Electron Microscopic Studies on the Mechanism of Vital Stain, as Compared with That of Phagocytosis*

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I) Introduction

The reticuloendothelial cells have been characterized by their marked ability
c) Electron microscopic observations. (Text-fig 1).

Mitochondria of normal peritoneal macrophages provided the ordinary structure; electron density of mitochondrial matrix was low, arrangement of the cristae was regular, and 1–2 mitochondrial granules were usually observable. Other structural details of normal peritoneal macrophage were described by this author in another report.\(^{74}\)

![Text-fig. 1. Mitochondrial changes of peritoneal macrophage vitally stained with Janus green. Left: mitochondria of untreated cell, upper: intramitochondrial vacuole formation and its pushing out. lower: reactive division of mitochondria. right: degeneration.](image_url)

Marked morphological alterations were observed within the mitochondria of cells stained with Janus green. The matrix of all mitochondria increased in electron density, and the arrangement of cristae became quite irregular. The dye stuff itself, however, could not be recognized because it had dissolved into the surrounding media during preparation. The mitochondrial granules became swollen and developed into vacuoles, the wall of which was very thick and markedly dense. These vacuoles were then pushed out of mitochondria into the cytoplasm (Fig. 1). Such vacuoles could be easily distinguished from those of cytoplasmic origin due to the thicker and denser wall of the former which was clothed with outer double membrane of mitochondria. Sixty minutes after injection, however, most mitochondria were swollen and markedly degenerated (Text-fig. 1, right).

d) Remarks

Mitochondrial changes which followed the injection of Janus green are schematically illustrated in Text-fig. 1. Some of such changes were also observed by Sasaki\(^{61,62}\) in supravital stain with Janus green.
The increase in electron density of the mitochondrial matrix means the increase in quantity of some of the matrix substances. Formation and pushing out of the intramitochondrial vacuoles may be regarded as the self-cleaning mechanism of mitochondria against the toxic substances with which mitochondria are diffusely filled up, whereby the substances are condensed in enormously higher concentration within such vacuoles. The origination of vacuoles from the mitochondrial granules seems to support Weiss’ idea that these granules are cation-segregating apparatus, although, contrary to his claim, the number of granules was not increased in vital stain. Another series of mitochondrial changes induced with Janus green is a sort of division figures of mitochondria, details of which will be reported elsewhere (Text-fig. 1, lower right).

It is noteworthy that the pinocytotic vacuoles are never observed to be connected with mitochondria during the whole course of the experiment. It means that dye stuffs exist freely in the cytoplasm, at least just prior to the entrance into mitochondria, and then penetrate the outer double membrane of the latter, and, therefore, that extracellular dye stuff should also penetrate the surface membrane of cell or limiting membrane of pinocytotic vacuoles to enter the cytoplasm.

According to Fautrez et Lison, Janus green particles have a size of 25.2Å, the largest one among the various vital dyes (Table II). That such large dye particles can penetrate the cell membrane without any structural changes of the latter is very suggestive for the understanding of the processes of vital stain.

### III) Carmine

a) Materials and methods

Adult mice were given subcutaneous injections of sodium carmine solution for 5 successive days according to the following schedule: the 1st day, 0.3 cc; the 2nd day, 0.5 cc; the 3rd day, 0.7 cc; the 4th day, 1.0 cc; the 5th day, 1.3 cc. Sodium carmine solution was prepared according to Ueyonahara, namely, 2 g of carmine was dissolved into 100 cc of 1% Na₂CO₃ solution. Lymph node cells were examined with a light as well as an electron microscope, 24 hours after each injection.

b) Light microscopic observations.

Macroscopically, coloring of the whole mouse body began 1 hour after a single injection, reached its maximum grade at the 3rd or 4th hour, and did not fade for a long time. Microscopically, however, cell stain was retarded much more than whole body coloring. Vital stain of the lymphatic reticulum cells were almost complete after the 5th injection, faintly positive after the 3rd injection, only partly positive after the 2nd injection, and negative after a single injection.

c) Electron microscopic observations.
After the 5th injection, the cytoplasm of reticulum cells was filled with numerous electron dense granules. These granules were markedly irregular in shape, approximately 1 μ or more in diameter, and provided a single distinct limiting membrane and diffuse internal matrix substance with granulo-fibrillar appearance. No findings were obtained to suggest the participation of mitochondria in forming such granules. Within the granules, carmine was identified as numerous fine particles with high electron density, several tens mμ in diameter. Such particles are considered to consist of thousands or hundreds of thousands of molecules of carmine. During the whole course of the experiment, carmine was not recognized as such anywhere except within such granules.

