear boundaries of the virions, which supposedly resulted from deposits of the specific reaction products, would support the view.

The reason why the nuclear amorphous materials were non-specifically stained in the paraformaldehyde-fixed cells remains to be solved. It is unlikely that either the anti-polyoma serum or the peroxidase-labeled antibody used by us immunologically crossreacted against some materials within the nucleus, because the simultaneously treated acetone-fixed cells were invariably stained

positively without any background staining. But there seems to be a slight possibility that some components of cells could be extracted or inactivated by acetone-fixation, resulting in the disappearance of the cross reactive materials with antibodies. It is also possible that free aldehyde groups left after the aldehyde-fixation could interact with the peroxidase-conjugated antibody leading to the non-specific false positive results as suggested recently [8]. It has been suggested, furthermore, that false positive reaction can also result from non-specific hydrophobic or ionic interactions of antibodies, tracers or reaction products with some tissue components [8].

Another remaining problem is why the intranuclear virions are hardly stained in the preparations of the paraformaldehyde-fixed cells. It is unlikely that specificity of the capsid antigen is lost by paraformaldehyde, because HA antigen titer of the virus after prolonged incubation with paraformaldehyde was hardly affected (Fig. 1). As the previous reports suggested, there is a possibility that some substance covering the surface of the intranuclear virions could obstruct the antigen-antibody reaction. The acetone-fixation might strip the intranuclear virions and facilitate the reaction between the capsid antigen and the corresponding antibody. An evitable problem concerning to the permeability of antibodies through the aldehyde-fixed cellular membrane will be mentioned later.

IV. ADENOVIRUS

In the above-described paper by Wicker and Avrameas [9], localization of the T-antigen and the structural antigen of adenovirus type 12 in infected cells has been briefly shown by using light microscopic immuno-enzyme techniques. The T-antigen appeared in the form of intranuclear fibers and agreed with those reported in both the previous fluorescein microscopic studies and an immuno-ferritin study. Although the fluorescein study revealed a specific staining in the form of cytoplasmic freck, whether or not the T-antigen of adenovirus is present in the cytoplasm is yet to be determined. Further studies at an electron microscopic level using the immuno-peroxidase method might clarify the site or the developmental process of the T-antigen of adenovirus within the cells.

The structural antigen of adenovirus type 12 has been shown as small intranuclear masses in infected cells by using an alkaline phosphatase-labeled antibody technique [9]. Since the structural antigen of adenoviruses have been recently analyzed in detail and now several types of the antigens related to the particles have been distinguished, the developmental site or localization of each antigen in infected cells should be further clarified by using the immuno-peroxidase method as well as the immuno-fluorescein method.

The electron microscopic immuno-peroxidase method has not yet been applied to the study of the structural antigens of adenoviruses. Since fine structural inclusion bodies, which were assumed to be related to an antigen of the viruses, have been known to be included in adenovirus-infected cells [26], the application of this method to adenovirus-infected cells might certainly bring advantages in that field. On the other hand, an immuno-ferritin study of the intracellular structural antigen of adenovirus type 12 was reported earlier by Levinthal *et al.* [27]. Their work has attracted us, since it contained the same problems as those seen in the studies on SV 40 and polyoma virus. Levinthal *et al.* pointed out in their paper that "the fact that little or no ferritin lodged in any consistent pattern on the adenovirus particles was a source of bafflement". In the considerably difficult speculation for that contradiction, they have insisted that what had been seen in the fluorescent antibody study was essentially the antibody reaction with stockpiles of unassembled structural antigens. It is noticeable here that the cells used for the fluorescent microscopic study were fixed in acetone, whereas the cells for the immuno-ferritin study were fixed in diluted formalin. We believe that the fixation procedure of cells for immuno-electron microscopic study should be the same as those of control preparations at the light microscopic level.

Lately, the result that did not agree with Levinthal *et al.* was reported by the use of a surface localizing method of the immuno-ferritin technique [28]. Canine adenovirus-infected cells were fixed in formalin and then embedded in glycol methacrylate. When a thin section of infected cells was treated with ferritin conjugated to the antibody of the canine adenovirus, a strong specific attachment of ferritin to the virus particles and inclusion bodies within the nucleus was shown. To our regret, however, the fine structure of cells illustrated in their electron micrographs was so damaged that the accurate localization of the antigen in cells could not be identified sufficiently.

