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Role of the cell surface in selection during transport of proteins from mother to foetus and newly born

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[Plates 16–21]

The transport of immunoglobulins from mother to foetus and newly born mammal involves selective events which are independent of molecular size, related to immunoglobulin class, structure, and species of origin, and involve considerable protein degradation. Such events are briefly described as background information to a discussion of how selection of proteins might take place during transport across the cellular barriers concerned, namely the yolk sac splanchnopleur, chorio-allantoic placenta, and small intestine. Until recently the Brambell hypothesis has been the most favoured explanation. This implies that selection occurs intracellularly, within endodermal cells of the yolk sac splanchnopleur and small intestine, and within the syncytiotrophoblast of the chorio-allantoic placenta, of certain species. It also suggests that specific receptors are present which give attached proteins protection from degradation when the vesicles containing them fuse with lysosomes; such protected proteins are then liberated from the vesicle by exocytosis. This hypothesis is examined in the light of what is now known about the mechanism of uptake and transport of proteins by the endodermal cells and syncytiotrophoblast. It is suggested that rather than being an intracellular event, involving protection from proteolytic degradation, selection takes place at the cell surface. Evidence is presented, some direct and some circumstantial, that proteins may be selectively endocytosed by coated micropinocytotic vesicles, and non-selectively endocytosed through a complex apical canalicular system leading to macropinocytotic vesicle formation. In the small intestine of the suckling rat these two processes appear to be segregated, selective uptake occurring in the proximal half and non-selective uptake occurring in the distal half. In the endodermal cells of the rabbit yolk sac splanchnopleur, and by implication in the syncytiotrophoblast of man and monkey, it is suggested that both selective, and non-selective, uptake of protein occurs. Non-selective uptake into macropinocytotic vesicles is regarded as an event leading to complete degradation of all contained protein and functioning so as to supply the foetus and newly born mammal with essential amino acids. Selective uptake into coated micropinocytotic vesicles is regarded as an event leading to the transport of immunoglobulins across the cell without any contact with lysosomes, and functioning so as to supply the newly born mammal with protection against invasive organisms. Specific receptors are still required but only for the initial uptake and segregation of proteins into coated micropinocytotic vesicles. The role which the glycocalyx might have in such selective binding of proteins is considered and possible difficulties in characterization of specific receptors brought to light in view of the likely overwhelming need for non-specific binding to effect non-selective uptake.

Introduction

The acquisition of maternal antibodies by foetal and newborn mammals provides a striking example of the ability of certain cellular barriers to allow large protein molecules to be transported across them, and the glycocalyx, which may justly be considered as part of the pericellular environment, could play an important regulatory role in this process. Such cellular barriers as I have alluded to are found at three sites: the yolk sac splanchnopleur, a foetal

membrane well established as the site for transmission of antibodies in the rabbit and guinea pig; the chorio-allantoic placenta, where transmission of antibodies would appear to take place in man and monkey; and the gut wall, across which antibodies contained in the colostrum and milk are transmitted when certain young mammals suckle, i.e. in the rat, mouse, hedgehog, cat, dog, pig, horse, sheep, goat and cow. The literature on this subject is extensive and has been reviewed in great detail by Brambell (1970); more recently I have reviewed literature pertaining to the cellular mechanisms involved (Wild 1973) and therefore in this introduction I shall make few references to published works, but merely give a summary of what I feel is appropriate background information.

Much interest has centred on the mechanism whereby antibodies are transported to the blood of the foetal and suckling mammal and especially on the process which enables some antibodies to be selected in preference to other proteins. Antibodies belong to different classes of immunoglobulin (principally IgG, IgA and IgM) which make up the γ-globulin fraction of the serum proteins. If one excludes ungulates, which show no evidence of selection during transport of proteins across the gut, and carnivores, on which few studies have been made, it is evident that in those other species mentioned γ-globulin is transferred in preference to other serum proteins. What is more, when the yolk sac splanchnopleur of the rabbit and guinea pig and the gut of the suckling rat, mouse and hedgehog are exposed to homologous and heterologous γ-globulins, selection operates in favour of the former type. Heterologous γ-globulins are transmitted to an extent which is dependent upon the species from which they originated. Selection also operates within classes of immunoglobulins and even within subclasses. Most species studied only show evidence of transport of IgG, but in the rabbit, IgM is also readily transported to the foetus (see Hemmings 1973). Recent investigations have also shown that little or no transport of IgA occurs to the rabbit foetus, or to the suckling rat (Hemmings, Jones & Williams 1973). In the mouse, where three subclasses of IgG are known to occur, IgG3 is transported more readily than IgG1 and IgG2 (Grey, Hirst & Cohn 1971; Gitlin 1971).

From our knowledge of the molecular masses of the serum proteins it is clear that size plays an unimportant role in their selection. For example, most studies have shown that albumin (mol. mass 60000) is transmitted less readily than IgG (mol. mass 150000). This has been further exemplified in studies of the transport of fragments of rabbit and human IgG to the rabbit foetus. Treatment of IgG with pepsin destroys the Fc piece but produces a smaller $F(ab')_2$ piece that still retains divalent antibody activity. Treatment of IgG with papain produces univalent Fab pieces (mol. mass 50000) and an intact Fc piece (mol. mass 80000). It can be inferred from studies carried out by Brambell, Hemmings & Oakley (1959) that $F(ab')_2$ is very poorly transmitted; on the other hand, Fc is transmitted very much better than Fab (Brambell, Hemmings, Oakley & Porter 1960). Immunoglobulins can also be split into heavy (H) and light (L) chains by cleavage of disulphide bonds. Human H chains from IgG and IgM (mol. mass 50000–75000) are readily transported to the rabbit foetus but L chains (mol. mass 22000–24000) hardly at all (Kaplan, Catsoulis & Franklin 1965). These findings have led to the conclusion that what determines the ability of immunoglobulins to be transported across cellular barriers of the type mentioned, is present, along with other adjunctive properties, in the C (constant) regions of the H chains that constitute Fc. At this point it is worth mentioning a further phenomenon, termed 'interference'. It has been shown that certain heterologous sera, when administered together with specific homologous antibodies to suckling

rats and mice, interfere with the transport of such antibodies to an extent which is again dependent upon the species from which the sera originated. Apart from IgG, other serum proteins do not exhibit interference properties. Morris (1963) has shown that when the Fc piece of rabbit IgG is administered to the suckling mouse along with IgG from other species, it interferes 3.5 times more readily with their transport than does the intact molecule; the Fab piece of rabbit IgG does not interfere at all. A detailed study of selection and interference during transport across the mouse gut has led Morris (1964) to conclude that selection and interference are probably different manifestations of a single mechanism.

Another feature relevant to the transmission of proteins must be mentioned in order to complete this background information. It concerns the amount of protein that reaches the foetal or newborn mammal from an administered dose. Hemmings (1957) found that of a given dose of rabbit immunoglobulins injected into the uterine lumen, and therefore available for transport across the yolk sac splanchnopleur to the rabbit foetus, only about 12 % was transmitted intact to the circulation. Similarly, it has been shown that only a small fraction (7.5–12 %) of rat IgG is transported intact from a given dose administered to the gut of the suckling rat (Bangham & Terry 1957; Brambell, Halliday & Hemmings 1961; Jones 1972). Much of the remaining protein can be accounted for by breakdown to amino acids within the yolk sac splanchnopleur and gut wall.

Somehow a balance has to be struck between the need to transport intact immunoglobulin to the foetal or newly born mammal in order to confer protection against invasive organisms, and the need to utilize proteins as a source of amino acids for the production of new, self proteins. What follows is a discussion of how this might be achieved which takes into account those other features of protein transport that have been referred to. I have tried to show that the evidence is pointing to selection operating at the cell surface through subtle differences in uptake of proteins, rather than by intracellular events as was formerly supposed.

Nature of the cell barriers

The yolk sac splanchnopleur and the gut of the suckling mammal are alike in that they have present an outer epithelium consisting of highly absorptive endodermal cells. Such cells are exposed to the proteinaceous contents of the uterine and gut lumen. In the haemochorial placenta of man and monkey there is present, in contact with the maternal blood, a layer of syncytiotrophoblast which from its ultrastructural appearance is also highly absorptive. Both cell types have a well developed microvillous border, a sub-apical canalicular system, and vesicles of varying sizes, the contents of which show varying degrees of electron density suggesting that some are phagolysosomes. Finer points concerning the structure of the apical regions and the nature of the vesicles within the cells are considered later. There are important differences between the proximal and distal regions of the small intestine which, to be more precise, are those regions of the gut most concerned in uptake and transport of proteins. These differences will also be considered in more detail later; suffice it to say at this stage that whereas the distal (ileal) region of the small intestine has long been considered to be the major site for transport of proteins, more recent evidence (Rodewald 1970, 1973) points to the proximal region.

The absorptive epithelial cells constituting this outer barrier have tight cell junctions at their apices which appear to preclude entry of protein through intercellular spaces. Proteins are in

fact endocytosed, both by macropinocytosis and by micropinocytosis. The absorptive cells rest upon a basement membrane which may also be regarded as part of the pericellular environment. Proteins that reach the basement membrane have to gain access to the underlying vessels and capillaries in the yolk sac splanchnopleur and chorio-allantoic placenta. These often lie in close apposition to the basement membrane but may also be separated by a loose mesenchymal layer consisting of collagen fibrils interspersed with fibroblasts and macrophages. In the small intestine, blood capillaries and lymphatic vessels abut closely on the basement membrane of the epithelium, but may also be separated by mesenchymal tissue. At all three sites, in order for proteins to reach the blood of the foetal and newly born mammal they must pass intercellularly or intracellularly, through endothelial cells. There are thus a number of regions at which selective processes could operate but the available evidence points only to the endodermal cells and syncytiotrophoblast.

The Brambell hypothesis

In 1958, Brambell, Halliday & Morris, proposed that selection could be most easily understood by assuming the presence of receptors within the absorptive endodermal cells of the small intestine since competition for such receptors would readily explain interference. The receptor hypothesis has since been reformulated to account for the degradation of much of the protein that takes place during transport and can equally be applied to the transport of proteins across the yolk sac splanchnopleur and chorio-allantoic placenta (see Brambell, Hemmings & Morris 1964; Brambell 1966, 1970). Elsewhere (Wild 1974) I have referred to this as the Brambell hypothesis and it is convenient to do so here.

In this hypothesis it is suggested that proteins enter the absorptive cells non-selectively by endocytosis. Proteins which will ultimately be transported across the cells without degradation and find their way to the circulation, become attached to receptors on the cell surface and are then carried inwards by surface invaginations (presumably along with non-attached proteins), so that ultimately they lie on the inner wall of the apical vesicles which characterize these cells. Since only a finite number of receptors can be present, when these are saturated the remaining protein molecules will lie free in the vesicle even though they may be capable of attachment. On fusion of such vesicles with lysosomes this free, unattached protein will be degraded by proteolytic enzymes, but in some way attached proteins gain protection and are ultimately discharged from the cell by exocytosis. Attachment to those receptors specific for immunoglobulins is presumed to be through part of the C region of the H chains.

It is easy to see how selection would operate in this hypothetical system. If the cells are exposed to IgG from a different species, transmission of this protein across the cell will depend upon how well the corresponding attachment site on the H chains is fitted to the receptor. Figure 1 depicts the transport of rabbit and bovine IgG across the endodermal cells of the rabbit yolk sac splanchnopleur as envisaged in the Brambell hypothesis. Compared to rabbit IgG, bovine IgG is transported at a very much lower rate (Hemmings 1956, 1961; Brambell 1966) and on this hypothesis the reason is that bovine IgG fits the receptors very badly so that virtually all remains free in the vesicle and is broken down in phagolysosomes. Interference is interpreted on the basis that molecules of IgG of one species occupy the receptors temporarily, so blocking access, during that period, to IgG of other species. Morris (1968) has suggested that the reason why rabbit Fc interferes so much more readily than the whole molecule is because

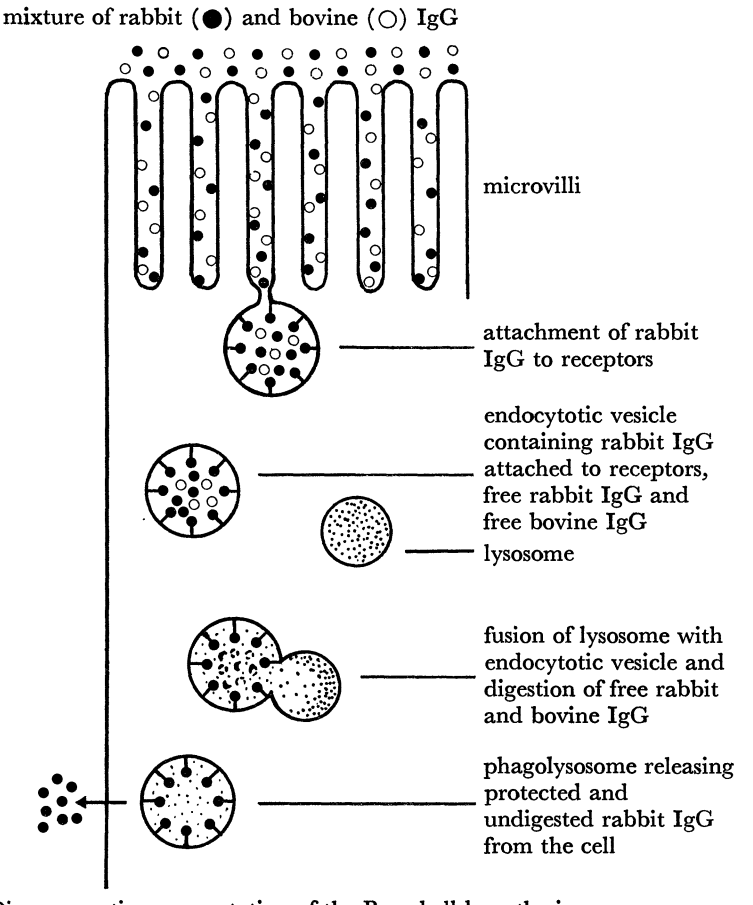


FIGURE 1. Diagrammatic representation of the Brambell hypothesis.

removal of the Fab piece brings about a reduction in steric hindrance, so leading to a greater affinity by Fc for the receptor.