After the 3rd injection, the granules were smoothly outlined, smaller in size, and sometimes slender in shape (Fig. 2). Carmine particles were also reduced in number, and most of them located peripherally within the granules. In this stage, larger dense masses, 100 mμ or more in diameter, were often observed beside carmine particles within the granules. These masses were often reduced in electron density in their central portion, and were considered lipoprotein in nature.

After a single injection, the granules were much smaller in size and often slender in shape. Carmine particles were hardly evident, whereas lipoprotein masses were found particularly in the ends of the slender granules.

Untreated reticulum cells sometimes provided no granular components. In most cases, however, granules like those of carmine stained cells were observable more or less in number even under physiological conditions.

Text-fig. 2. Phagocytosis, pinocytosis and membrane-penetration
Possible pathways of extracellular dye stuffs to the inside of segregomes.
Left: phagocytosis, middle: pinocytosis, right: membrane penetration.
d) Remarks (Text-fig. 2)

According to a serial examination of carmine stain, it is clear that carmine is precipitated in aggregated form within the non-specific cytoplasmic granules; some of them preexist and the others are newly formed. Such granules develop through hypertrophy by themselves and by fusing with each other into typical vitally stained granules.

Then, which is the mechanism of transporting the extracellular carmine to the inside of the granules, phagocytosis, pinocytosis, or some other way? If phagocytosis means the phenomenon, whereby extracellular “particulate” matters are captured by cells within the cytoplasmic vacuoles, vital stain is not phagocytosis, because dye stuffs can be recognized as particulate matters only within the granules. Does pinocytotic vacuole develop into the granule, whereby condensation of its content proceeds? From the morphological standpoint of view, it may not be the case for vital stain, because of following reasons: 1) pinocytotic vacuoles are not increased in number, 2) transitional states between carmine-stained granules and pinocytotic vacuoles are not observed, 3) some of vitally stained granules provide no limiting membrane (see Neutral red), 4) even dye stuffs of larger particles such as Janus green can penetrate the cell membrane within a few minutes (see Janus green).

Therefore, it is highly presumable that carmine also penetrate the cell membrane to enter into the cytoplasm, and then is collected in high concentration within the granules. Such an assumption will be again discussed later from the viewpoint of staining kinetics, because it is so fundamental for the vital stain.

In my opinion, the process, whereby any substances scattered diffusely in the cytoplasm are collected and condensed in higher concentration within the non-specific cytoplasmic granules, is nothing but segregation phenomenon, and so I should like to propose for these granules the name “segresomes”. Segregated materials can be recognized only when they are preserved with the usual fixatives and, in addition, provide enough electron density.

IV) Trypan blue

a) Materials and methods

0.5 cc of 1% trypan blue solution was injected daily subcutaneously into adult mice for 3 successive days. Approximately 24 hours after each injection, lymph node cells and ascites were examined according to methods similar to the case of carmine.

b) Light microscopic observations.

Macroscopic coloring of the whole mouse body developed in the same manner as that observed in the case of carmine. No cells were stained 24 hours after
the 1st and 2nd injection. The 3rd injection induced the positive cytoplasmic stain of the lymphatic reticulum cells. Trypan blue-stained granules were, in general, larger than those stained with carmine.

c) Electron microscopic observations (Fig. 3)

Numerous segresomes were observed in the cytoplasm of the lymphatic reticulum cells 24 hours after the 3rd injection. The segresomes were considerably large, and provided a limiting membrane as well as internal matrix substance. Trypan blue itself can hardly be identified probably because of its low electron density. Lipoprotein masses were also hardly observable.

d) Remarks
Some morphological differences can be pointed out indeed between carmine- and trypan blue-induced segresomes. Such differences, however, may not be fundamental, but only modifications of the structure of the same nature, the segresomes.