V. HERPES VIRUS GROUP

Recently, EB virus, cytomegalovirus, herpes simplex virus and Mareck disease virus have been widely recognized as oncogenic, or tumor-related viruses. Although both immuno-fluorescein and immuno-ferritin methods have been frequently utilized to study intracellular localization of those viral antigens, the immuno-peroxidase technique has been applied only to the studies of EB virus [29] and cytomegalovirus [5]. Although herpes zoster virus antigen has been briefly studied using this technique [17], it is doubtful whether the virus antigen could be successfully stained, because we could hardly identify the specific staining product on the virus particles in the

electron micrographs. The highly magnificated electorn microphotograph was somewhat out of focus. On the other hand, Suzuki *et al.* [29] have successfully stained the mature EB virus envelopes or the nuclear chromatin of the P3HR-1 cells which were derived from Burkitt's lymphoma. They used the immuno-peroxidase conjugated with the human gammaglobulin of a patient with Burkitt's lymphoma. It has been known through the recent fluorescent microscopic studies that the sera from patients with nasopharyngeal carcinoma or Burkitt's lymphoma contain high titer antibody to early antigen (EA), membrane antigen (MA), and complement-fixing antigen (EBNA) in addition to viral capsid antigen (VCA) [30]. Further studies with more specialized antibody should be neccesary for certification of intracellular localization of such various EB virus related antigens. The work in such a direction of EB virus has been recently published, although by the use of the immuno-ferritin technique [31]. More recently, Shamoto *et al.* [32] successfully demonstrated EBNA in the nuclei of P3HR-1 cells with the immuno-peroxidase method at an electron microscopic level. The cells were fixed with a mixture of neutral formalin and glutaraldehyde and were not followed with any freezing or thawing. The remaining free radicals of aldehyde, which might presumably cause a nonspecific reaction, were reacted with L-lysine added to the washing solution after fixation.

Kurstak [5] has illustrated in his review that the cytomegalovirus in infected human fibroblasts could be stained in the immuno-peroxidase method. He described that his revelation of the enzymatic activity of peroxidase by staining with benzidine had been omitted in that study. Since we do not have a detailed description of the methods and results of his study, no criticism can be made on that work.

It is interesting that Nii *et al.* [3] could hardly tag intranuclear capsids with antibodies specific for the virus in the studies on herpes simplex virus with the ferritin antibody technique. There is a possibility that it might be due to the use of formalin as the fixative of cells, as suggested above by us. In a succeeding study, on the other hand, Miyamoto *et al.* [34] seem to have successfully tagged the capsids located in nuclei by using more specialized antibodies. But it remains to be certified whether what is described as ferritin-tagged nuclear capsids by them is virtually localized inside the nucleus, since only a part of the cells was shown by them.

VI. DISCUSSION AND SUMMARY

Satisfactory and reproducible results have been obtained when the immuno-peroxidase method was applied for the ultrastructural localization of cell surface antigens [35, 36, 37]. This technique also could be excellent

for the detection of intracellular antigens at the electron microscopic level. As indicated above through the studies on DNA tumor viruses, however, several technical problems remain to be settled, and this method might not yet be suitable for the routine use. We and others have often encountered unsatisfactory and inconsistent results in the process for detection of intracellular viral antigens.

In summary, almost all of the problems appear to have resulted from fixation procedures of cells. This seems equally true for the immuno-ferritin method. To obtain reliable results in the immuno-peroxidase staining, fixatives employed should stabilize and preserve a cellular fine structure, and should prevent the extraction and secondary relocation of antigens. At the same time, they should not inhibit the ability of antigens to interact with antibodies. These two sets of requirements lead to an apparent paradox, since the fixation procedures currently used for electron microscopic studies seriously interfere with antigenecity. To circumvent this problem, paraformaldehyde (formalin) has been widely used for immuno-electron microscopic studies.

But there seems to be another problem in fixation procedures with paraformaldehyde. The problem is whether or not the antibody or the conjugate can penetrate the interior of the fixed cells. Although no reliable experimental data are available, it has been believed by many workers that the conjugate could hardly permeate through the cellular membrane fixed with aldehyde fixatives. To allow for or enhance the penetration of the conjugate into cells, the cells were often frozen and thawed after fixation; consequently, the cellular structures were largely damaged. There are some reports which describe the intranuclear and/or intracytoplasmic positive staining with the immuno-peroxidase method without any freezing and thawing of cells after paraformaldehyde fixation [9, 15, 30, 38]. In the process of immuno-peroxidase study on polyoma structural antigen in infected cells, we have also encountered frequent intranuclear stainings in paraformaldehyde-fixed cells with no freezing and thawing. Although we obtained the specific staining in the cells fixed with acetone, we could not regard the nuclear staining in paraforaldehyde-fixed cells as to be specific. It is probable that the problem of cells fixed with paraformaldehyde might not only be the permeability of cell membranes but rather unavoidable false-positive and false-negative staining of cells. Part of this erroneous staining could probably result from free aldehyde groups remaining in cells after aldehyde fixation. It might be required to reexamine, from such a viewpoint, the mysterious and unexplicable results which had been described in the immuno-electron microscopic studies on SV 40, adenovirus and herpes viruses. It has been recently suggested that the treatment of cells with sodium borohydride which