LOCALIZATION OF PROTEINS DURING TRANSPORT

A number of attempts have been made to localize proteins during their transport across the yolk sac splanchnopleur, intestinal wall and chorio-allantoic placenta: in some cases at the light microscope level using fluorescent protein tracing, in others at the electron microscope level using electron dense proteins or reaction products in the case of enzymes; inferences have also been made about protein transport simply from the normal ultrastructural appearances of the absorptive cells (reviewed in Wild 1973). My own investigations have been concerned with transport across the yolk sac splanchnopleur. By injecting proteins labelled with fluorescein isothiocyanate (FITC) or normal proteins, into the uterine lumen of pregnant rabbits, and detecting the proteins in sections of the yolk sac splanchnopleur by means of the attached fluorescent label, or by means of the specific fluorescence produced after treatment of sections with FITC or Lissamine Rhodamine B 200Cl (RB200Cl) labelled antibodies, it has been possible to confirm that the yolk sac endoderm is the site of protein selection. A wide variety of proteins were found to become localized in vesicles (now recognized as macropinocytotic vesicles) within the yolk sac endoderm, but only proteins readily transported to the foetal blood

(rabbit and human IgG) were found in the basement membrane and below it in the vascular mesenchyme and vitelline vessels (Wild 1970). Human and bovine IgG have been injected simultaneously into the uterine lumen and both proteins subsequently detected in the same sections of yolk sac splanchnopleur by applying a mixture of FITC and RB200Cl-labelled antibodies directed against either human or bovine IgG. The red or green fluorescence indicative of one or other proteins can be viewed sequentially by use of appropriate filter systems and sections treated in this way reveal an identical localization of both immunoglobulins within vesicles in the yolk sac endoderm (figure 3, plate 16). Unlike human IgG, bovine IgG cannot be detected in or below the basement membrane or in the foetal blood, at least after the short duration (1–5 h) of these experiments.

Previously, I have interpreted these findings as evidence for the intracellular selection of proteins during their transport across the yolk sac endoderm, but in the light of further evidence, other explanations now seem more likely. There is no doubt that these results demonstrate the non-selective uptake of proteins into vesicles: indeed, we have confirmed this at the ultrastructural level. Ferritin, which is not transported to the foetal rabbit circulation but which is readily endocytosed by the yolk sac endoderm (Slade & Wild 1971), was injected together with ^{125}I-labelled human IgG into the uterine lumen. By combining electromicroscopy with autoradiography, both proteins were subsequently detected in the yolk sac endoderm and shown to be present in the same vesicles (Wild, Stauber & Slade 1972). Such proteins had a distribution within the vesicle similar to that seen by fluorescence microscopy, i.e. closely associated with the vesicle wall or diffusely distributed within the vesicle. What is questionable however, is whether or not such vesicles liberate any of the protein they contain. In all of our studies we have never, to our satisfaction, observed fusion of protein-containing vesicles with the lateral or basal plasmalemma, although as will be discussed later, we have frequently observed fusion of much smaller, coated micropinocytotic vesicles. Assuming the Brambell hypothesis to be the correct explanation, we had hoped to find a difference in distribution of 'transmitted' and 'non-transmitted' proteins within the vesicles, but this was not the case. We had also expected those vesicles lying closer to the basement membrane to contain only the protein to be transmitted, the rest having been degraded, but again this was not the case.

Localization and role of proteolytic enzymes

If selection were to operate as suggested in the Brambell hypothesis one might also expect proteolytic enzymes, as well as protected proteins, to be exocytosed and appear in the basement membrane since enzymes also ought not to be degraded. We have attempted to discover whether or not such transport of proteolytic enzymes takes place by looking at the distribution of Cathepsin D (the major proteolytic enzyme) in the yolk sac splanchnopleur. Antisera to rabbit Cathepsin D (see Weston & Poole 1973) were conjugated to FITC and applied to frozen or wax embedded sections of normal tissue or tissue that had been exposed to heterologous immunoglobulins. Specific antiserum and antiserum which was less specific but with a higher antibody activity, were employed. Specific fluorescence indicative of Cathepsin D was confined to vesicles present predominantly in the yolk sac endoderm (figure 4, plate 17). Cathepsin D could not be detected in the basement membrane or in the vascular mesenchyme. No fluorescence could be detected in these sites with the less specific but more potent antiserum. As far as could be determined, all vesicles containing immunoglobulin contained Cathepsin D. These

results suggest that vesicles containing proteolytic enzymes and proteins, never fuse with the basal or lateral plasmalemma; in fact this might be considered a hazardous process, unless of course the proteolytic and other hydrolytic enzymes were neutralized in some way.

The relevance of proteolytic enzymes in the process of selective transmission of proteins across the suckling rat gut has also been questioned on the grounds that ^{125}I-labelled PVP (polyvinyl pyrrolidone) K60 mol. mass 160000, is not transmitted to the circulation (Clarke & Hardy 1969). Transmission fails to occur despite the fact that PVP K60 is readily endocytosed by ileal cells and is not broken down by enzymes. Similarly, ^{125}I-labelled PVP K60 is not transported to the circulation of the foetal rabbit in significant amount (less than bovine IgG, for example) when injected into the uterine lumen (Wild 1974). Human IgG injected into the uterine lumen along with PVP is readily transported however, showing that PVP has not in any way interfered with the normal transport mechanism. PVP is readily endocytosed by the yolk sac endoderm and it seems likely that it enters the same vesicles as immunoglobulins.

From the evidence outlined here I think it is reasonable to conclude that macropinocytotic vesicles, which have been involved in non-selective uptake of proteins and have fused with lysosomes, are not involved in selective transport of proteins. We must therefore look to some other mechanism for their transport and selection.

Role of coated micropinocytosis vesicles in selection

As mentioned previously, it has often been assumed that the distal (ileal) region of the small intestine of the suckling rat (and mouse) is the site principally involved in transport of proteins to the circulation. Since a number of workers have located a wide range of proteins and other macromolecules in macropinocytotic vesicles in ileal epithelial cells, it follows that this should have also been assumed to be the site for their selection and the mechanism to be an intracellular one. However, as with studies on transport of proteins across the yolk sac splanchnopleur, evidence that such vesicles fuse with the lateral or basal plasmalemma and discharge their protein has not been obtained. By isolating proximal and distal regions of the small intestine *in situ*, and introducing antibodies to ferritin into the lumen, Rodewald (1970) found that the proximal region, contrary to current opinion, transported more antibody to the circulation than did the distal region. Rodewald (1973) has subsequently investigated the transport of homologous and heterologous ferritin conjugated immunoglobulins (IgG-Ft) and antibodies to peroxidase, across the proximal region of the small intestine, localizing such proteins at the ultrastructural level by virtue of the attached electron dense ferritin or by virtue of the electron dense reaction product produced by peroxidase after it had been allowed to combine with antibody and appropriately treated. His results provide strong evidence that selection takes place during the initial stage of protein uptake, and not as a result of any intracellular process. Rat and bovine IgG-Ft and rat antibodies to peroxidase (but not chicken IgG-Ft, bovine serum albumin-Ft, or chicken antibodies to peroxidase, or free ferritin), became attached to the amorphous coating (glycocalyx) on caveolae formed at the apical cell surface. These proteins were also found to become localized in apical tubules and coated micropinocytotic vesicles, the latter sometimes budded off from the former, and coated micropinocytotic vesicles were observed fusing with the lateral plasmalemma apparently discharging their contents. Rodewald also looked at the transport of rat IgG-Ft, free ferritin, and rat antibodies to peroxidase, across the distal region of the small intestine. Here, proteins became localized in larger

apical macropinocytotic vesicles and in the large supranuclear vesicle which is so characteristic of these distal cells. There was no evidence of discharge of proteins from the vesicles into the intercellular space. Few coated vesicles were observed in distal cells, and few large macropinocytotic vesicles in proximal cells. It seems reasonable to suppose that had they been investigated, those other proteins that did not enter proximal cells would have entered distal cells through non-selective uptake. Rodewald's results show that the capacity for selective and non-selective uptake of proteins is segregated in different regions of the small intestine. He concludes that non-selective uptake of proteins by macropinocytotic vesicles results in degradation of all contained protein. It is interesting that Rodewald found no inhibition of transport of immunoglobulin by conjugating it to ferritin, since in our studies on the yolk sac splanchnopleur, such a process appeared to prevent completely the transmission of human IgG, although it did not prevent its uptake into macropinocytotic vesicles (Slade & Wild 1971).

We have frequently observed coated micropinocytotic vesicles in the yolk sac endoderm, most often apparently fusing with the basal plasmalemma (figure 4*b*, plate 17) and also apparently forming at the base of microvilli (figure 5*b*, plate 18). In view of Rodewald's findings on proximal epithelial cells of the small intestine a new significance attaches to their presence and it is tempting to believe that here, too, they are engaged in selective uptake and subsequent discharge of protein. Their small size (0.07–0.15 μm) would make any protein they contain difficult to visualize by fluorescence microscopy and even the silver grains observed in electronmicrographs after e.m. autoradiography would probably have obscured them. It would seem that in the yolk sac endoderm, unlike the situation pertaining in the epithelium of the small intestine, coated micropinocytotic vesicles and macropinocytotic vesicles exist together in the same cells. Assuming that coated micropinocytotic vesicles have a selective role to play in transport of proteins across the yolk sac endoderm I have depicted in figure 2 how this might operate when the cells are exposed to a mixture of rabbit and bovine IgG. Virtually all of the bovine IgG and a large part of the rabbit IgG is taken up non-selectively into macropinocytotic vesicles which subsequently fuse with lysosomes. All the protein contained in the phagolysosomes is digested and the resulting amino acids transported to the foetus for incorporation into proteins. A small percentage of rabbit IgG and virtually no bovine IgG is taken up into coated micropinocytotic vesicles. These escape fusion with lysosomes and on fusing with the lateral or basal plasmalemma discharge their contained protein into the basement membrane or lower regions of the intercellular space. Such a mechanism would more satisfactorily explain the findings for protein transport in the yolk sac endoderm than would the Brambell hypothesis.