V) Neutral red

a) Materials and methods
2 cc of 1% neutral red solution was injected subcutaneously into adult mice, and lymph node cells as well as ascites were examined in the light microscope supravitally 1/2, 1, 2, 4, 10, and 24 hours thereafter. Electron microscopic specimens were obtained from the lymph node and ascites fluid 1 or 2 hours after injection.

b) Light microscopic observations.
Development of whole body coloring was the same as that in the case of acid dyes in the early stage, but after 24 hours marked fading appeared. Contrary to
the case of acid dyes, all cells formed typical neutral red vacuoles as early as 2 hours after injection, although findings were rather variable at the 1st hour. Neutral red vacuoles were observable as well at the 10th hour, but all cells except reticulum cells faded completely 24 hours after injection.

c) Electron microscopic observations.

For the precise study of morphogenesis of neutral red-segresomes, it is very profitable to utilize lymphocytes, because these cells are not phagocytic and poor in granular components in the cytoplasm.

The first change observed in lymphocytes after the administration of neutral red was the appearance of a small group of fine particulate matters in the vicinity of the centrosphere (Text-fig. 3). Such fine particles are not neutral red dyes because the dye stuff had dissolved away into the surrounding media during preparation and, therefore, these particles must be the matrix substance of segresomes, without which the dye stuff could not be localized in high concentration in the cytoplasm. The formation of a limiting membrane was observed somewhat later. This means neutral red exists out of vacuoles in the cytoplasm, at least in the early stage of stain.

In the well-developed neutral red-segresomes, there often appeared electron dense, lamellated ring structures consisting of many thin membranes arranged concentrically; these are the so-called myelin-like figures presumably of lipoprotein nature (Fig. 4). Well-developed segresomes often showed very complicated structures such as open rings of myelin-figures or vacuolizations.

Neutral red-segresomes of plasma cells consisted of a fine granular matrix and complicatedly wavy myelin-like structures, and provided no limiting membrane. Neutral red-segresomes of reticulum cells were morphologically very similar to those induced with trypan blue, but were more rich in lipoprotein masses than the latter. Myelin-like figures were also observed in neutral red-segresomes of peritoneal macrophages.

d) Remarks

It is clear that the ultrastructure of segresomes, although induced with the same agent, neutral red, is more or less modified according to the kinds of cells and the stages of segregation. Moreover, the kinds, dosis and fixability of dye stuffs also may exert influence upon the morphology of segresomes. Nevertheless, such variations are only modifications of the structure of the same origin and nature, namely, the segresomes.

VI) Discussion

a) The nature and origin of the vitally stained granules.

Vital stain is established with the precipitation of dye stuffs within the non-specific cytoplasmic granules which are designated as segresomes in this paper. Overflow saturation of dye stuffs in the cytoplasm is avoided by segregation and
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condensation of them within the segresomes. Existence of such granular components in the cytoplasm was already postulated by some well-experienced light-microscopists such as Renaut\textsuperscript{55}) (grain de segregation), Kiyono\textsuperscript{40}) (chromophil granules) or Evans and Scott\textsuperscript{19}) (segregation-apparatus).

Some of segresomes preexist prior to vital stain, but most of them are newly formed by the cell at the time of dye stuff invasion. When dye stuffs enter into the cytoplasm, they are forced to be localized within certain small areas in high concentration. The substantial basis of such a process is given by the mobilized substances of cytoplasmic origin which combine with dye stuffs and later develop