reduces aldehyde to alcohol, could eliminate residual aldehyde groups [8, 39]. Thus, to detect intracellular antigens further studies would be necessary for the improvement of the fixation procedures in the immuno-electron microscopic study. The immuno-peroxidase method at the light microscopic level seems to have been established already by using acetone or alcohol as fixatives.

Although the surface localizing method in the electron microscopic immuno-peroxidase method was hardly referred to here, it would prepare a new step for the study of detection of intracellular antigen [8, 40]. By using this method we may expect to circumvent some technical difficulties by which the above-mentioned diffusion method is confronted. In the case of the surface localization procedure, no diffusion restrictions are to be expected because the immunologic reagents are applied directly to the surface of thin sections. In addition, free aldehydes are probably blocked by protein conditioning of the section surface which preceding localization. In spite of such definite advantages, the surface localizing method has been applied only to a few studies in virological fields. This is probably because of the complicated procedure of the technique and the low sensitivity to antibody reactions. Recently, various embedding meaterials which do not seriously compromise antigenicity have been introduced for this technique, and their disadvantages might be overcome in the near future. In conclusion, it would appear that the immuno-peroxidase technique at the electron microscopic level has not yet been completely established for the detection of intracellular antigens. But this technique is certainly a potentially important tool for the resolution of a wide variety of problems involving cellular and viral antigens. Accordingly, for all pratical purposes of this technique, the works at the light microscopic level (both immuno-fluorescein and immuno-peroxidase studies), an attempt to investigate various fixatives and the strict criticism of the control experiments should always be taken into consideration. Furthermore, the combined staining with the surface localizing method would also be recommended.

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EXPLANATION OF FIGURES

Fig. 2, 3. Polyoma structural antigen in mouse kidney cultured cells 38 hr after polyoma virus infection. Fig. 2: A flluorescence micrograph of acetone-fixed cells. Fig. 3: Immuno-peroxidase staining of acetone-fixed cells.

Fig. 4, 5. Light micrographs of thick sections from Epon-embedded materials for EM study stained by immuno-peroxidase method. Fig. 4: Acetone fixation. Fig. 5: 2% paraformaldehyde fixation.

Fig. 6, 7, 8. Electron micrographs from the same material as shown in Fig. 5. 2% paraformaldehyde fixation. No post-staining.

Fig. 9, 10. Electron micrographs from the same material as shown in Fig. 4. Acetone fixation. An arrow at Fig. 10 shows a negatively stained polyoma virus particle. A black line indicates 0.1 μm. No post-staining.