Coated micropinocytotic vesicles have been described in the syncytiotrophoblast of the guinea pig chorio-allantoic placenta and shown to be involved in uptake and discharge of ferritin during its transport across this cell layer, albeit on a small scale (King & Enders 1971). They also feature in human syncytiotrophoblast, along with many other types of vesicles and multivesiculate bodies that might reasonably be presumed to be involved in protein digestion (for example see plate 1 (2), in the paper by Martin & Spicer 1973). They are a prominent feature at the surface of the mosquito oocyte, where according to Roth & Porter (1964) they function so as to transport selectively yolk proteins, manufactured in the midgut region and secreted in the haemolymph, to the vitellus. They have also been implicated in the selective uptake of haemolymph proteins by the pericardial cells of the aphid (Bowers 1964); in the uptake of proteins by the epithelium of the rat vas deferens (Friend & Farquhar 1967) and in the uptake of ferritin by macrophages (Lagunoff & Curran 1972), to mention just a few examples

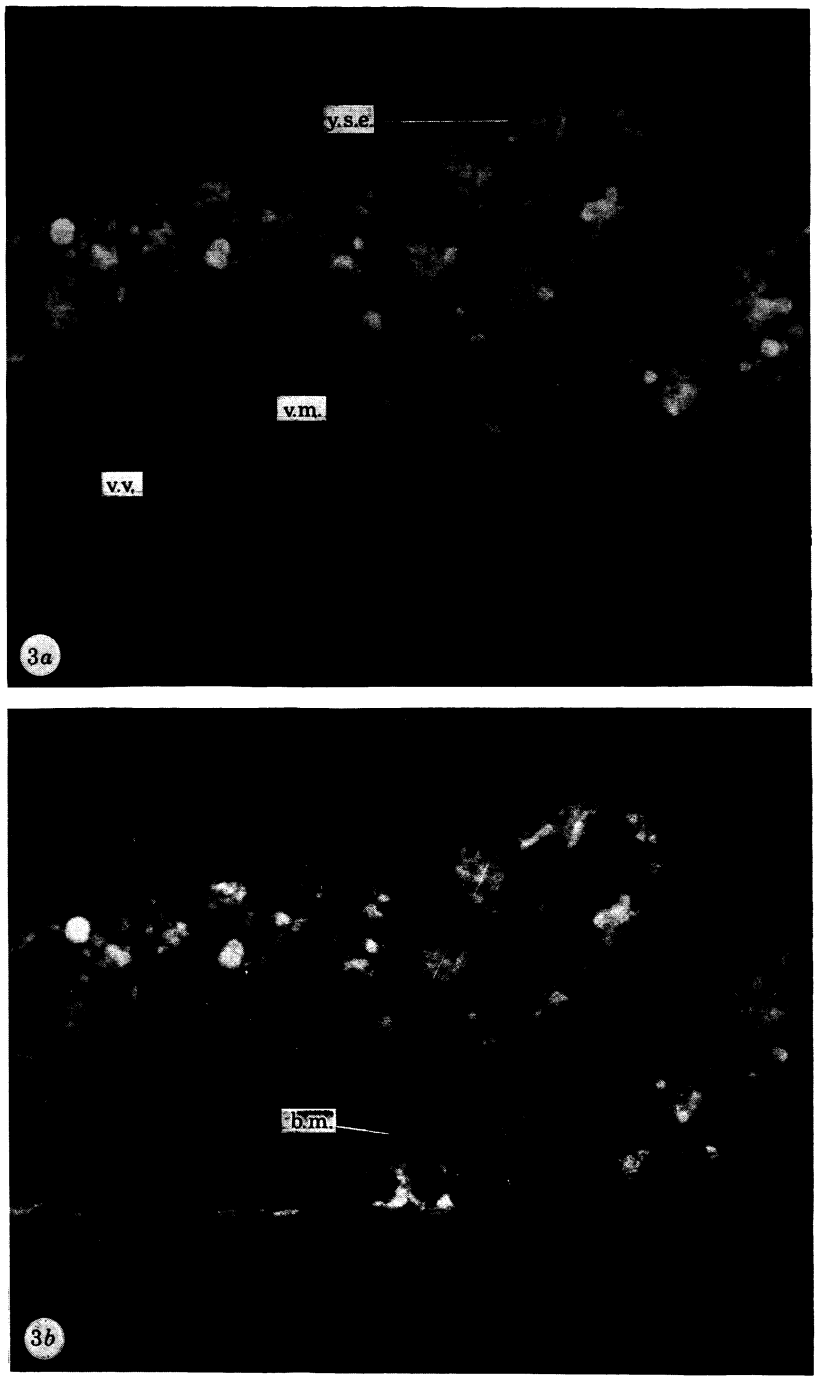
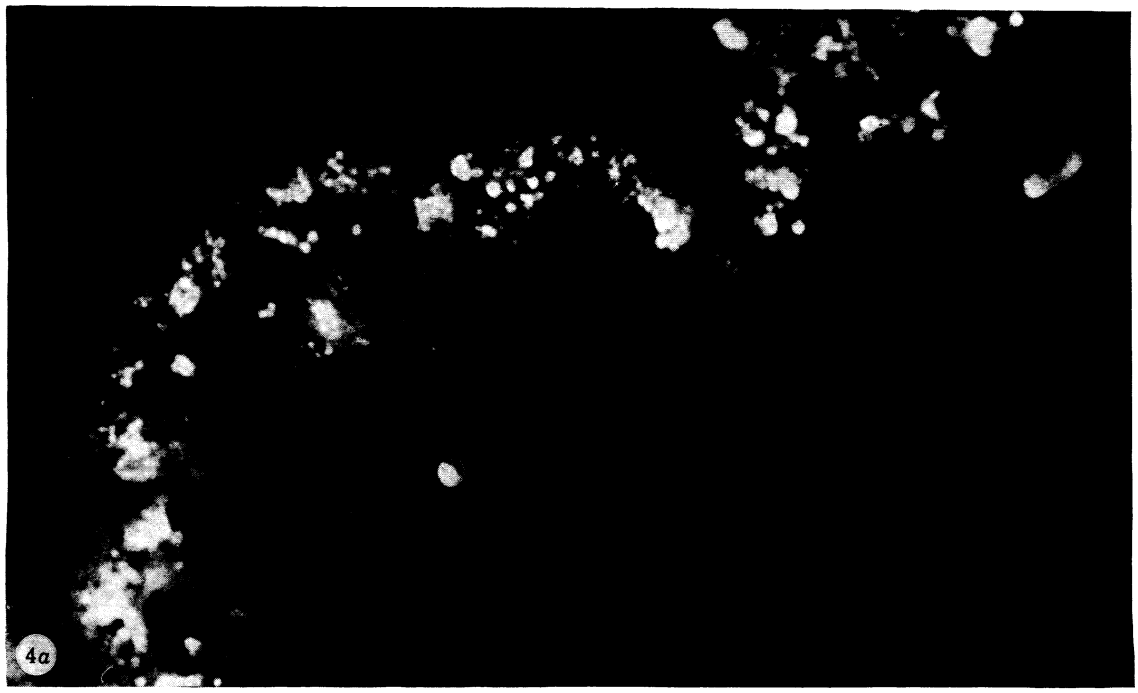


FIGURE 3(*a*). Section of yolk sac splanchnopleur from a 26 day old rabbit conceptus that had been exposed to a mixture of 1% bovine IgG and 1% human IgG for 4 h. The section was treated with both FITC-labelled rabbit anti-human IgG and RB200Cl-labelled rabbit anti-bovine IgG. Specific fluorescence due only to RB200Cl-labelled antibodies was visualized in a Leitz Ortholux fluorescence microscope fitted with a Ploem vertical illuminator (filter combination S 546+BG 36/K 610). Bovine IgG can be seen to be present in macropinocytotic vesicles in the yolk sac endoderm (y.s.e.). Other abbreviations: v.v., vitelline vessel; v.m., vascular mesenchyme. (Magn. × 375.) (From Wild 1974.)

(*b*). The same section as shown in (*a*) but now visualized for specific fluorescence due only to FITC-labelled antibodies (filter combination 2 KP 490/S 525). Note that human IgG has the same localization as bovine IgG in macropinocytotic vesicles in the yolk sac endoderm, but in addition is present in the basement membrane (b.m.), vascular mesenchyme and within the vitelline vessels. (Magn. × 375.) (From Wild 1974.)

(*Facing p.* 402)



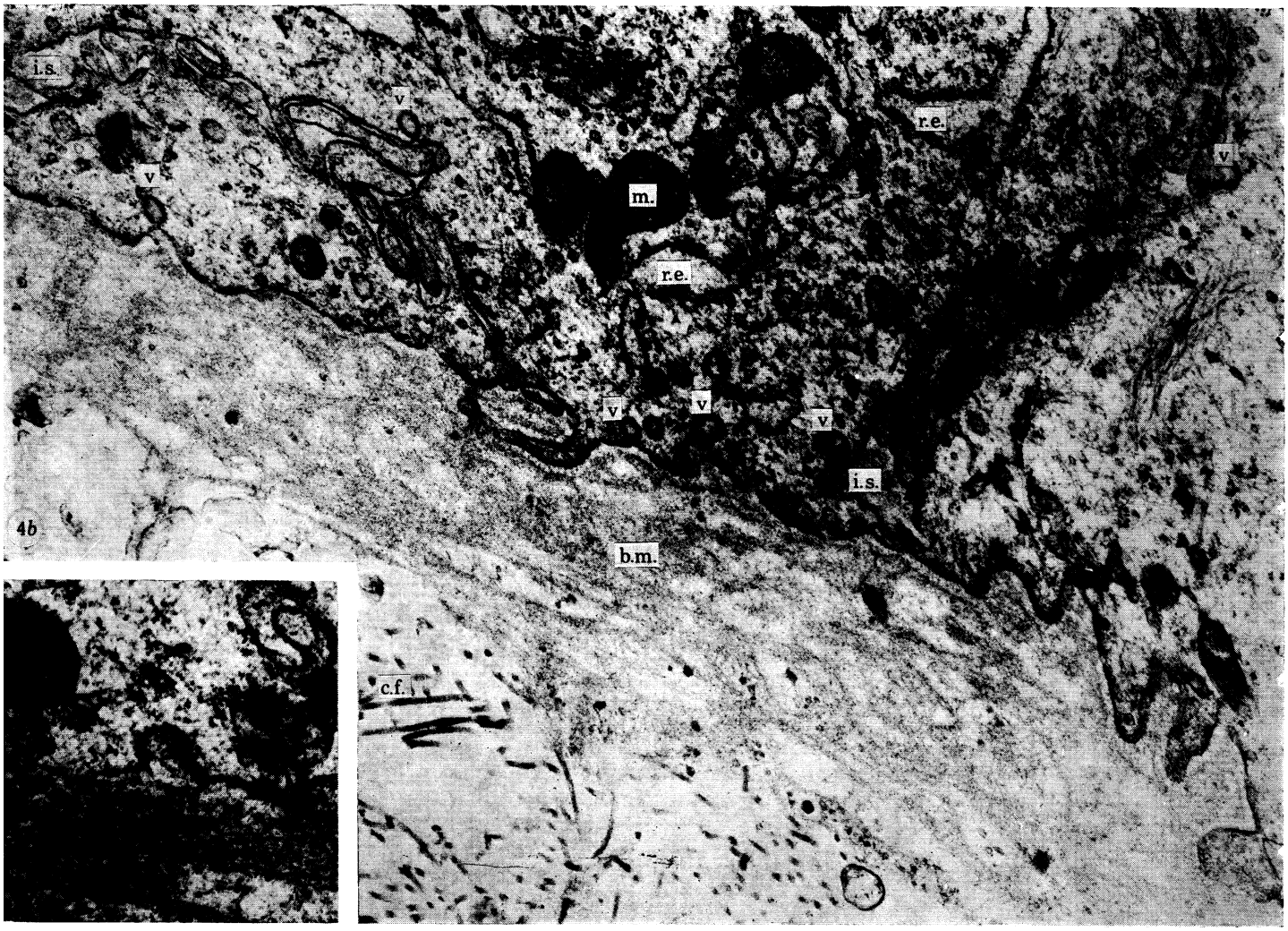


FIGURE 4. For description see opposite.

of their wide occurrence in protein absorbing cells. Bowers (1964) observed coated vesicles in surface view and described them as being composed of hexagonal structures. Kaneski & Kadota (1969) have made a detailed study of coated micropinocytotic vesicles isolated from nerve endings of guinea pig brain, and shown by shadowing techniques that they consist of an inner vesicle surrounded by a spherical polygonal 'basketwork'. The projections which make up the coat, referred to as 'bristles' by some workers, are regarded by Kaneski & Kadota as the superimposed images of the regular hexagons and pentagons which make up the basketwork.

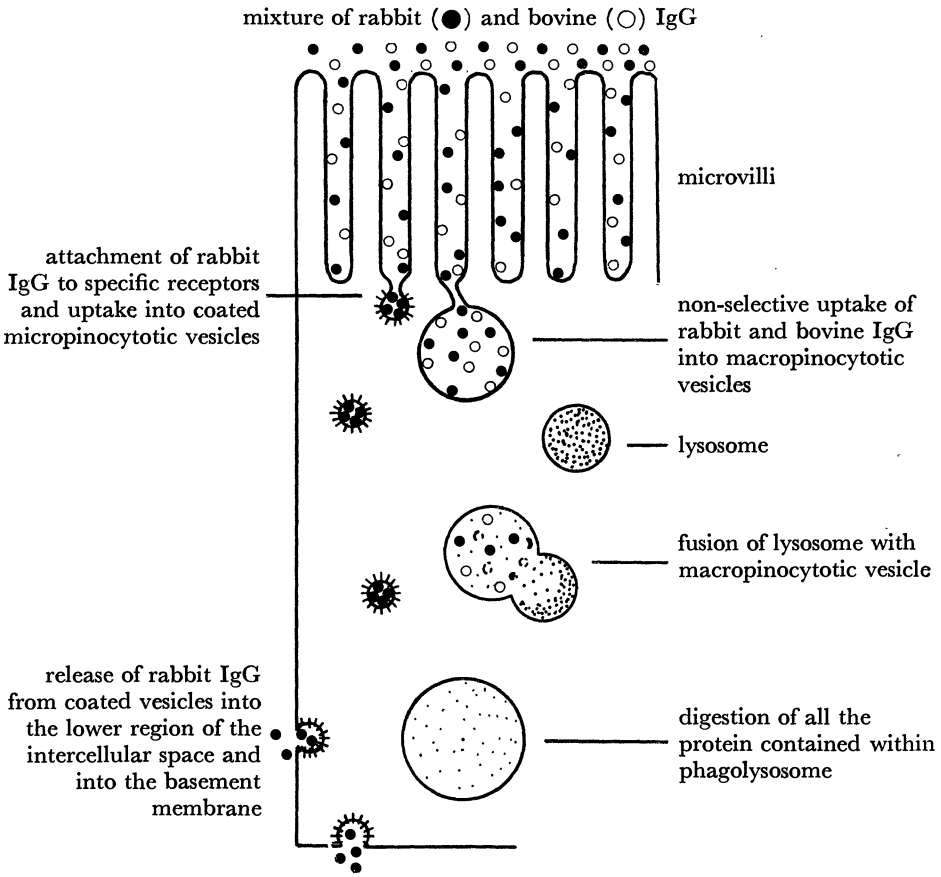


FIGURE 2. Possible mechanism of selection involving selective and non-selective uptake of proteins into endodermal cells in the yolk sac splanchnopleur.

