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By

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(With 5 Text-figures)

The ability of victoria blue to stain lipid material has long been under discussion by several authors. Robinow and Murray (1953) reported that dilute aqueous solutions of victoria blue 4R and B stained selectively the superficial layer of fixed bacterial and of yeast cells: under a similar condition ordinary basic dyes stained intensely cytoplasm only, and this staining reaction was due to the avidity of the cytoplasmic membrane for victoria blues. It seems probable that the cytoplasmic membrane contains a relatively high concentration of lipids. Murakami (1954) has questioned in his experiment on the blood cells of newts whether victoria blue 4R stains lipid only, though this dye does stain both fatty acids and lecithines.

When a fixed *Paramecium* was stained with victoria blue 4R (V. blue), a basic dye of triphenolmethane series, its superficial layer showed an evidently selective staining. To learn whether the staining ability of V. blue is effective with lipid material only, some experimental observations have been made with *Paramecium* as material.

The work was done under the direction of Prof. S. Makino to whom the author's cordial thanks are due for his keen interest in the subject. The author is also grateful to Mr. T. A. Okada and Mr. S. H. Hori for their valuable advice in the course of this study.

Material and method

For the study *Paramecium caudatum* was mostly used due to the fact that other species of *Paramecium* showed a similar pattern in results.

Fixation: Specimens grown in culture medium were fixed in a depression slide and transferred to slide glasses with a glass pippete. The fixatives used are 5% formalin, formalin-calcium, absolute alcohol, dilute Bouin's, Carnoy's, Schaudinn's and Worcester's solutions.

Staining: Selective staining important for analysis of the cell structure is not obtainable with the ordinary solution of stains, since the specimens become

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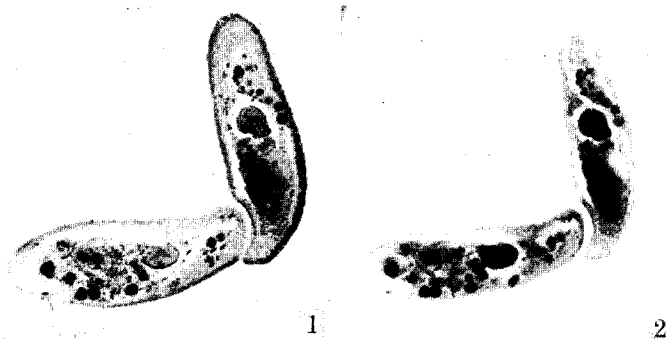
deeply and rather homogeneously stained in a very short time. The V. blue solution when it is used in a dilute solution, shows a differentiation of cell structure. Fixed specimens were stained in 0.05% aqueous solution of V. blue for 2 to 3 seconds. They were compared with those stained for the same length of time in 0.01% aqueous solution of thionine: V. blue can diffuse (5-10 min.) from the cell surface into the cytoplasm sooner than thionine.

Pyridin-test: The specimens were fixed with dilute Bouin's fluid for 15 minutes, immersed in 70% alcohol for one hour, transferred to pyridin fluid, and kept at 60°C for 24 hours. Then they were rinsed with tap water for 30 to 40 minutes and stained.

RNase-treatment: After fixation with Bouin's solution, the specimens were put into 70% alcohol for one hour, and immersed in 0.02% RNase solution warmed previously at 60°C: they were kept for 30, 40 and 60 minutes in groups.

Experimental observations

When the fixed *Paramecium* were stained with V. blue, its superficial layer, so-called "ectoplasm" initially shows a selective staining with coloration of clear blue (Fig. 1). After about 5 to 10 minutes, the dye diffuses immediately from the ectoplasm into the endoplasm and nuclei without selective staining. The material stained with V. blue can be destained by the use of alcohol and aceto-alcohol. The material can be repeatedly stained by the application of this procedure. Also the specimens which were stained with thionine and then are destained through the above procedure can be stained again with V. blue (Fig. 2).



Figs. 1. and 2. Photographs of *Paramecium caudatum*; comparison after staining with victoria blue 4R (Fig. 1) and thionines (Fig. 2). Carnoy-fixation. $\times 300$.

Influence of fixation upon the stainability was studied with many kinds of fixative. Such fixatives as 5% formalin, formalin-calcium, absolute alcohol, dilute Bouin's solution and Carnoy's fluid were found to be satisfactory in that

respect while the fixatives containing mercuric chloride such as Schaudinn's and Worcester's fluids showed neither staining specificity for the surface layer of *Paramecium* nor staining of its cytoplasm and nuclei except for the fact that the adsorption of some particles of the dye occurred in the cytoplasmic and nuclear membrane. Of the fixatives used, Carnoy's solution was favorable in staining sharply the contour of the specimen. The V. blue solution showed a remarkable surface activity: its staining reaction is either adsorption or chemical association. The staining reaction was then examined in the specimens which were pretreated with sugar and glycerin so as to disturb the surface activity of V. blue: the adsorption in the surface region was reduced, whereas the peculiar staining ability of V. blue was not disturbed. For the same purpose the material was exposed to absolute alcohol which produced a remarkable plasmolysis (Fig. 3-4): numerous particles of dye were arrested in the surface layer, while the ectoplasm stained deeply. A similar pattern of stainability was also found following the acetone vapor treatment which ensued in the raising up of cytoplasmic membrane (Fig. 5). From the results of the above experiments it is thus apparent that the ability to take a stain seems to be due to some staining reaction involving a chemical procedure (or procedures). A question arises whether or not the dye now under consideration stains lipids only. Then a pyridin-test was made by extracting the lipid material with pyridin. This resulted in finding that the staining ability of V. blue remained unchanged, and further that V. blue is not always the dye to stain lipid only.