<table>
<thead>
<tr>
<th>Cell</th>
<th>Designations</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) Epithelium Hepatic cells</td>
<td>Microbodies</td>
<td>Rouiller et al\textsuperscript{50})</td>
</tr>
<tr>
<td>&quot;</td>
<td>Lysosomes</td>
<td>Hagihara\textsuperscript{24})</td>
</tr>
<tr>
<td>&quot;</td>
<td>Granules</td>
<td>Novikoff et al\textsuperscript{49})</td>
</tr>
<tr>
<td>&quot;</td>
<td>Electron-dense body</td>
<td>Essner\textsuperscript{18})</td>
</tr>
<tr>
<td>Kidney epithelia</td>
<td>Microbodies, Big granules</td>
<td>Yura\textsuperscript{64})</td>
</tr>
<tr>
<td>&quot;</td>
<td>Osmiophilic inclusions</td>
<td>De Man et al\textsuperscript{44})</td>
</tr>
<tr>
<td>&quot;</td>
<td>Lysosomes</td>
<td>Rhodin\textsuperscript{44})</td>
</tr>
<tr>
<td>&quot;</td>
<td>Large round cytopol. bodies</td>
<td>Weiss\textsuperscript{83})</td>
</tr>
<tr>
<td>Pancreatic epithelia</td>
<td>Osmiophilic inclusions</td>
<td>Novikoff\textsuperscript{50})</td>
</tr>
<tr>
<td>Intestinal epithelia</td>
<td>Dense bodies, Inclusions</td>
<td>Clark\textsuperscript{4})</td>
</tr>
<tr>
<td>&quot;</td>
<td>Inclusions</td>
<td>Weiss\textsuperscript{83})</td>
</tr>
<tr>
<td>Uterus surface ep.</td>
<td>Dark granules, Areas, Bodies</td>
<td>Clark\textsuperscript{4})</td>
</tr>
<tr>
<td>Adrenocortical ep.</td>
<td>Microbodies</td>
<td>Woodsdell et al\textsuperscript{84})</td>
</tr>
<tr>
<td>&quot;</td>
<td>Globules, Pigment bodies</td>
<td>Karre\textsuperscript{54})</td>
</tr>
<tr>
<td>Thyroid epithelia</td>
<td>Granules</td>
<td>Nilsson\textsuperscript{47--48})</td>
</tr>
<tr>
<td>B) Muscular tissue Heart muscle cells</td>
<td>Cytosom</td>
<td>Bel\textsuperscript{21})</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td>Pigmentgranula</td>
<td>Zelander\textsuperscript{83})</td>
</tr>
<tr>
<td>of uterus</td>
<td></td>
<td>Ekhholm et al\textsuperscript{15})</td>
</tr>
<tr>
<td>C) Nervous tissue Spinal ganglia cells</td>
<td>Pigment</td>
<td>Lindner\textsuperscript{41})</td>
</tr>
<tr>
<td>Axon and Schwann cell</td>
<td>Osmiophilic granules</td>
<td>Lindner\textsuperscript{42})</td>
</tr>
<tr>
<td>D) Mesenchymal cells and others Reticulum cells</td>
<td>Segresomes</td>
<td>Hess\textsuperscript{51})</td>
</tr>
<tr>
<td>Alveolar macrophages</td>
<td>Cytosom</td>
<td>Elfvin\textsuperscript{16})</td>
</tr>
<tr>
<td>Peritoneal macrophages</td>
<td>Inclusion bodies</td>
<td>Tanaka\textsuperscript{75--77})</td>
</tr>
<tr>
<td>Histiocytes</td>
<td>Neutral red vacuoles</td>
<td>Schul\textsuperscript{64})</td>
</tr>
<tr>
<td>&quot;</td>
<td>Phagocytic vacuole</td>
<td>Karrer\textsuperscript{77--80})</td>
</tr>
<tr>
<td>&quot;</td>
<td>s-granules, NR vacuoles</td>
<td>Tanaka\textsuperscript{70})</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>Odd granules</td>
<td>Kajikawa et al\textsuperscript{85})</td>
</tr>
<tr>
<td>&quot;</td>
<td>Neutral red vacuoles</td>
<td>Tanaka\textsuperscript{74})</td>
</tr>
<tr>
<td>Plasma cells</td>
<td>a-granules</td>
<td>Tanaka\textsuperscript{71})</td>
</tr>
<tr>
<td>Basophil leucocytes</td>
<td>Segregation apparatus</td>
<td>Low et al\textsuperscript{13})</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>LK-granules</td>
<td>Tanaka\textsuperscript{73})</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Round dense bodies</td>
<td>Ito\textsuperscript{94})</td>
</tr>
<tr>
<td>Sertoli cells</td>
<td></td>
<td>Beams et al\textsuperscript{1})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ubukata et al\textsuperscript{80})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zebrum et al\textsuperscript{87})</td>
</tr>
</tbody>
</table>
into matrix substances of segresomes. Surrounding such a complex aggregation, the limiting membrane is formed secondarily. A quite similar process was observed by Richter\textsuperscript{57} in the uptake experiment of iron compounds. Myelin figures or lipoprotein masses which are often formed within developed segresomes were observed even in extracellular spaces where materials such as hemoglobin were found in extremely high concentration.\textsuperscript{45}

Structures identifiable as segresomes have been described in numerous electron microscopic reports, and their representatives are summarized in Table I. As demonstrated in Table I, segresomes are formed in almost all sorts of cells, and designated as lysosomes, microbodies, cytosomes, etc. It is true that these structures provide a more or less characteristic morphology for themselves. In my opinion, however, such characteristics are only morphological modifications of the structure of the same nature and origin; the segresomes.