DESCRIPTION OF PLATE 17

FIGURE 4(*a*). Section of normal yolk sac splanchnopleur from a 26 day old rabbit conceptus. The section was treated with FITC-labelled specific sheep antiserum to rabbit Cathepsin D. Specific fluorescence indicative of Cathepsin D is present only in vesicles in the yolk sac endoderm. Bright, non-specific fluorescence due to red blood cells is present in a vitelline vessel (top right). (Magn. × 350.)

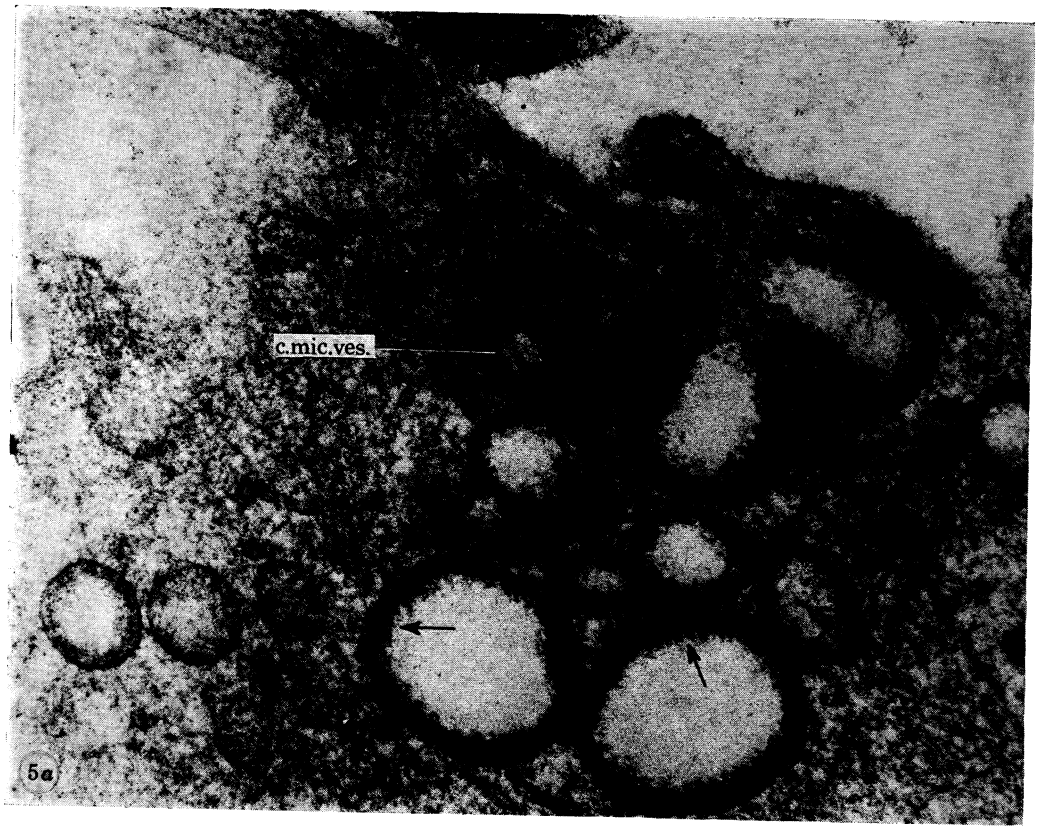
(*b*). Electronmicrograph of yolk sac splanchnopleur from a 26 day old rabbit conceptus showing the basal region of three adjoining endodermal cells. Coated micropinocytotic vesicles (arrow heads) can be seen confluent with the basal plasmalemma and the lower regions of the lateral plasmalemma. In this particular area the intercellular spaces (i.s.) do not appear to be occluded by tight cell junctions. The basement membrane (b.m.) displays characteristic vacuities and is underlined by collagen fibrils (c.f.) which together with macrophages and fibroblasts (not evident) make up the vascular mesenchyme. Other abbreviations: m., mitochondrion; r.e., rough endoplasmic reticulum. (Magn. × 34 300.)

Inset: a higher power view of a coated micropinocytotic vesicle in confluence with the basal plasmalemma. Note the characteristic 'bristles' on the cytoplasmic surface of the vesicle membrane. (Magn. × 82000.)

Once the vesicles have entered the vitellus in the mosquito oocyte the coating is lost, the vesicles fuse together, and crystalline yolk bodies are formed from the contained yolk protein (Roth & Porter 1964). Loss of the coat from micropinocytotic vesicles has been reported in other absorptive epithelia, for example in aphid pericardial cells (Bowers 1964) and in proximal kidney tubule cells (Maunsbach 1966). However, as in the case of the mosquito oocyte, these are sites where one would suppose that little subsequent export of endocytosed protein took place. The coat appears to be retained in yolk sac endoderm, proximal epithelium of the small intestine, and syncytiotrophoblast, and I suggest that its presence here is needed in order to prevent vesicles from fusing with lysosomes.

The cell surface and receptors

So far I have made little mention of the cell surface, but it follows that if the mechanism of selection is as I have outlined here, then the cell surface is the controlling factor. Differences must exist between the cell surface of proximal and distal cells in the suckling rat small intestine to account for the different mechanism of protein uptake. It would also seem that differences exist between the cell surface of proximal cells of the 22 and 10 day old rat, since at the older age, when antibody transmission has virtually ceased, Rodewald (1973) could find no selective binding or uptake of rat IgG-Ft. If the mechanism of uptake and transport of proteins operates in the rabbit yolk sac endoderm in the way I have outlined, then differences must exist here between different regions of the same cell. In order for coated micropinocytotic vesicles to select proteins, it is still necessary to assume the presence of receptors as originally envisaged by Brambell and co-workers, and the most likely place for them to be found is in the glycocalyx. This mucopolysaccharide coating can be readily demonstrated by ruthenium red labelling and in the rat (Jollie & Triche 1971) and rabbit (Wild 1973) yolk sac endoderm, where such treatment has been applied, the glycocalyx appears as a thin coating over the surface of microvilli and as a dense accumulation on caveolae formed at the base of microvilli (figure 6, plate 19). In the sub-apical region of these cells, and also in the ileal cells of the young rat intestine, there is an extensive system of tubules which are lined by a mucopolysaccharide coat and which ultimately terminate in the larger macropinocytotic vesicles. As can be demonstrated by treating fixed tissue with ruthenium red, much of this apical canalicular system is in open communication with the exterior, and it is through this system of tubules that non-selective uptake of proteins appears to take place. Wissig & Graney (1968) have made a detailed study of the coating present on the luminal surface of the extensively branching and anastomosing apical tubular canalicular system of suckling rat ileal cells and shown it to consist of an ordered array of plaques, each with a dense particle on it. Evidence of an ordered but less well defined structure can be discerned in the coating lining the apical vesicles and tubules of rabbit yolk sac endoderm (figure 5*a*, plate 18). Ferritin (Slade 1970; King & Enders 1970; Lambson 1966) and other macromolecular substances such as thorotrast (Carpenter & Ferm 1969) become closely associated with this material on the microvilli and especially so with that lining the caveolae where ruthenium red labelling shows it to be most dense. Whether or not it is essential for proteins to become firmly attached to the glycocalyx in order to be carried through the apical canalicular system by some membrane flow system (Bennett 1956), or whether 'mixed' and/or 'fluid' endocytosis (Jacques 1969) can occur, is still unknown. If attachment is necessary, it would appear to be non-specific in nature and the suggestion made by Wissig & Graney (1968),



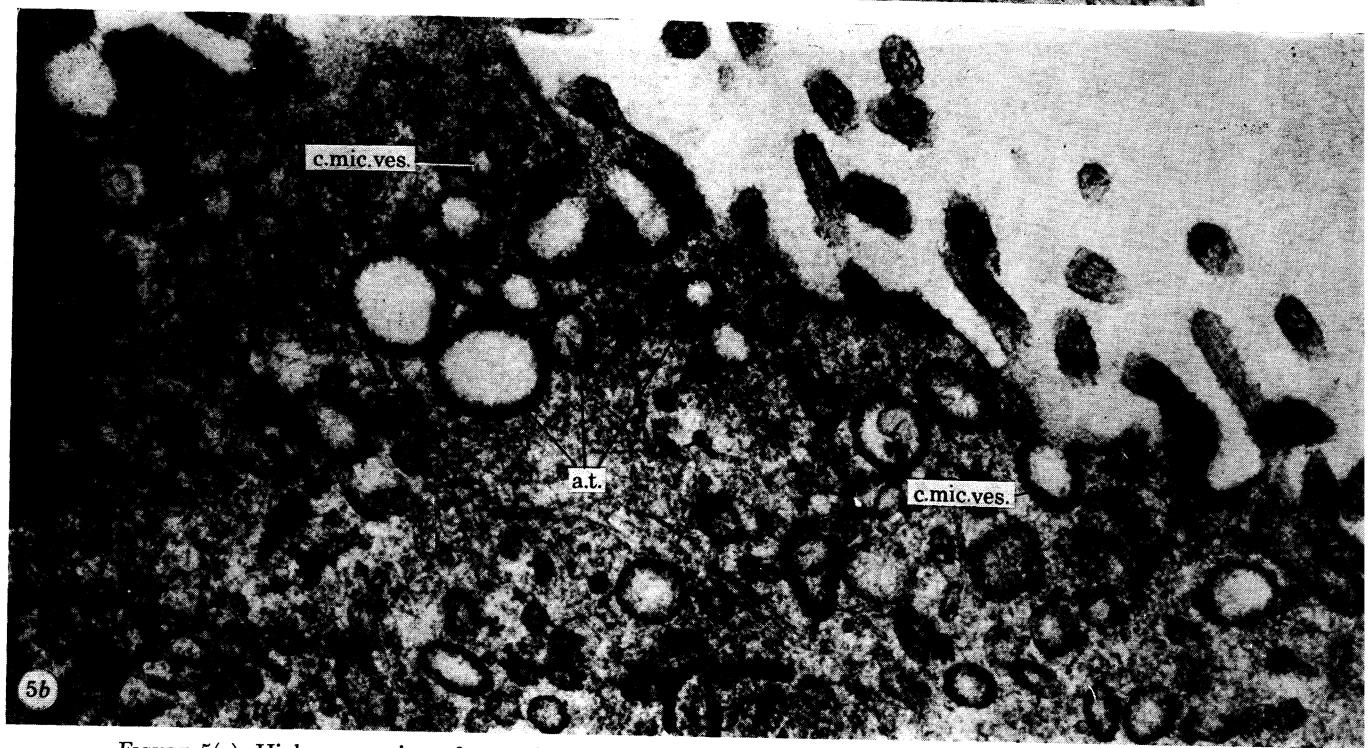


FIGURE 5(*a*). High power view of part of the apical region of the rabbit yolk sac endoderm, showing the structured glycocalyx on the luminal surface of apical tubules (arrowed), and a coated micropinocytotic vesicle (c. mic. ves.) lying close to the cell surface. (Magn. × 79800.)

(*b*). Apical region of the yolk sac endoderm showing apical tubules (a.t.) which make up the canalicular system and coated micropinocytotic vesicles (c. mic. ves.), one of which is apparently forming from the surface membrane. (Magn. × 50544.)