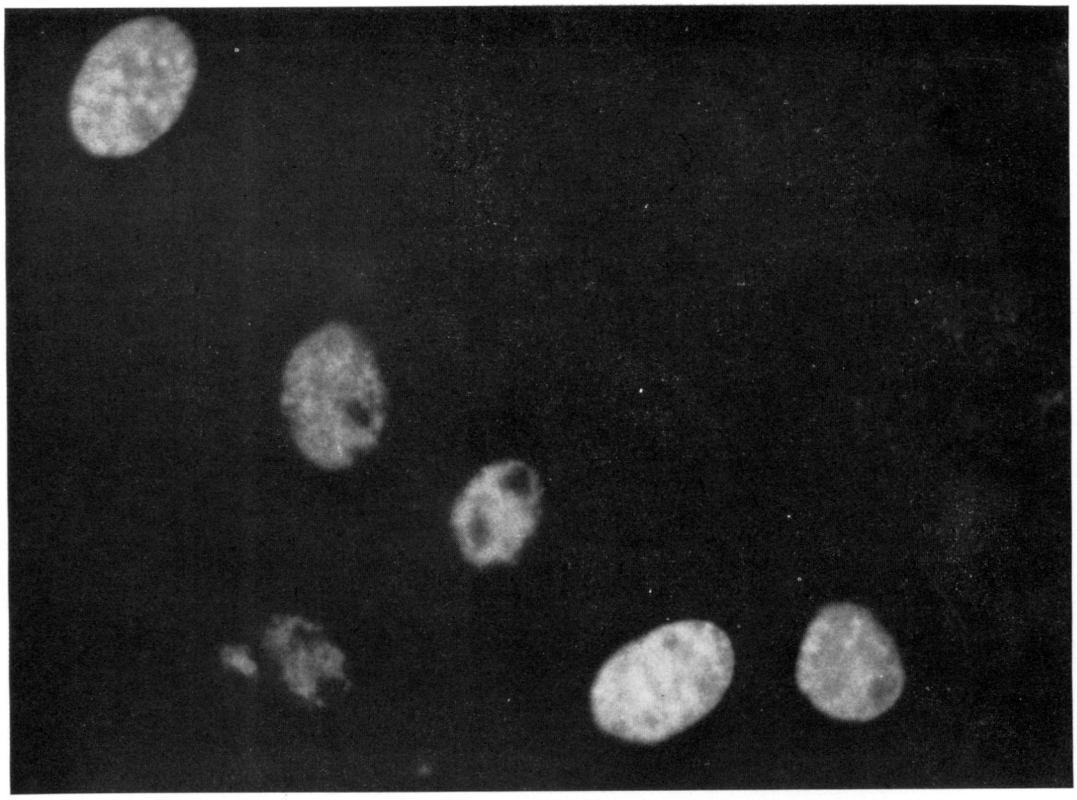


Fig. 2.

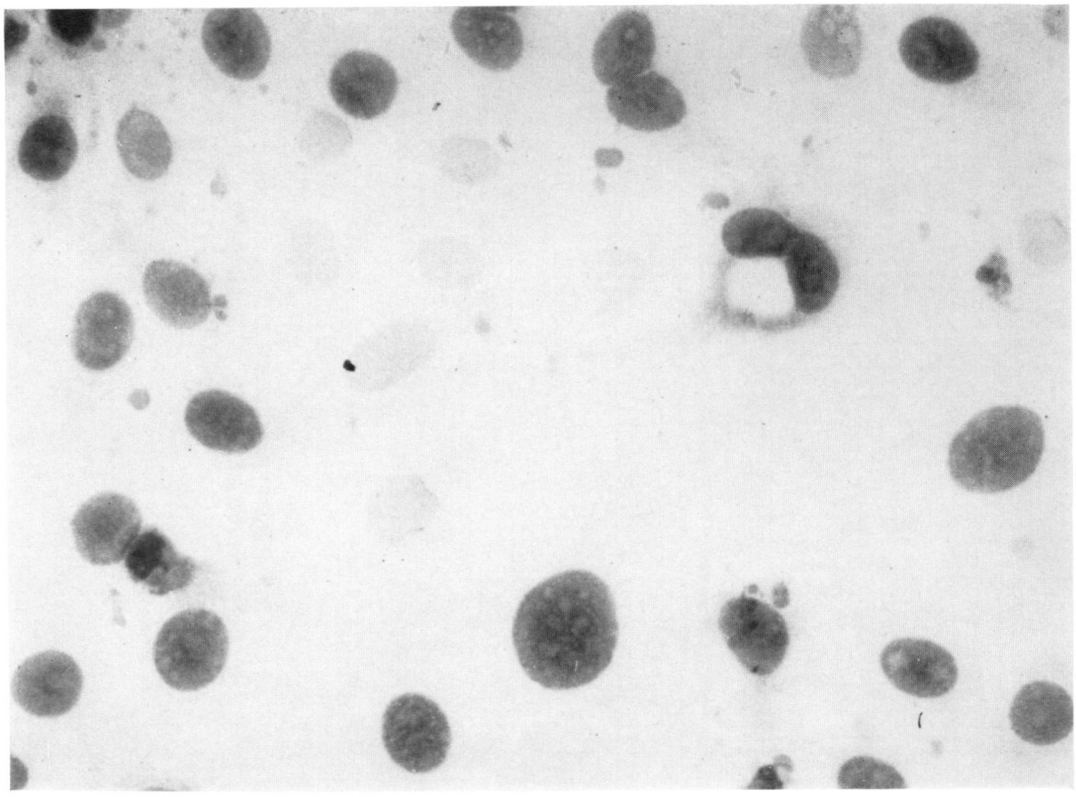


Fig. 3.