De Duve, Novikoff and others not only described the classical morphology of the lysosomes of liver cells,\textsuperscript{49} but also recently attempted to include so many other structures within the category of lysosomes that the concept of lysosomes in the most recent sense\textsuperscript{7,8,14,18,32,51} are, to a certain extent, similar to that of segresomes. But details of morphogenesis and of structural modifications of lysosomes were still not given by them.

Microbodies\textsuperscript{25,55,59}, cytosomes\textsuperscript{41,94} and many other structures described in Table I have often been considered precursors or derivatives of mitochondria. Such mistakes might arise from the misunderstanding of myelin-figures often observed within segresomes as signs or remnants of mitochondrial cristae.

From the investigation of experimental conditions in Table I, it is reasonable to assume that segresomes are formed by the cell not only against the exogenous foreign bodies such as dye stuffs, but also more often against many substances of internal origin, for example, pigments of various natures.\textsuperscript{18,31,41,88} Therefore, it is natural that in the reticuloendothelial cells segresomes are found even under physiological conditions\textsuperscript{35,37,38,64,74} because management of toxic or useless substances is one of the major functions of these cells.

b) The mechanism to transport the extracellular dye stuffs to the inside of the segresomes.

The assumption that the process of vital stain is neither phagocytosis nor pinocytosis, but segregation following membrane penetration of extracellular dye stuffs is repeatedly described above. It is necessary to trace whether or to what extent such an assumption can interpret most of important kinetics of vital stain.

In Table II, various sorts and sizes of substrates which were utilized hitherto in phagocytosis or pinocytosis experiments by means of the electron microscope are summarized. It is very interesting to note that all sorts of substrates except acid dyes are taken into the cells within minutes or hours, whereas days are necessary for cells to take up acid dyes. Rapid uptake of macromolecules was
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TABLE II. Approximate Size of Various Substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Diameter (Å)</th>
<th>Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large bodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>80,000</td>
<td>17, 65,</td>
</tr>
<tr>
<td>Bacteria</td>
<td>10,000</td>
<td>24, 25, 85</td>
</tr>
<tr>
<td>Colloidal particles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>India ink</td>
<td>1,000</td>
<td>38, 65, 79</td>
</tr>
<tr>
<td>ThO₄ and HgS</td>
<td>200</td>
<td>29, 52, 60</td>
</tr>
<tr>
<td>Colloidal gold</td>
<td>140</td>
<td>30</td>
</tr>
<tr>
<td>Ferritin</td>
<td>100</td>
<td>4, 20, 21, 39, 57</td>
</tr>
<tr>
<td>Width of cell membrane</td>
<td>90 (fresh)</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>75 (fixed)</td>
<td>58</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>75 × 50</td>
<td>45</td>
</tr>
<tr>
<td><strong>Vital dye stuffs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Janus green</td>
<td>25.2*</td>
<td>61, 62, 66, 77, 78</td>
</tr>
<tr>
<td>Carmin</td>
<td>20.4*</td>
<td>75, 76, 77</td>
</tr>
<tr>
<td>Trypan blue</td>
<td>13.2*</td>
<td>77</td>
</tr>
<tr>
<td>Neutral red</td>
<td>8.2*</td>
<td>66, 73, 77, 83</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>?</td>
<td>12, 13</td>
</tr>
</tbody>
</table>

*: Cited from Fautrez and Lison.²²

Also supported by the fluorescent antibody methods⁵⁶,⁷⁷,⁷⁸,⁷⁹ and histochemistry.⁸⁰

Pinocytosis is vaguely assumed to be performed by all sorts of cells, whereas phagocytosis is observed in only a limited range of cells. Although the range of cells capable to uptake acid dyes is roughly the same as that of phagocytosis, some phagocytes such as granulocytes and monocytes are not stained vitally with acid dyes, whereas basic dyes are taken up by all sorts of cells.⁴⁰ (Instainability of some phagocytes with acid dyes may be interpreted by the fact that the time duration necessary for vital stain is longer than the life span of these cells.)