(*Facing p.* 404)

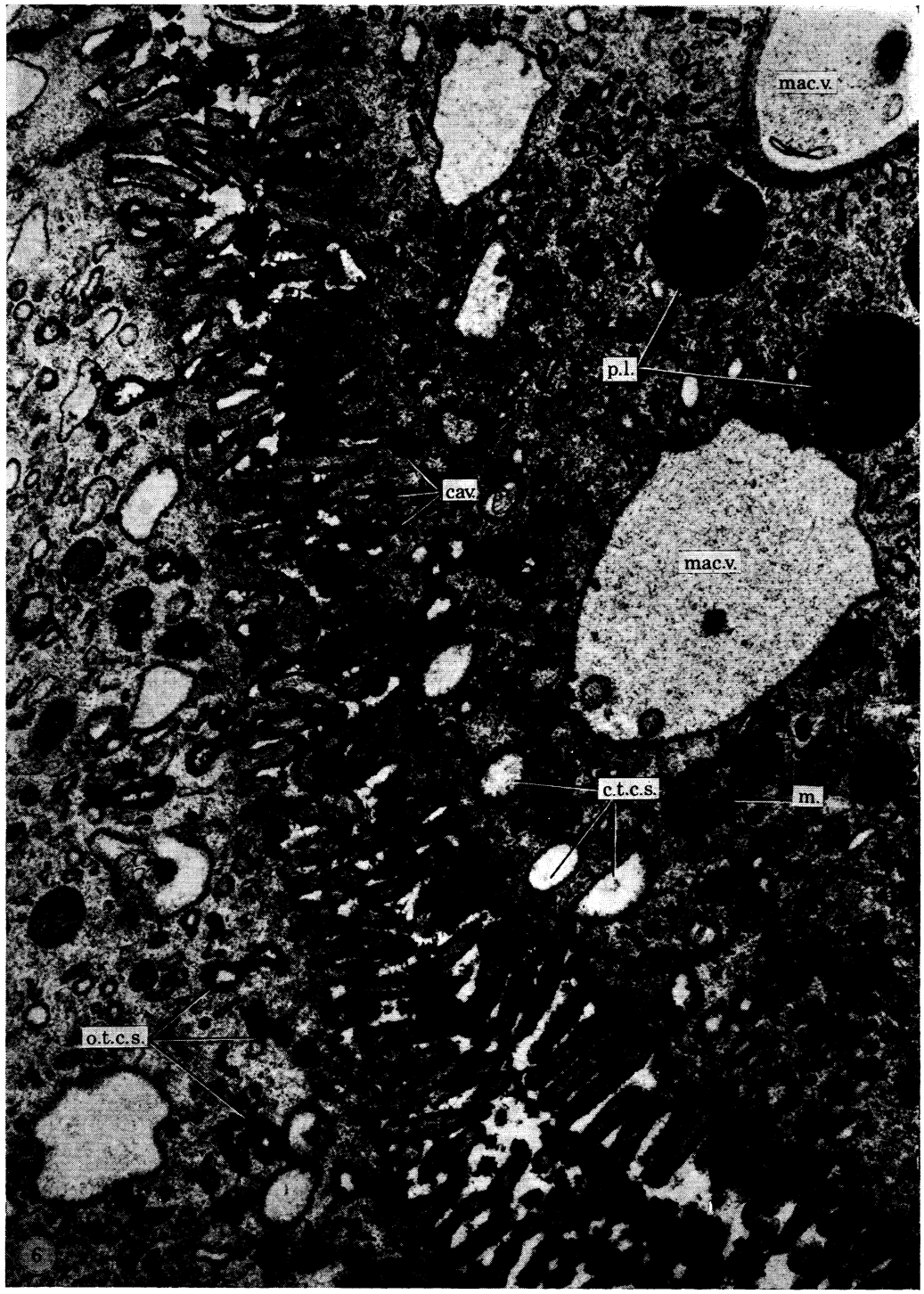


FIGURE 6. Appearance of the apical region of the rabbit yolk sac endoderm after treatment with ruthenium red by the method of Jollie & Triche (1971). Prior to osmification, the yolk sac splanchnopleur was fixed in 3% cacodylate buffered glutaraldehyde containing 0.03% ruthenium red (ruthenium red does not cross the intact plasmalemma). Osmium, indicative of ruthenium red bound to the glycocalyx, is deposited as a thin coat over the microvilli of two adjacent cells. This coating is particularly dense on the caveolae (cav.) formed at the base of the microvilli and on the luminal surface of those elements of the tubular canalicular system in open communication with the exterior (o.t.c.s.). Elements of the apical tubular canalicular system not in communication with the exterior (c.t.c.s.) lie alongside these open tubules, and beneath these lie closed, macropinocytotic vesicles. Two densely staining bodies probably representing primary lysosomes (p.l.) lie close to the macropinocytotic vesicles (mac.v.). m., mitochondrion. (Magn. × 31 200.) (From Wild 1973.)

FIGURE 7(*a*). Direct deposition autoradiograph of ^{125}I-labelled rabbit IgG in an endoderm cell of the 24 day pregnant rabbit yolk sac.

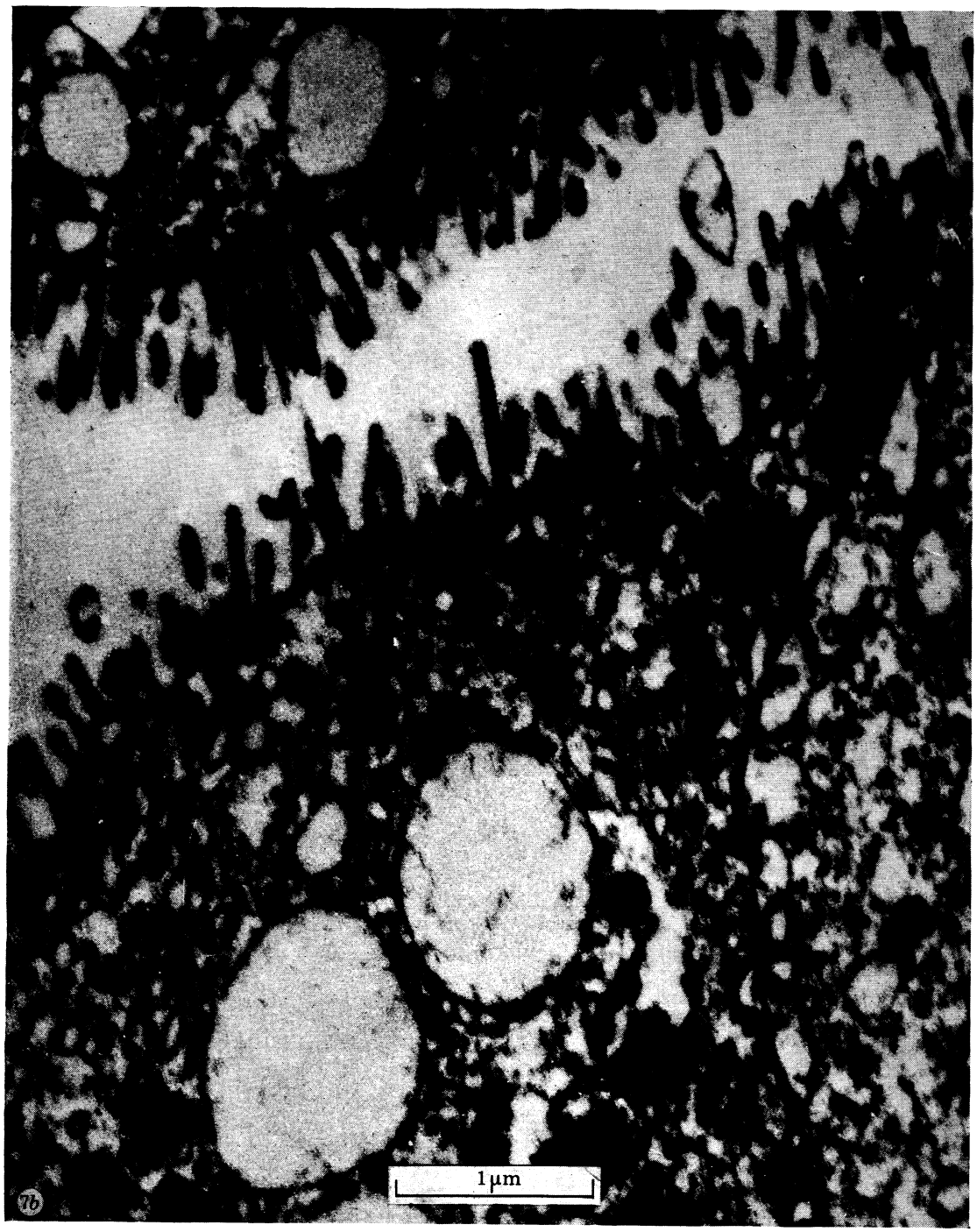


FIGURE 7(*b*). Control section of a similar cell which had not been exposed to the isotope but which was submitted to the same development process.

that the structural modifications seen on parts of the surface membrane and apical tubules of rat ileal cells represent specific receptor sites, does not seem tenable in the light of Rodewald's (1973) findings. Added to this is the fact that similar structural modifications are present on the ileal cells of the suckling pig (Hardy, Hockaday & Tapp 1971) and in this species, selection of proteins does not take place during transport. From fluorescent protein tracing studies (Payne & Marsh 1962) and ultrastructural observations (Hardy *et al.* 1971) it would appear that macropinocytotic vesicles *are* involved in transport in this species. Coated micropinocytotic vesicles have also been observed fusing with the lateral plasmalemma, but any selective transport they might possibly have been engaged in would no doubt be overshadowed by the non-selective transport mediated through macropinocytotic vesicles.

Hemmings & Williams (1973) have recently studied the extent to which bovine IgG labelled with ^{131}I and rabbit IgG labelled with ^{125}I bind to different components of the rabbit yolk sac splanchnopleur. All components studied (nuclear, mitochondrial, microsomal and cell sap) bound rabbit and bovine IgG to about the same extent and from this Hemmings and Jones have concluded that selectivity in transport cannot be explained at the stage of attachment to cellular receptors. However, it seems unlikely that proteins would come into contact with nuclear or mitochondrial membranes during their transport across the cells and these results probably reflect only non-specific binding of the sort that might be mediated through negative-positive charge interactions. The glycocalyx would presumably be present in the microsomal and cell sap fractions, but again non-specific attachment might overshadow any specific attachment that occurred. Sonada & Schlamowitz (1972) on the other hand, have produced evidence that specific binding of rabbit IgG and albumin does take place to the surface of the yolk sac endoderm. *In vitro* preparations of rabbit yolk sac splanchnopleur were fixed with formaldehyde so as to prevent endocytosis from occurring, and arranged so that the endodermal surface could react with ^{125}I-labelled rabbit IgG or ^{125}I-labelled rabbit serum albumin. Specific binding of these proteins to the surface was established by the fact that bound ^{125}I-labelled rabbit IgG was displaced by unlabelled rabbit IgG and not by albumin, and bound ^{125}I-labelled albumin was displaced by unlabelled albumin and not by IgG. These workers rightly point out that such apparent specificity may simply reflect differences in charge, shape and size between the two proteins and not the specificity required in the receptor hypothesis. A further criticism of experiments of this type is that specific binding to the cell membrane may be something generated only during the formation of coated micropinocytotic vesicles and might therefore not be apparent in such non-living preparations.

It has been pointed out by Bowers (1964) that the dimensions of the coated micropinocytotic vesicles, in aphid pericardial cells at least, are such that the cavity of the vesicle, if one takes into account the glycocalyx adhering in that region, is essentially filled when the vesicle is formed. Thus only proteins attached to the glycocalyx would be incorporated and there would be little possibility of fluid endocytosis occurring. This would seem to be just the sort of selective mechanism required for transport of proteins across endodermal cells and syncytiotrophoblast if fusion of vesicles with lysosomes did not occur. In macrophages (Lagunoff & Curran 1972) it has been suggested that exhaustion of coated sites on the plasma membrane (i.e. those regions where coated micropinocytotic vesicles will form) may be a factor limiting pinocytosis. This could also be of importance in regulating how much protein is transmitted intact to the blood of the foetal or newborn mammal. Increasing the concentration of protein to which the protein absorbing cells are exposed may simply drive more of the protein into the macropinocytotic

system when coated vesicle sites are exhausted. There has been some speculation as to whether the 'bristle coat' might be involved in specific binding of proteins during pinocytosis (Roth & Porter 1964) but other workers (Kaneski & Kadota 1969) are firmly of the opinion that it is exclusively an apparatus to control the infolding and fissioning mechanism of the membrane.

Except for strong circumstantial evidence that they occur on the surface of protein absorbing cells, and with strong indications that they are associated with coated micropinocytotic vesicles, little is known about the receptors. What does seem to emerge however, is that any search for them in the yolk sac endoderm and possibly also in the syncytiotrophoblast, may be fraught with difficulties in view of a possible overwhelming non-specific attachment of proteins to that part of the glycocalyx involved in non-selective protein uptake. The proximal cells of the small intestine of the suckling rodent, which seem to be engaged almost exclusively in selective uptake of proteins, would seem to offer the best possibilities for their isolation and characterization.

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Discussion

J. B. Lloyd (*Biochemistry Research Unit, Keele University, Staffordshire*). My colleagues and I at Keele have recently made a quantitative study of pinocytosis in the rat yolk-sac maintained in organ culture (Moore, A. T., Williams, K. E. & Lloyd, J. B. *Biochem. Soc. Trans.* 1974, in the Press). The anatomical relationships in the rat placenta are similar to those described by Dr Wild for the rabbit, and the 17 day rat yolk sac can easily be dissected free from other tissues; it survives for many hours when cultured in a simple tissue culture medium (TC-199) containing 10% calf serum. By adding radiolabelled macromolecular substrates to the culture medium it has been possible to measure the rates of their pinocytic uptake. ^{125}I-labelled polyvinylpyrrolidone (PVP) and colloidal [^{198}Au]gold are captured at approximately the same rate, whereas ^{125}I-labelled bovine serum albumin or human fibrinogen are taken up at much higher rates (up to 50 times the rate for ^{125}I-labelled PVP). The rate of uptake of these proteins varies considerably with changes in their conformational state, presumably because such changes expose or mask determinants on the molecule necessary for its uptake.