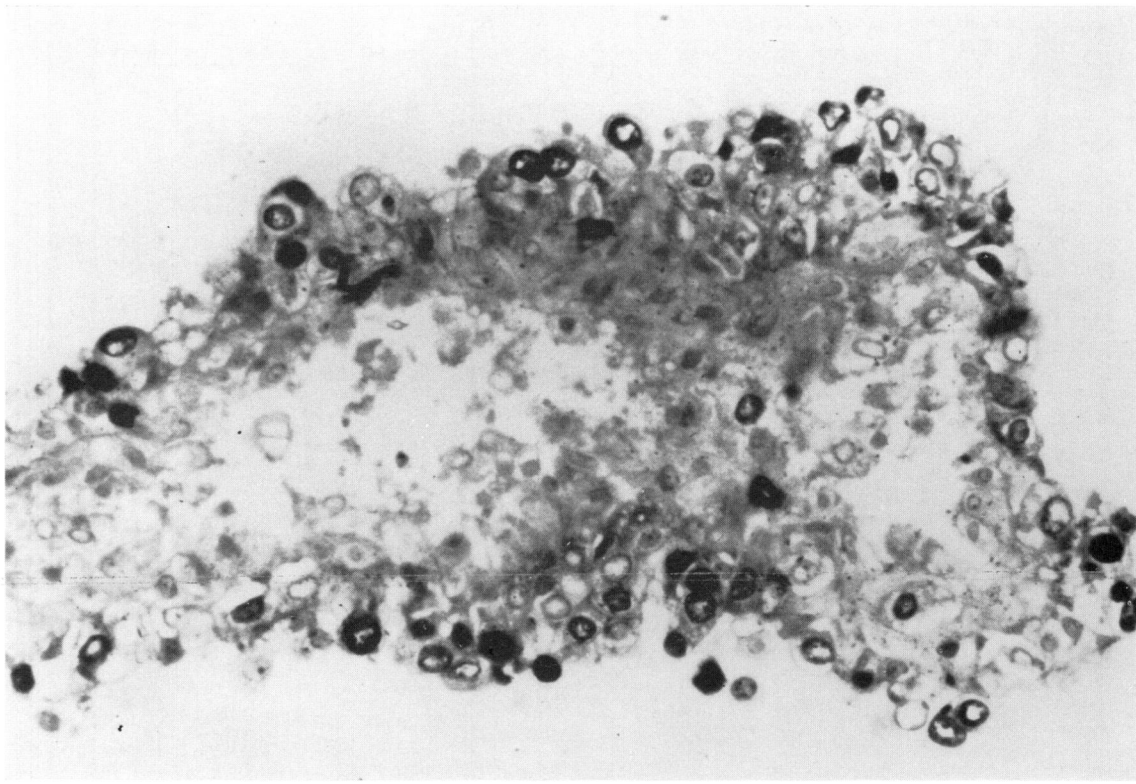


Fig. 4.