Figs. 3 and 4. Showing plasmolysis due to treatment with absolute alcohol, and staining ability of V. blue. $\times 400$.

Fig. 5. Showing elevation of the cytoplasmic membrane with acetone vapour. $\times 400$.

Next, for the purpose of digesting protein and nucleic acid, some experiments were carried out with the use of depolymerizers such as hot water at 60°C , 1N HCl and 5% trichloroacetic acid. The experiments resulted in not only

disappearance of stainability but also the decrease of coloration in cytoplasm: the staining ability of V. blue which was not disturbed by lipid solvents was completely lost by the application of depolymerizers. The evidence obtained seems to suggest the essential role of nucleic acid, particularly of ribonucleic acid (RNA), in determination of avidity for V. blue because of the fact that desoxyribonucleic acid (DNA) and histone were not present in the cytoplasm. To confirm the significance of RNA, an experiment with RNase (depolymerase of RNA), was next performed: the material that stained with V. blue following complete degradation of RNA with RNase, did not show the peculiar staining. On staining with toluidine blue according to Lison's technique (1953) after the application of RNase for 30 minutes at 60°C, the material exhibited no visible change, but the material exposed to RNase for 40 minutes or more showed the abolishment of the coloration of the ectoplasm. Further, a thorough color change was obtained in the material exposed for 60 minutes. This seems to indicate that the degradation of RNA by the use of RNase starts in the ectoplasm prior to starting in endoplasm. It seems probable that the disappearance of the peculiar staining ability of V. blue by RNase takes place in association with the degradation of RNA. It has been inferred from these results that in fixed and stained specimens the peculiar staining power of V. blue is connected with an affinity for RNA occurring in the surface layer of the material.

Discussion

Robinow and Murray (1953) stated that the dilute aqueous solution of V. blue passed readily through the cell wall of bacteria, staining it lightly, and that it was arrested at the surface of the protoplast, and that this dye had an affinity for the cytoplasmic membrane. It was early recognized that cytoplasmic membrane contains a relatively high concentration of lipids. Then it has been generally considered that V. blue is effective in the staining for lipids. The V. blue solution differs from most of the basic dyes in general use, because they are almost completely separated from aqueous solutions when shaken up with lecithines or fatty acids in ether as shown by Schumacher (1936).

Murakami (1954) reported that V. blue was used effectively to stain exceedingly fatty acids and lecithines, but not either neutral fats nor mineral oils. Concerning the selective staining initiated by V. blue in the ectoplasm of fixed *Paramecium*, the question regarding adsorption or staining was solved by some trials which lead to a separation of the cytoplasmic membrane from the cytoplasm with absolute alcohol and acetone before staining. When the lipids are extracted with pyridin the staining ability of V. blue remains unchanged: all of the stained substances are not always lipid, although V. blue does stain lipid material. In the experiments with substances which dissolve lipids such as chloroform or ether, the staining reaction shows some retardation but gradually comes to show the characteristic reaction shortly. On this basis, it is difficult to conclude that

V. blue is a specific stain for lipids. Sudan III and Sudan black B used as tentative reagents for lipids did not show such staining reaction as did appear in the case of the use of V. blue. In the experiments with depolymerizers of protein and nucleic acid, hot water at 60°C digests nucleic acid especially RNA, 1N HCl digests histone and RNA, and 5% trichloroacetic acid digests DNA and RNA. Histone and DNA, however, certainly do not exist in the cytoplasm, and therefore the affinity of V. blue appears to be associated with RNA existing in the ectoplasm.

The ability of V. blue to stain nucleic acid has already been shown by Sibatani (1948). Murakami (1954) has shown that the staining reaction of basophilic granulocytes to V. blue also disappears following the pretreatments with depolymerizers of nucleic acid, protein and polysaccharides, and that the staining with V. blue is regarded as a sort of group reaction in these components.

Since the pretreatment of the material with depolymerizers of nucleic acid results in the disappearance of the peculiar staining ability of V. blue, it is apparent that the avidity of the ectoplasm for V. blue is attributable to the existence of RNA on the surface layer of the material.

It has been shown that the stain reaction of the cytoplasm to toluidin blue is gradually decreased by the presence of RNase: the former was completely abolished by the exposure of the material to the latter for one hour. The results obtained indicate that the degradation of RNA seems to go on from the ectoplasm into the endoplasm, or else that RNA present in the ectoplasm may be of depolymerization. If RNase were purified, the substance stainable with V. blue would be RNA itself.

Summary

The dilute aqueous solution of V. blue stains the superficial layer, so-called "ectoplasm", of fixed *Paramecium*. Some experiments on the staining ability of V. blue were performed following pyridin-test and with the use of lipid solvents. The results obtained indicate that the peculiar staining ability of this dye is not connected with lipids only because it was demonstrable even if the lipid material was extracted by pyridin. However, the staining power of V. blue was abolished with a depolymerizer of nucleic acid, particularly of RNA. It is considered that the staining ability of V. blue exists in association with RNA.

References

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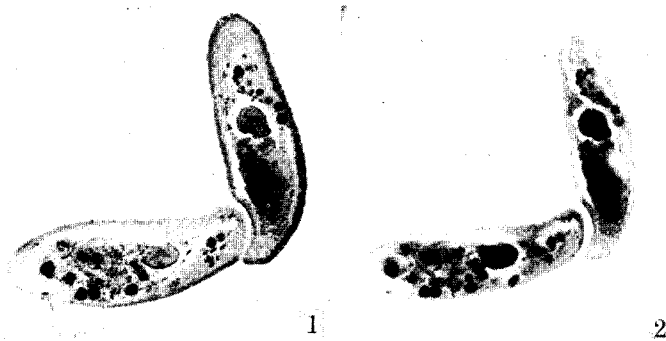
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