These experiments are relevant at a number of points to the Brambell hypothesis and to the alternative proposal by Wild. First, the proteins we have used were fully degraded to amino acids by the yolk-sac tissue after uptake. The kinetics revealed that endocytosis was the rate-determining step in the overall process of uptake, digestion, and release of digestion products. The catabolic machinery of this tissue is very great and its capacity much greater than demand. Secondly, uptake that is followed by digestion, although non-selective in Wild's sense (not specific for γ-globulins), is highly selective, capturing some macromolecules much more efficiently than others. Thirdly, it has been shown that this selectivity is not achieved by changes in the rate of internalization of plasma membrane; hence it must reflect uptake of different substrates within distinct types of vesicle or, much more likely, by differential adsorption of substrates on plasma membrane 'receptors'.

Dr Wild, you are suggesting that two distinct types of pinocytic vesicle are being formed from the apical plasma membrane of yolk sac cells. Do you postulate a micro-heterogeneity of the plasma membrane, so that some regions can give rise to one type of vesicle and some to the other? This seems a necessary corollary of your proposal, but it sounds a difficult feat for a cell to achieve, particularly a cell in which internalization of membrane by pinocytosis is constantly taking place.

A. E. Wild. I am postulating a micro-heterogeneity of the plasma membrane and I think there are precedents for this in other cell types. For example, one only has to think of macrophages; these have specific receptors for IgG (Huber *et al.* 1968). There is some evidence that IgG and haemoglobin become localized in different vesicles in macrophages and it is presumed that this is because of a different localization of the Fc receptor site on the surface of the cell (Dingle, Poole, Lazarus & Barrett 1973).

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W. A. Hemmings (*A.R.C. Zoology Department, University College of North Wales, Bangor*). I am a little worried by Dr Wild's reliance upon Rodewald's (1973) work, because Edgar Williams & I have attempted (1974) to confirm his main conclusions using quantitative as well as electron microscopic methods, and our findings are quite contrary to Rodewald's. Rodewald claims that heterologous protein (e.g. ferritin) is not taken up by the duodenal cells of the young rat gut, and that while both homologous and heterologous proteins are taken up by the ileal cells, neither are transmitted, but both are broken down *in situ*. We have administered a mixture of ^{131}I-labelled ferritin and ^{125}I-labelled rat IgG to isolated segments of the intestine of young rats, estimating the total protein-bound activity of the carcase (less the injected gut segment) three hours after the operation. Table 1 shows the results of these experiments, the values being the means of groups of five animals each. You will see that the entry, expressed as the percentage of the dose transmitted per centimetre of gut exposed, is greatest for both proteins in the ileal segment. More IgG than ferritin crosses the ileum; that is, it is still selective. These findings are contrary to Rodewald's conclusion about the ileum. Then in the duodenum it is seen that there is a considerable uptake of ferritin, in fact about one-third of the uptake of IgG: a surprising result when one bears in mind the disparity of size between the molecules. So here again our result is contrary to Rodewald's conclusion from his ultrastructural studies. Mr Williams has, also, many electron micrographs showing ferritin in, and leaving, both duodenal and ileal cells.

Table 1. Percentage of the injected dose/cm gut length absorbed into the carcase

(Mean of five animals.)

	% dose absorbed/cm gut								
	duodenum			jejunum			ileum		
protein	mean	± S.E.	*P*	mean	± S.E.	*P*	mean	± S.E.	*P*
^{131}I-ferritin	1.25	0.068		2.98	0.38		3.01	0.49	
			< 0.001			< 0.01			< 0.005
^{125}I-rat IgG	3.17	0.11		5.3	0.82		4.75	0.23	
ratio 131/125	0.39			0.56			0.63		

I should like now to turn briefly to the rabbit yolk sac, and show a part of an endoderm cell that had been exposed to rabbit IgG labelled with ^{125}I (figure 7, plates 20 and 21). Because the appearance of this radio-autograph may be unfamiliar to you, I must explain that it was developed by the direct deposition technique (Normandin 1973). It was in fact treated by immersion of the sections in dilute gold chloride for 20 s, washing them and air-drying before examination in the microscope. The great advantage of the technique, apart from this simplicity and speediness, is as you can see that the particles are small and very specifically localized. In this vacuole, which is a fraction of a micrometre in diameter and may be one of Wild's coated vacuoles, there is a close group of particles, which are rolled up as a bolus in a thin background material which I suggest may be glycocalyx. The particles are not spread out round the periphery of the vacuole in the way predicted by Brambell's hypothesis. I may say that this distribution is typical of many vacuoles we have examined, containing both homologous and heterologous protein. This seems to be a severe blow to that hypothesis. Please note also that there are several groups and discrete singlet particles distributed through the cytoplasm in this field. This again is typical, and holds true also for heterologous protein. When we carried out our first experiments with

ferritin a decade ago we were immediately impressed with the amount of free molecular ferritin in the cytoplasm, as well as that packed into vacuoles. We could not tell whether this pattern held for native IgG however, or was idiosyncratic to ferritin, until we had available the direct deposition technique. The classical technique is not precise enough to be sure a track is originating in the cytoplasm and not in one of the many small vacuoles. It appears now to be true however, that both homologous and heterologous IgG are found in quantity in the cytoplasm, and this fact needs to be taken into account in formulating our hypothesis. In case Wild claims this vacuole as one of his transport vesicles, I must add that I could have shown the identical appearance from an experiment with bovine IgG. I think it is only when we have available a double labelling autoradiographic technique that we shall be able to resolve these questions. I hope that will not be long coming.

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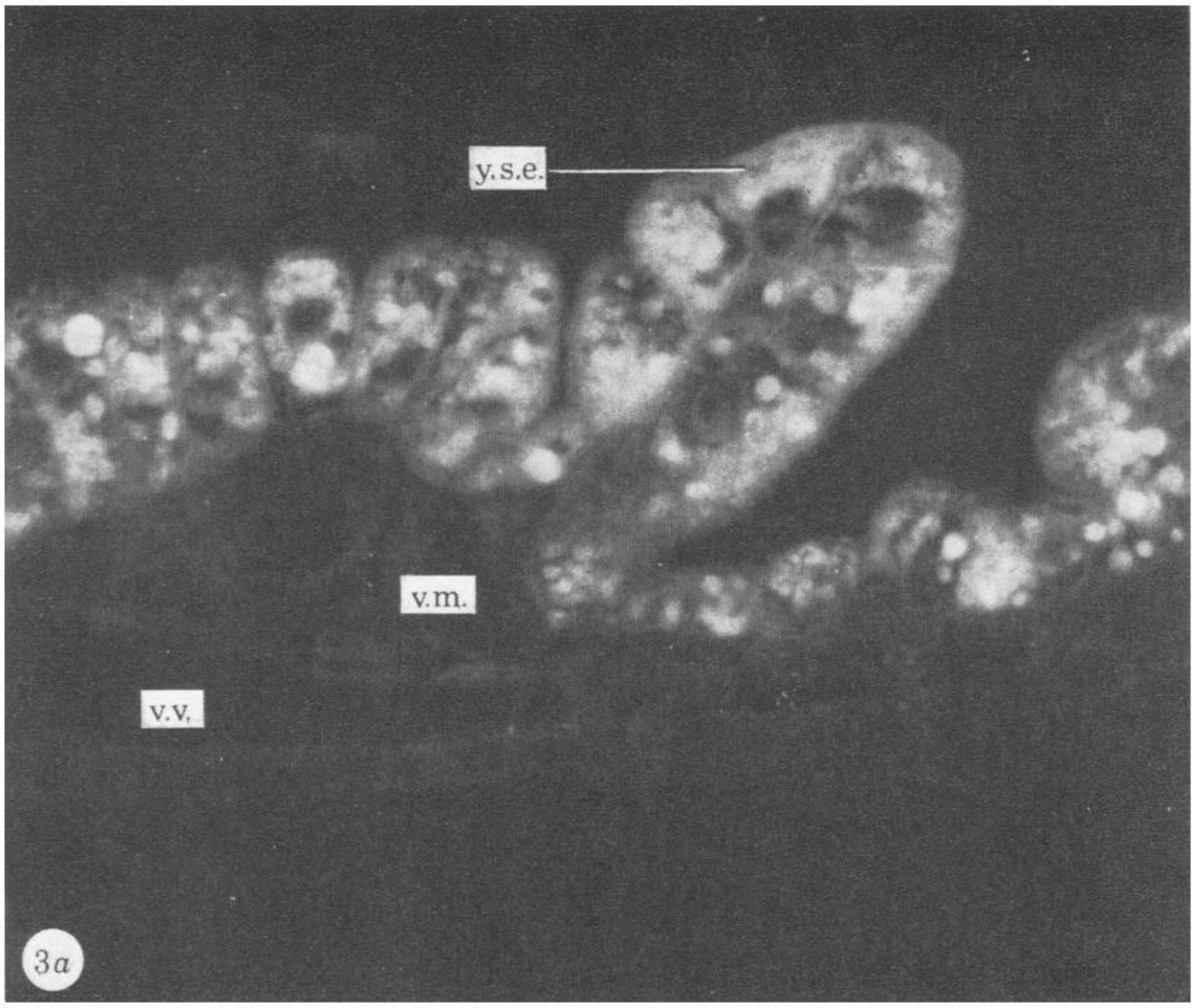


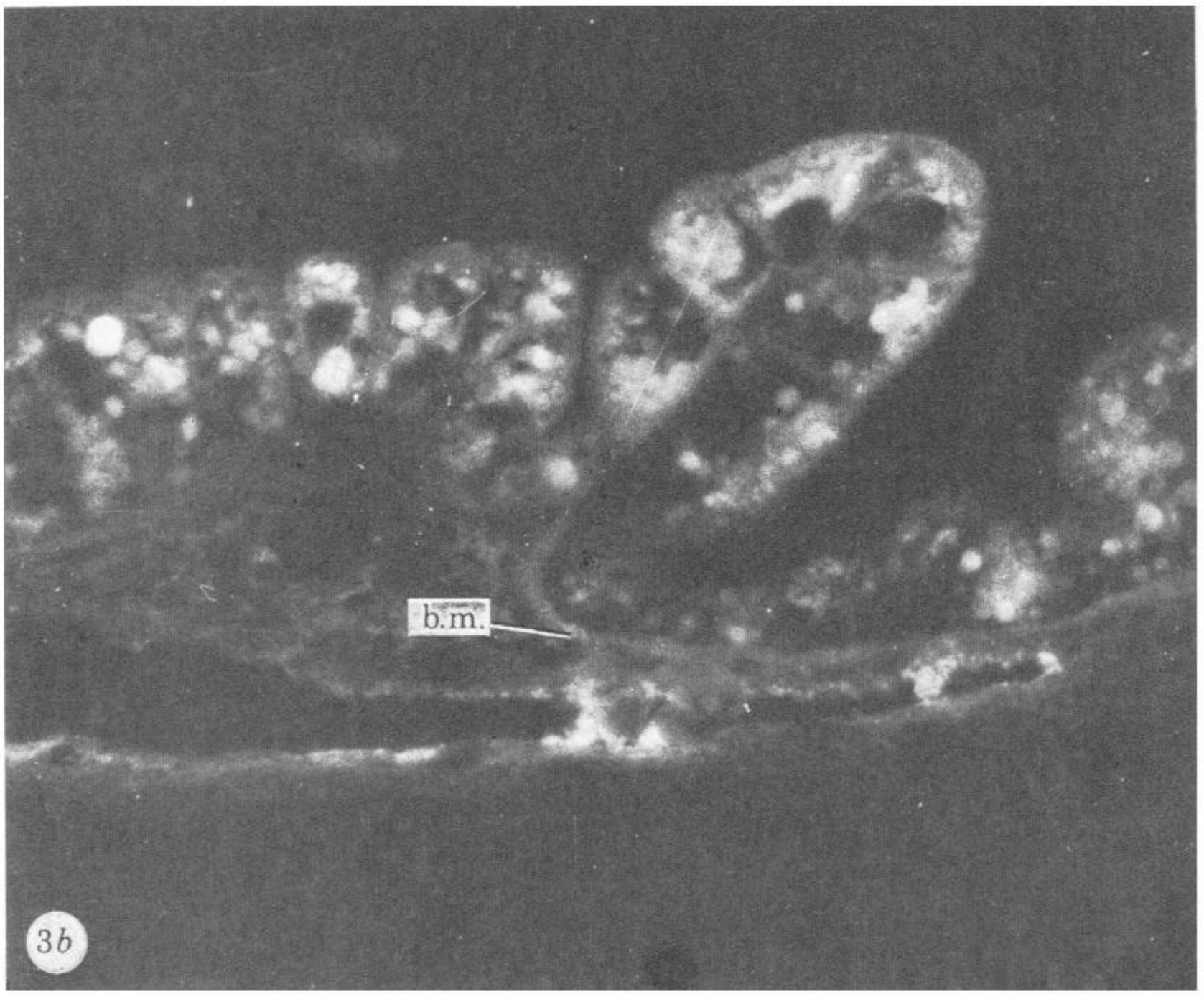


FIGURE 3(*a*). Section of yolk sac splanchnopleur from a 26 day old rabbit conceptus that had been exposed to a mixture of 1 % bovine IgG and 1 % human IgG for 4 h. The section was treated with both FITC-labelled rabbit anti-human IgG and RB200Cl-labelled rabbit anti-bovine IgG. Specific fluorescence due only to RB200Cl-labelled antibodies was visualized in a Leitz Ortholux fluorescence microscope fitted with a Ploem vertical illuminator (filter combination S 546 + BG 36/K 610). Bovine IgG can be seen to be present in macropinocytotic vesicles in the yolk sac endoderm (y.s.e.). Other abbreviations: v.v., vitelline vessel; v.m., vascular mesenchyme. (Magn. × 375.) (From Wild 1974.)