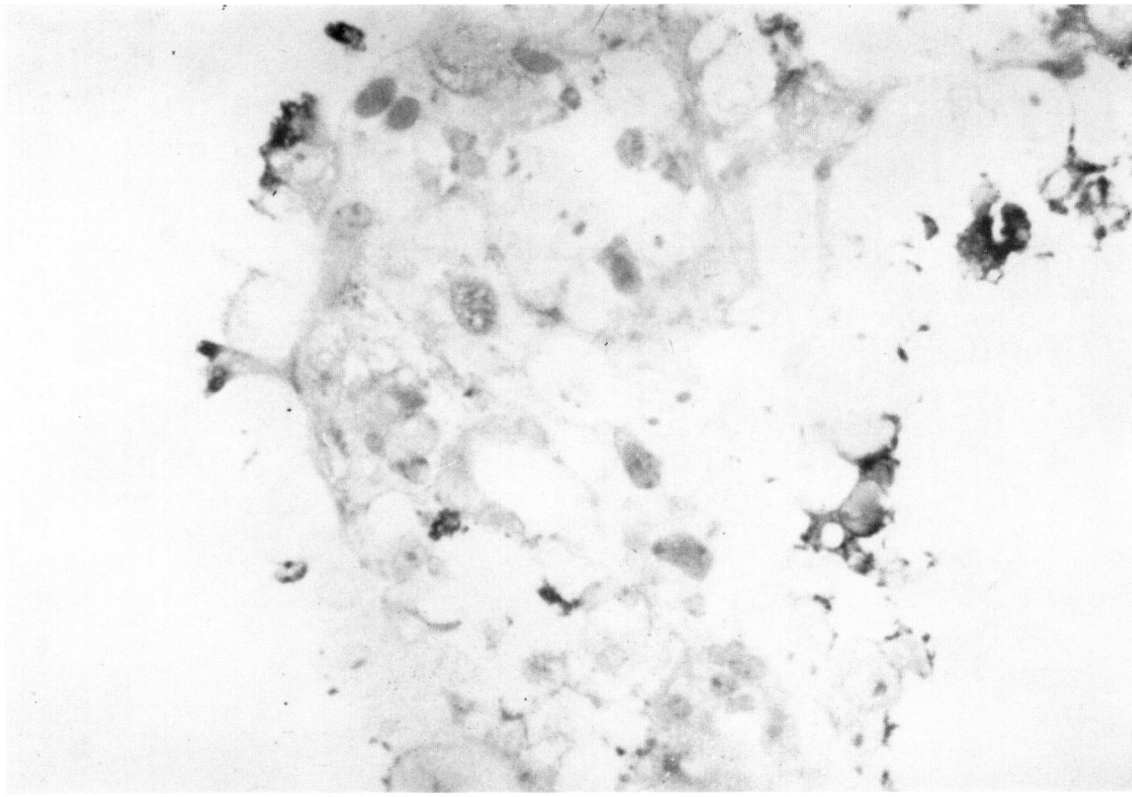


Fig. 5.

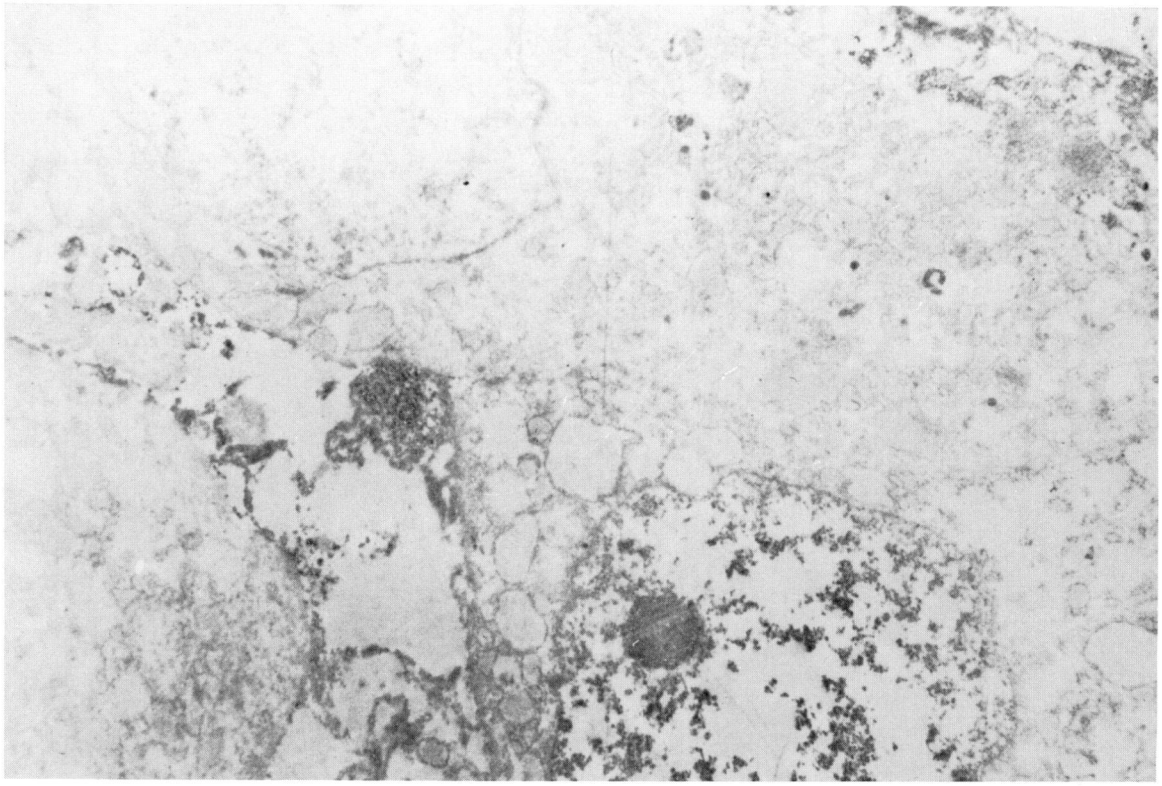
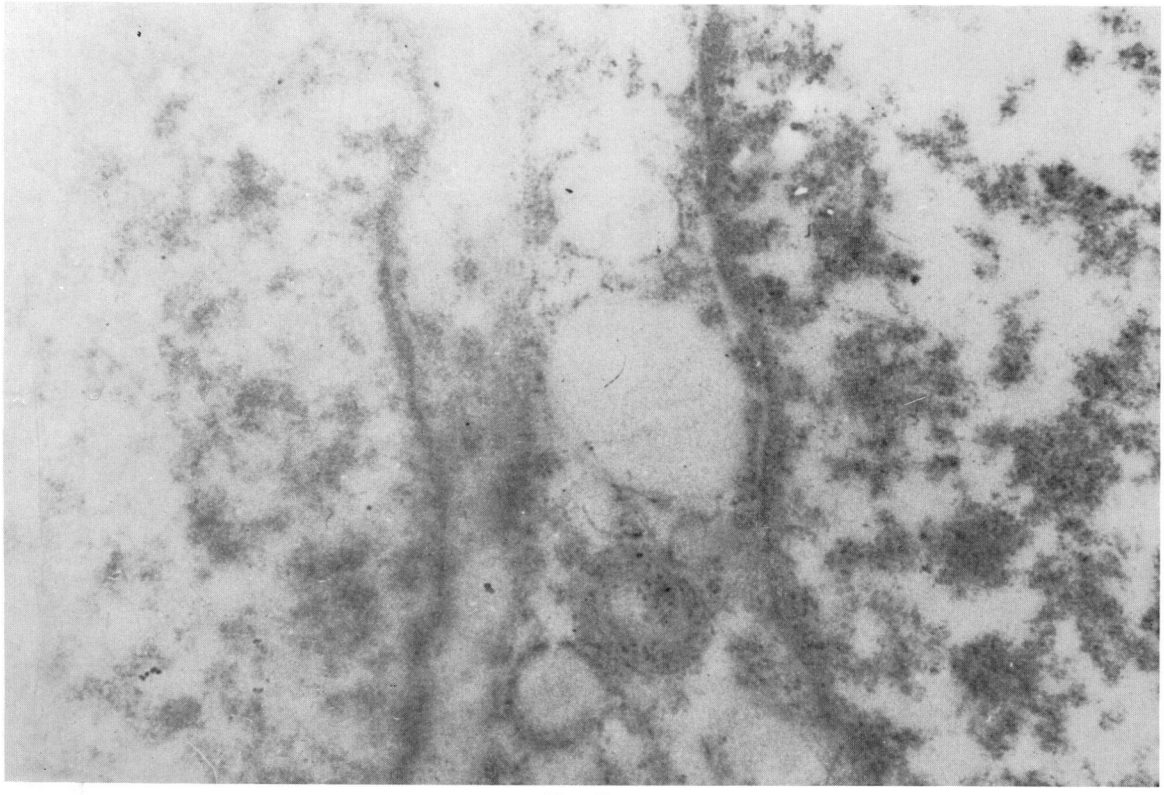