Moreover, the diffusibility, or in other words, the particulate size of dye stuffs has the fundamental effect upon the range of stained cells and time duration necessary to stain cells, not only in the case of acid dyes (Schulemann⁴³, Kiyono et al.⁴⁰) but also in that of basic dyes (Sugiyama).⁸⁸,⁹⁹ As coloring of the whole body and ascites develop in an approximately similar manner regardless of diffusibility of dyes, the latter may be significant at the time of the penetrating cell membrane of dye stuffs. Pohle⁵³ reported that uptake and excretion of acid dyes were accelerated (retarded) by acid (base) administration, whereas those of basic dyes were influenced in the opposite way.

From the study of membrane permeability to ions, Mond⁴⁸ concluded that the erythrocyte membrane consisted of protein with an isoelectric point of pH 8–8.3. Although his experiment itself was later criticized by Davson¹¹, Mond’s idea is, to my opinion, quite suggestive for the interpretation of vital stain.

All the fundamental kinetics of vital stain described above indicates that vital stain in general cannot be regarded as simple phagocytosis or pinocytosis.
The assumption of membrane penetration and of secondary segregation, however, seems to be profitable to interpret the process of vital stain: acid dye stuffs as anions are, as a rule, not allowed to penetrate the cell membrane because of a highly negative charge of the latter, whereas some sorts of cells such as reticuloendothelial cells can uptake acid dyes after several days because of only weakly negative (nearly neutral) charge of their cell membrane. Such a postulate may also throw some light upon the nature of the reticuloendothelial cells.

Davson and Danielli assumed that the cell membrane consisted of a thin layer of lipide covered with unrolled porteins on both sides (paucimolecular theory). More recently, this assumption was supported also morphologically by Robertson by means of the electron microscope; he advocated a triple layered structure as the general morphology of cell membrane (unit membrane). Most of kinetics of vital stain, therefore, seem to be substituted by the problems of interactions between the electric charges of dye stuffs and of proteins of cell membrane on the one hand, and the dynamics for dye stuffs of various molecular sizes to pass through the pores of network of polypeptide chains at the surface of cell membrane on the other.

c) Relations between phagocytosis, pinocytosis and vital stain (Text-fig. 2)

It is well known pseudopods formation, invagination of surface membrane, and formation of intracytoplasmic vacuoles are observed in phagocytosis and pinocytosis.

The difference between phagocytosis and pinocytosis seems to depend upon whether substrate is "formed" or fluid, and, therefore, is problem of definition to a certain extent. If the word "formed" means that individual particle of substrate is identified in the electron microscope, the under limit of "formed" substrate to be phagocytized is ferritin, 100Å in diameter. Such a distinction, however, is quite artificial and arbitrary. Nevertheless, distinction of these two phenomena is not meaningless, because of the difference of cell range in which this or that phenomenon is observed. It may be necessary to establish a more profitable definition of phagocytosis and pinocytosis.

Substrates in particulate size as large as or larger than macromolecules remain within phagocytic or pinocytotic vacuoles at least for the time being, and do not penetrate the limiting membrane of vacuoles unless digestion and disintegration occur. Whether substrates can penetrate membrane or not depends upon their particulate sizes, and the border line may be found at approximately 30Å in diameter because Janus green can easily enter into the cytoplasm whereas hemoglobin can not. It is noteworthy that most of metabolites are smaller than 30Å in size.

Although the intracellular fate of substrates in vital stain is different from that in phagocytosis (or pinocytosis), formation of phagocytic or pinocytotic
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Vacuoles is very useful to establish vital stain, because 1) it increases the membrane area for the dye to penetrate, 2) it makes possible a mass emigration of dye stuffs with a certain orientation (membrane flow of Bennett\(^3\)), and 3) the permeability of vacuole membrane is much higher than that of surface membrane of the cell as pointed out by Holter.\(^3\)

In the typical process of phagocytosis, nothing but phagocytized substrate is observed within the phagocytic vacuoles.\(^6\)\(^,\)\(^7\)\(^,\)\(^9\) In some cases, particularly in the late stage of phagocytosis, amorphous, dense materials begin to appear and often fill up the phagocytic vacuoles.\(^17\)\(^,\)\(^25\)\(^,\)\(^29\)\(^,\)\(^52\) In some cases, moreover, typical myelin figures are formed there.\(^3\)\(^8\) Essner\(^17\) reported that acid phosphatase is as highly active in phagocytic vacuoles as in the lysosomes. Such evidences mean that some phagocytic as well as pinocytotic vacuoles in the late stage can not be distinguished form segresomes morphologically and functionally. It has also long been well known that vital dyes stain not only segresomes but also phagocytic vacuoles.\(^4\)\(^9\) In this case, however, dye stuffs are not phagocytized, but are segregated within phagocytic vacuoles as well as within segresomes, after the entrance into the cytoplasm.