(*b*). The same section as shown in (*a*) but now visualized for specific fluorescence due only to FITC-labelled antibodies (filter combination 2 KP 490/S 525). Note that human IgG has the same localization as bovine IgG in macropinocytotic vesicles in the yolk sac endoderm, but in addition is present in the basement membrane (b.m.), vascular mesenchyme and within the vitelline vessels. (Magn. × 375.) (From Wild 1974.)

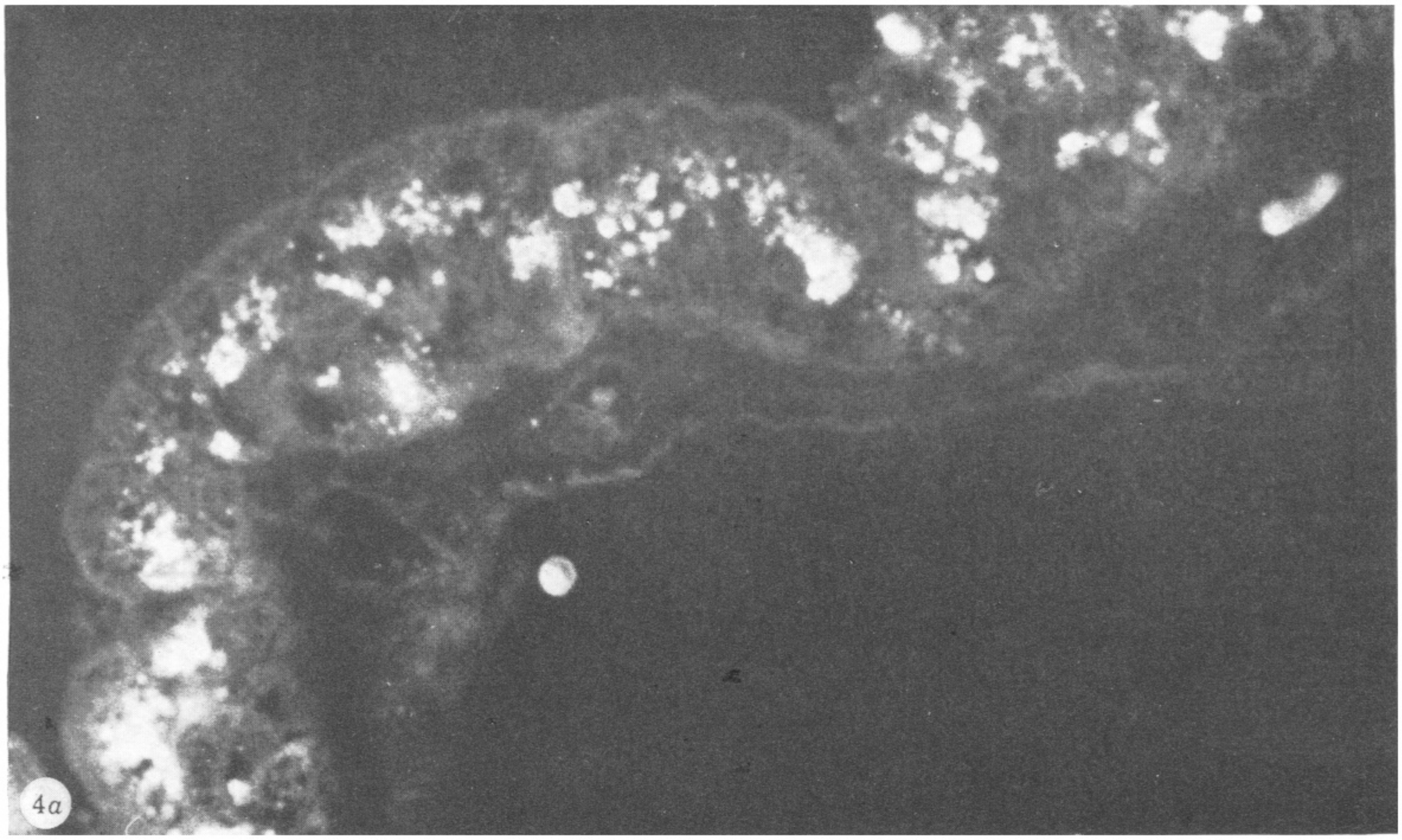
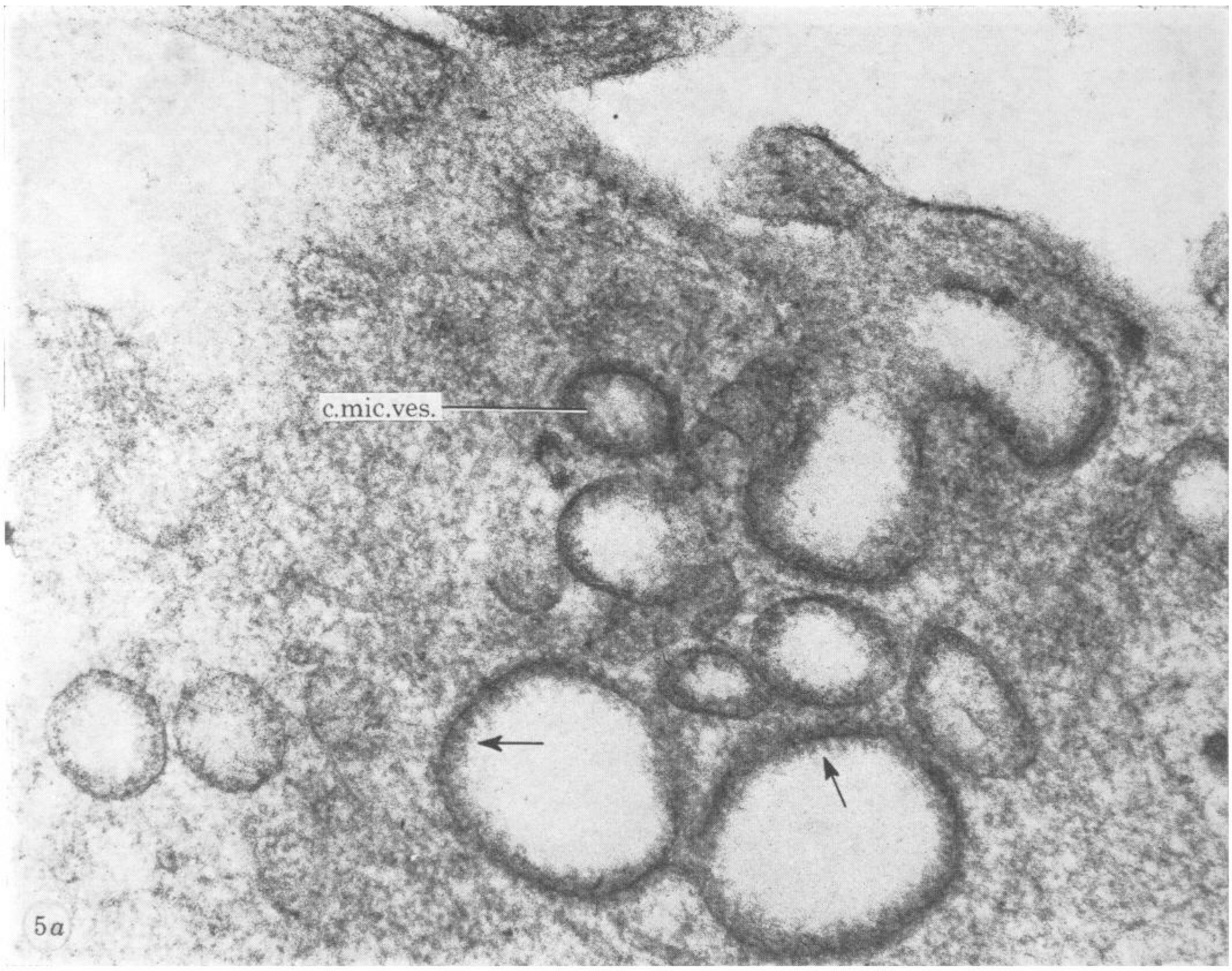


FIGURE 4. For description see opposite.



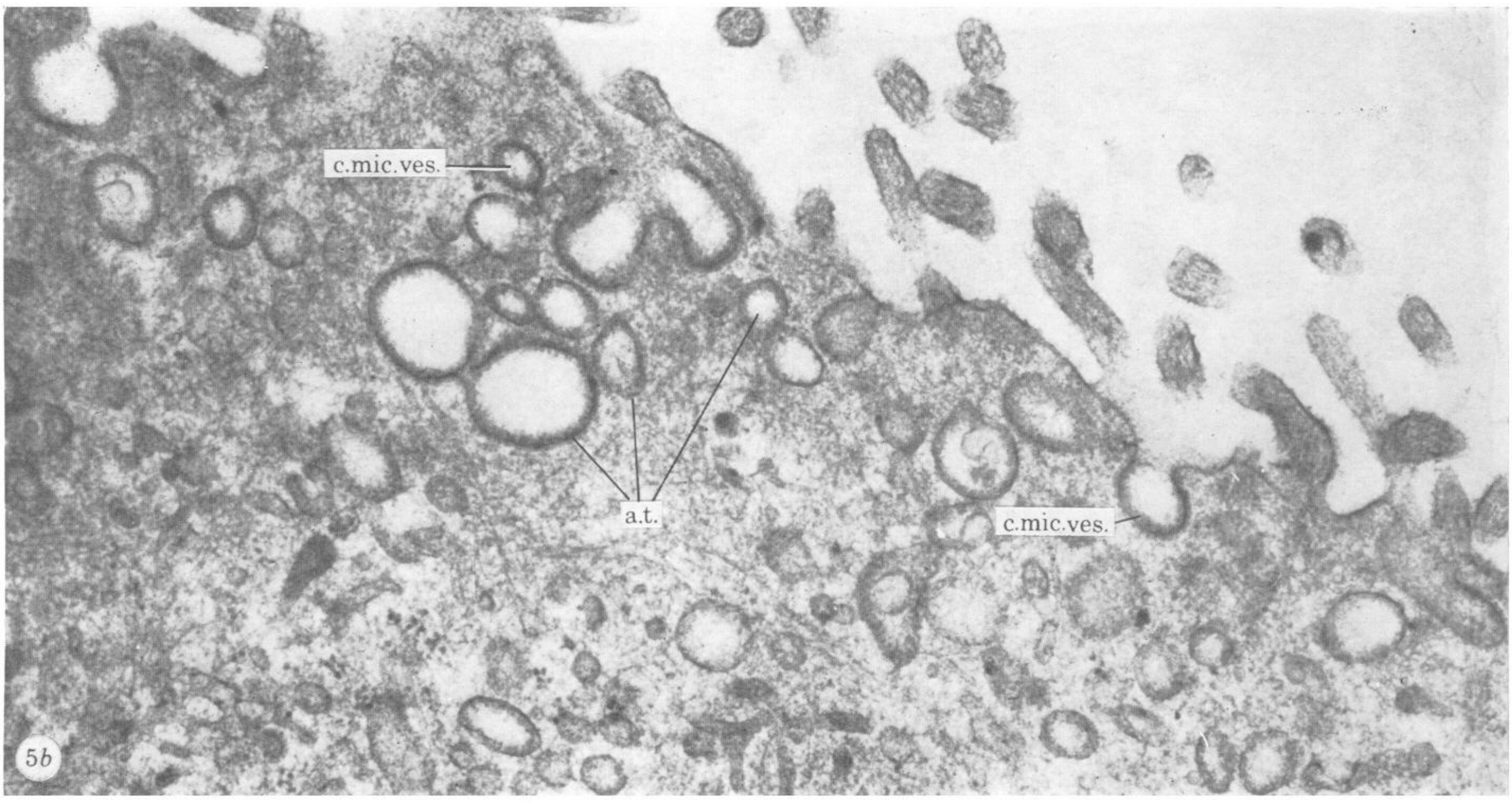


FIGURE 5(*a*). High power view of part of the apical region of the rabbit yolk sac endoderm, showing the structured glycocalyx on the luminal surface of apical tubules (arrowed), and a coated micropinocytotic vesicle (c. mic. ves.) lying close to the cell surface. (Magn. × 79800.)