Fig. 6.



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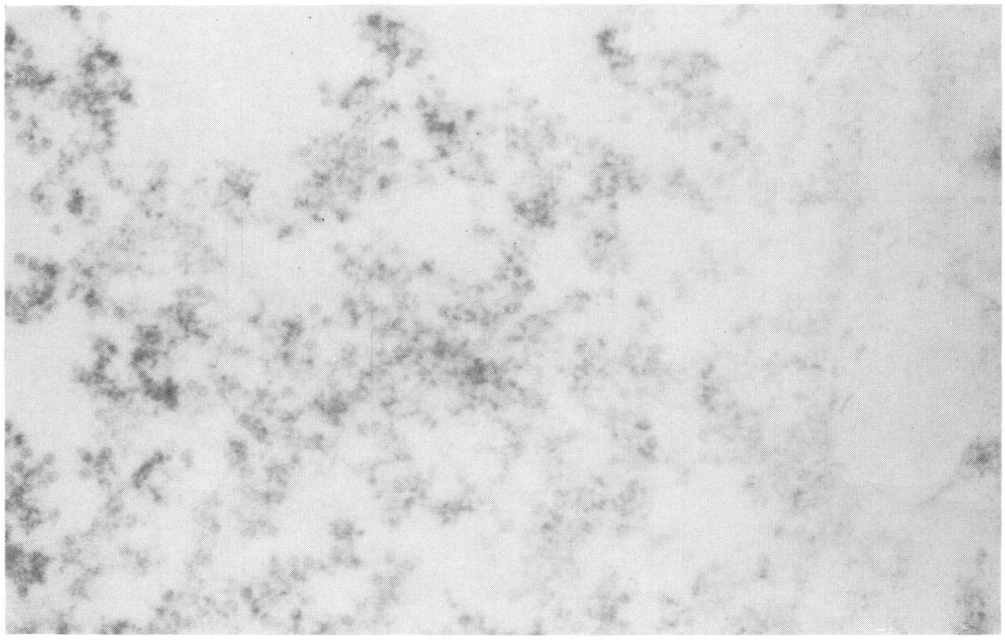

Fig. 8.