Phagocytosis, pinocytosis and vital stain are indeed fundamentally different processes, however, in practice, these are so closely connected with each other as indicated above.

VII) Conclusions

1) Electron microscopic observations of vital stain with acid (carmine and trypan blue) and basic (Janus green and neutral red) dyes were described and discussed.

2) Vitally stained granules originate from the non-specific cytoplasmic granules: some of them preexist prior to, and the others are newly formed at the time of dye stuff invasion into the cytoplasm. Dye stuffs are segregated within these granules in high concentration. Such granules are designated as segresomes, and characterized by their single limiting membrane, diffuse internal matrix and lipoprotein masses often observed within them. The ultrastructure of segresomes, however, is more of less modified according to the sorts of segregating cells and segregated materials as well as the dosis and fixability of the latter.

3) Segresomes are formed in almost all cells under various conditions, and are designated by each author as lysosomes, microbodies, cytosomes, etc. Segresomes are formed not only against foreign materials of external origin, but also against various materials of internal origin such as pigments.

4) Extracellular dye stuffs penetrate the surface membrane of cell or limiting membrane of intracytoplasmic vacuoles to enter into the cytoplasm, and then are segregated within the segresomes, whereas particles as large as or larger than macromolecules remain, unless digestion occurs, within phagocytic or pinocytotic...
vacuoles. The upper limit of particulate size of substrates capable of penetrating cell membrane is assumed to be approximately 30Å.

5) Acid dye stuffs do not enter, in general, into the cells, because dye stuffs as anions can not penetrate the cell membrane on account of the highly negative charge of the latter. Reticuloendothelial cells and some others, however, can uptake acid dyes after a long time because of only a weakly negative (nearly neutral) charge of the cell membrane of these cells.

6) Phagocytosis, pinocytosis and vital stain (segregation) are performed by cells in close connection with each other, although these are fundamentally different phenomena.

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Addendum.
Following the reception of this manuscript for publication, a study was reported by Moore et al (J. Ultrastr. Res., 1961, 5, 244.) indicating that colloidal iron, 40-50Å in size, could penetrate the surface membrane of cell rather easily to enter into the cytoplasm.

References
Mechanism of Vital Stain

28) Hamajima, Y., Allergy (Jap.), 1960, 9, 122.
46) Mond, R., Pfliigers Arch., 1927, 217, 618.
61) Sasaki, H., Okayama-Igakkai-Zasshi, 1960, 72, 1789.
63) Schulze, W., Biochem. Zschr., 1917, 80, 1.
Fig. 1. A part of the cytoplasm of peritoneal macrophage 30 minutes after Janus green administration. An intramitochondrial vacuole is being pushed out of mitochondria. The outer double membrane of mitochondria is seen to continue over the surface of the vacuole (arrow.)

Fig. 2. A part of the cytoplasm of reticulum cell 24 hours after the 3rd injection of carmine solution. Segresomes provide a single distinct limiting membrane and diffuse internal matrix of granulofibrillar appearance. Carmine is identified as numerous fine particles within segresomes (1), whereas lipoproteins are observed as larger masses (2), central portion of which is sometimes less electron-dense as a result of extraction (3).
Fig. 3. A part of the cytoplasm of reticulum cell 24 hours after the 3rd injection of trypan blue solution. The structure of segre-
omes is fundamentally the same as those induced with carmine. Trypan blue, however, is not identifiable probably because of its low electron density, lipoprotein masses are also hardly recognizable.

Fig. 4. A part of the cytoplasm of lymphocyte 2 hours after the injection of neutral red solution. Within a segresome (neutral red "vacuole"), myelin-figure which consists of many thin membranes arranged concentrically is observed.