(*b*). Apical region of the yolk sac endoderm showing apical tubules (a.t.) which make up the canalicular system and coated micropinocytotic vesicles (c. mic. ves.), one of which is apparently forming from the surface membrane. (Magn. × 50544.)

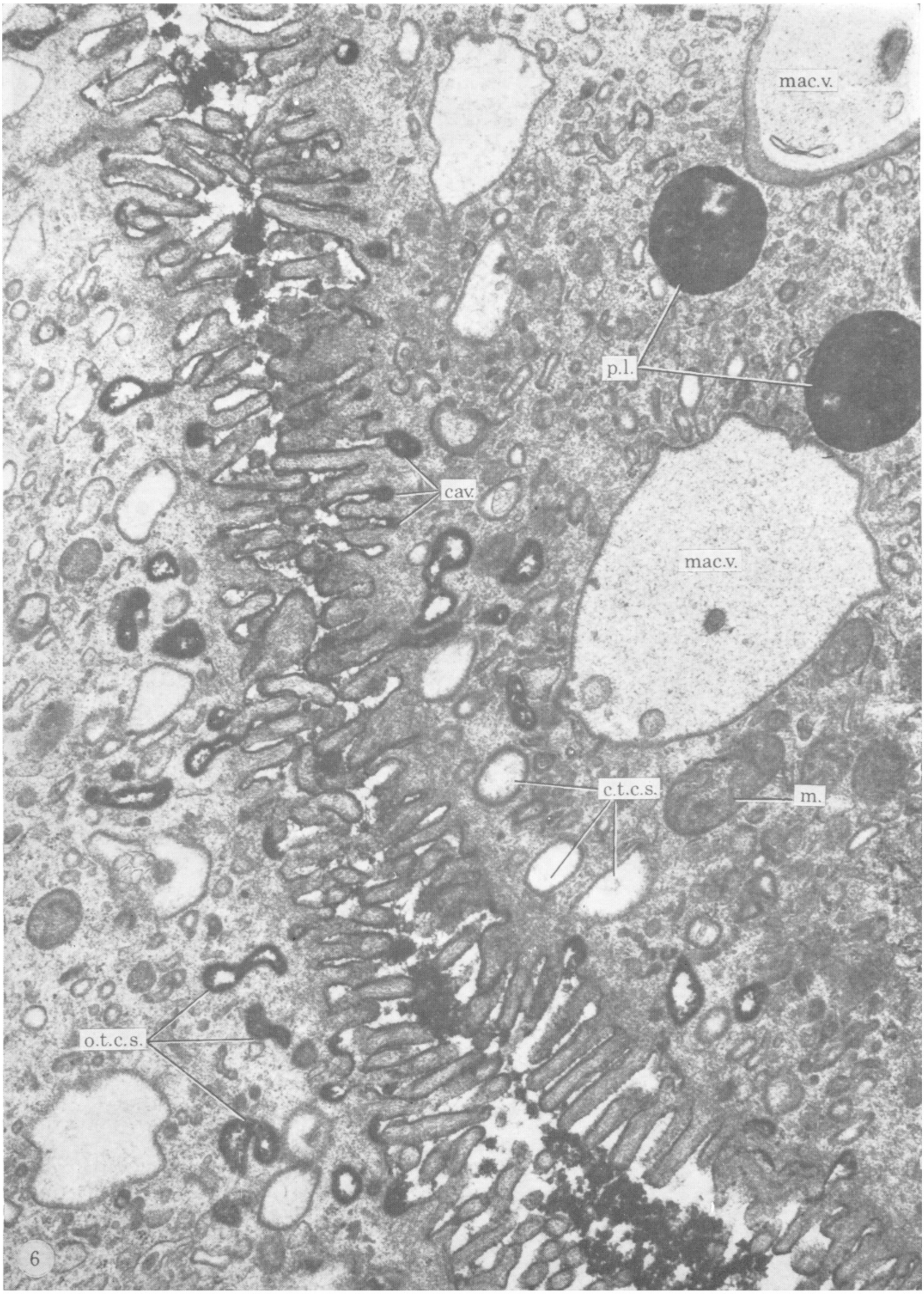


FIGURE 6. Appearance of the apical region of the rabbit yolk sac endoderm after treatment with ruthenium red by the method of Jollie & Triche (1971). Prior to osmification, the yolk sac splanchnopleur was fixed in 3% cacodylate buffered glutaraldehyde containing 0.03% ruthenium red (ruthenium red does not cross the intact plasmalemma). Osmium, indicative of ruthenium red bound to the glycocalyx, is deposited as a thin coat over the microvilli of two adjacent cells. This coating is particularly dense on the caveolae (cav.) formed at the base of the microvilli and on the luminal surface of those elements of the tubular canalicular system in open communication with the exterior (o.t.c.s.). Elements of the apical tubular canalicular system not in communication with the exterior (c.t.c.s.) lie alongside these open tubules, and beneath these lie closed, macropinocytotic vesicles. Two densely staining bodies probably representing primary lysosomes (p.l.) lie close to the macropinocytotic vesicles (mac.v.). m., mitochondrion. (Magn. × 31 200.) (From Wild 1973.)

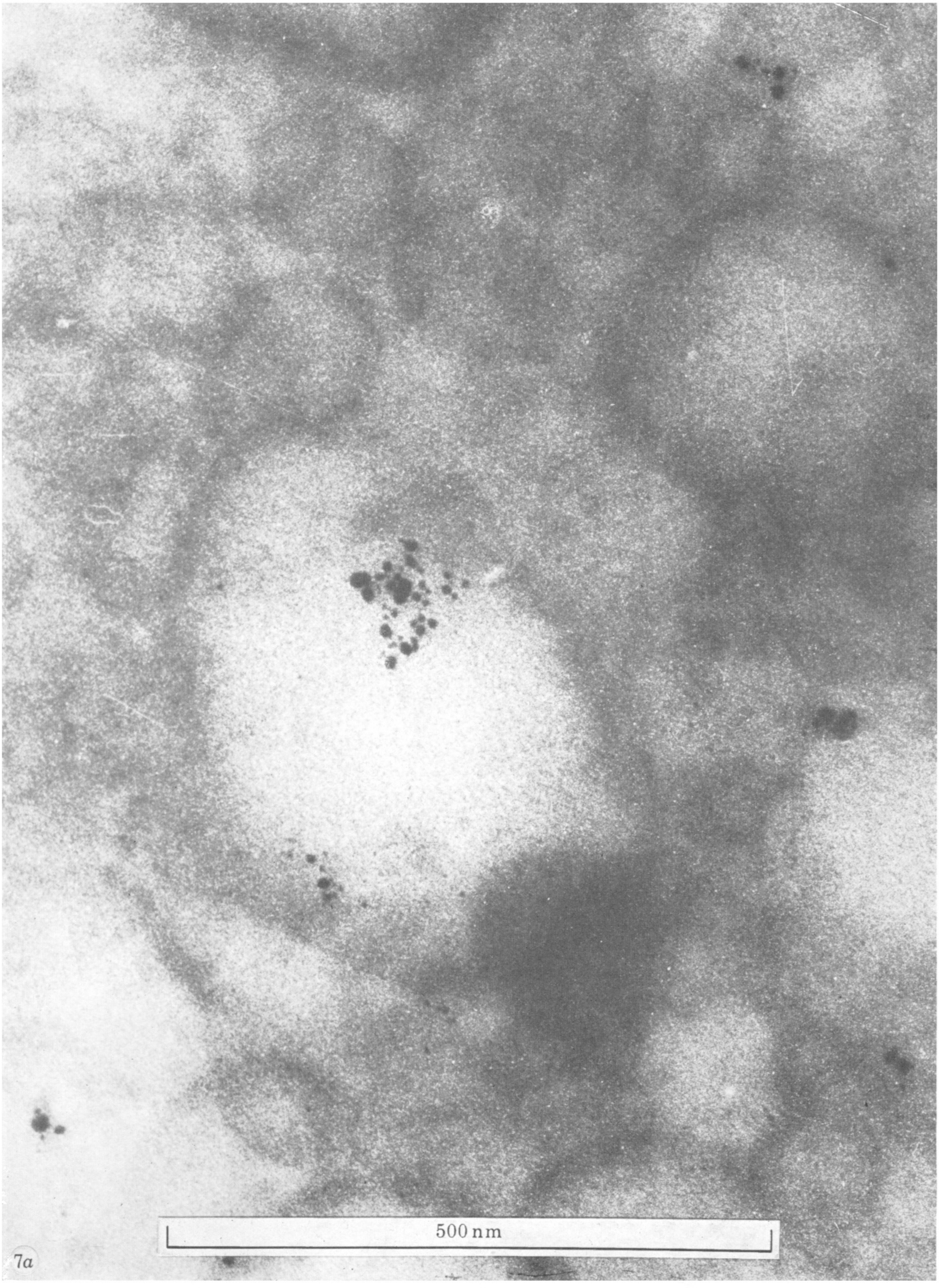


FIGURE 7(*a*). Direct deposition autoradiograph of ^{125}I-labelled rabbit IgG in an endoderm cell of the 24 day pregnant rabbit yolk sac.

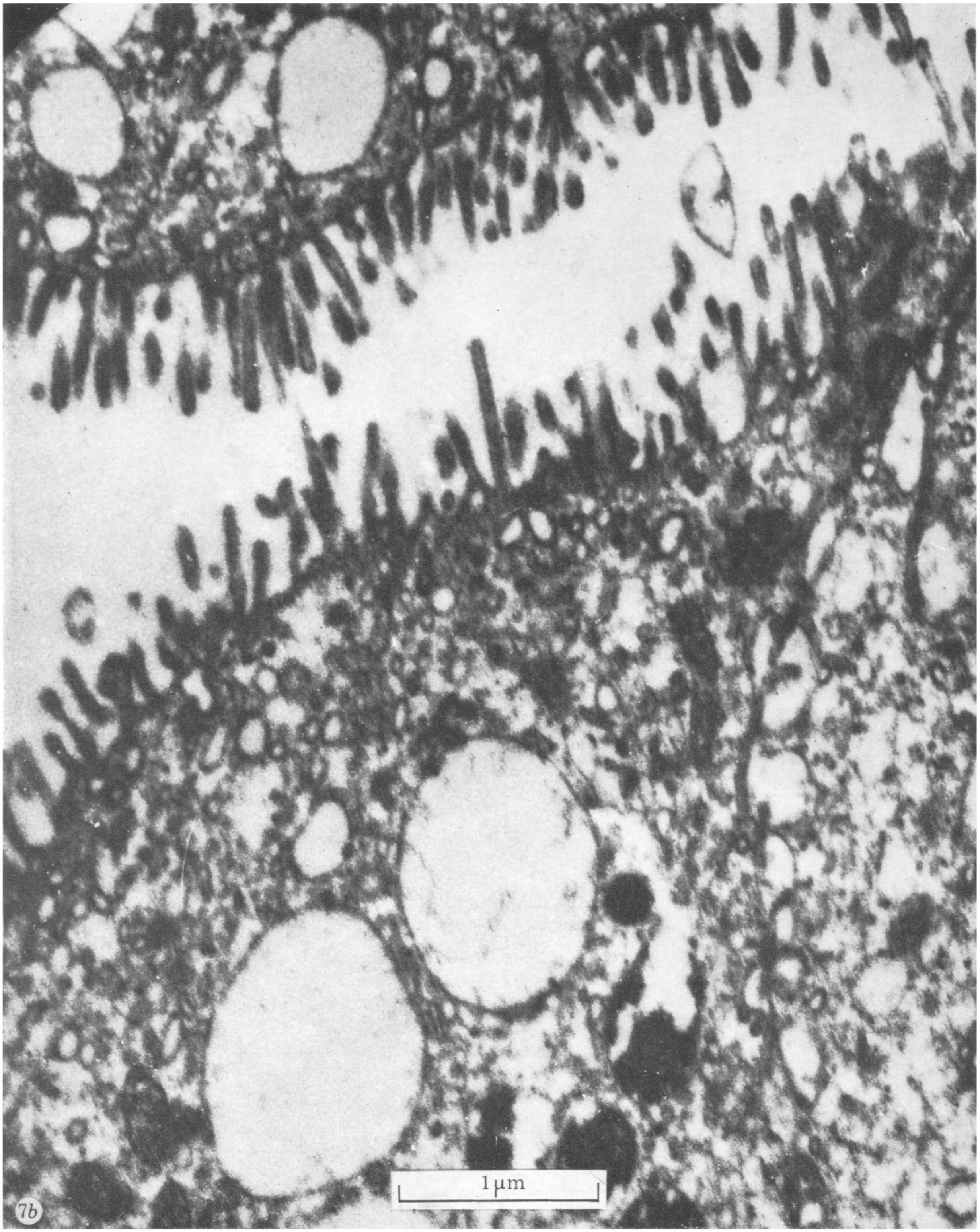


FIGURE 7(*b*). Control section of a similar cell which had not been exposed to the isotope but which was submitted to the same development process.