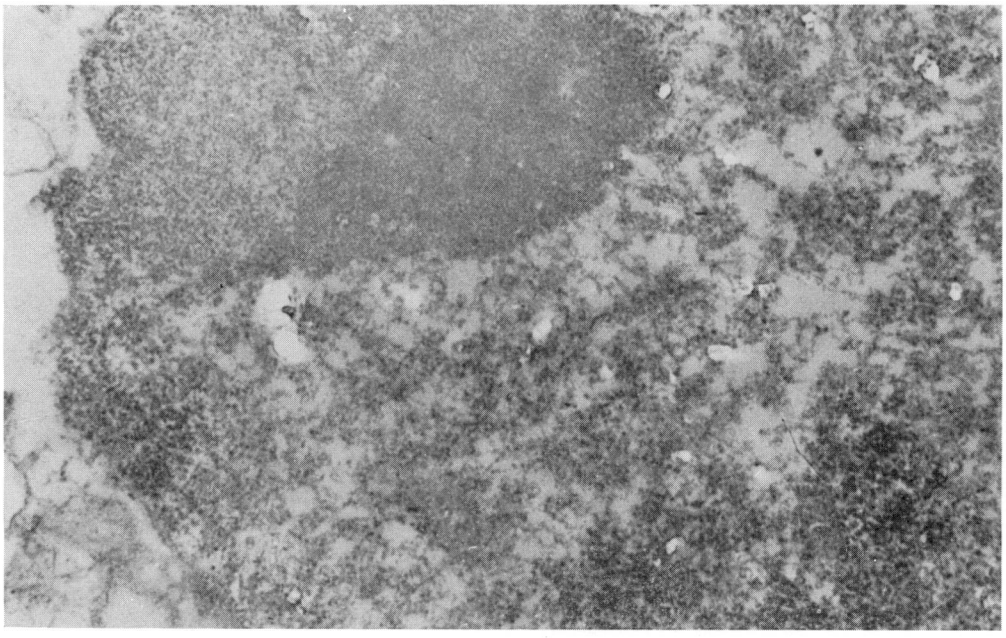

Fig. 9.

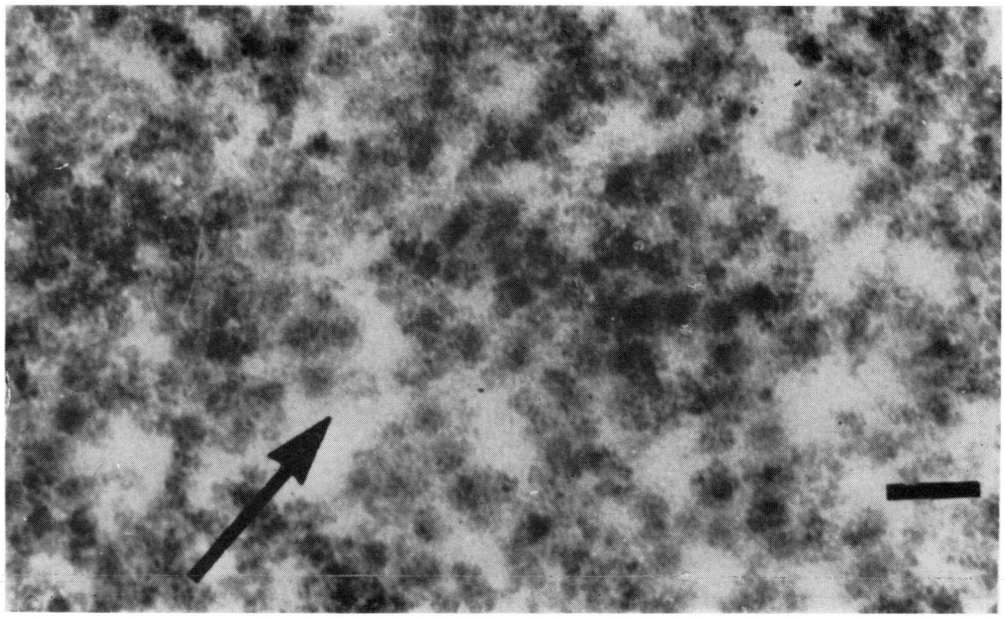

Fig. 10.