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Some cytochemical observations on *Zschokkella auerbachi* (Weill) Chakravarty, 1940 (myxosporidia, protozoa)

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With 10 figures

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Summary

Cytochemical observations have been made on the different developmental stages of *Zschokkella auerbachi* (Weill) Chakravarty, 1940, a coelozoic parasite in the gall bladder of *Bufo melanostictus* Schneider. Feulgen-positive substance (DNA) is found to be present in the nucleus in all stages of life cycle. Extra-nuclear DNA is also present in the spore coat, polar capsules and polar filaments. PAS-positive substances occur in varying concentration in all developmental stages and these remain, in lesser intensity, even after digestion with saliva. Toluidine Blue metachromatic (gamma) substances are present only in spore coat and polar capsules. Alcian Blue positive substances are localised in all stages with the strongest intensity in spores. Bromophenol Blue positive substances are localised in all the stages with strongest intensity in spore. Bound lipids are deposited in all stages in varying degrees of concentration.

Introduction

Mykosporidia is a group of animals parasitic in fishes, amphibians and in some reptiles. Cytochemical studies on these organisms have been done but little. Only the studies by Erdmann (1917), Kudo (1921), Bond (1937), Lom and Vavra (1961), Chakravarty, Maity and Ray (1962), Lom (1964) and Maiti, Chakravarty and Ray (1964) are related to the cytochemical make up of these parasites, and as such not much is known about the biochemistry and physiology of these coelozoic and histozoic parasites of lower vertebrates. In the present communication cytochemical localization of deoxyribonucleic acid (DNA), polysaccharides (including glycogen, muco- and acid muco-polysaccharides), general protein and bound lipids in the different stages of the life cycle of *Zschokkella auerbachi* from gall bladder of the toad, *Bufo melanostictus* Schneider, have been reported.

Material and methods

Toads, *Bufo melanostictus*, were collected from the Ballygunge Science College campus and also from other local areas. The gall bladder of freshly killed toad was taken out and smears made of its contents after diluting with a few drops of 0.6 % saline solution. The trophozoites and spores were found floating freely in the bile.

A few slides were stained by usual histological procedure in Heidenhain's haematoxylin for cytomorphic observations. All the cytochemical techniques were followed after PEARSE (1961). DNA was localised by Feulgen reaction. For polysaccharides Periodic Acid Schiff (PAS) technique, and for acid muco-polysaccharides Toluidine Blue and Alcian Blue methods were followed. Bound lipids were detected by Acetone Sudan Black B method, and general protein by Bromophenol Blue method.

Observations

A comprehensive idea about the morphological characters of *Z. auerbachi* was given by CHAKRAVARTY (1940). In the present study it has been further observed that in the formation of the spore valves, the valvular nuclei of the monosporoblast play an important role. After proper orientation of the two developing spores in the mother pansporoblastic membrane, and after differentiation of polar capsules, the two valvular nuclei in each monosporoblast first enlarge and then break down into smaller particles of varying diameters, which appear to be arranged in rows conforming to the shape of the spore. Gradually these particles, which take intense haematoxylin stain, diesappear and the spore coat now becomes basophilic (Fig. 1).

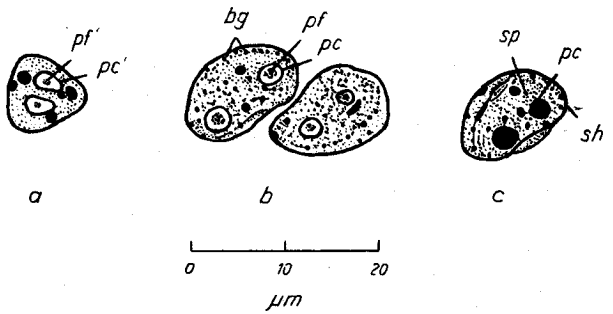


Fig. 1a, b and c. Showing later stages in the development of spore; stained with Heidenhain's haematoxylin (Camera lucida drawings). *bg* — basophilic granules; *pc* — polar capsules; *pc'* — developing polar capsules; *pf* — polar filament; *pf'* — developing polar filaments; *sh* — shell valves; *sp* — sporoplasm.

Detestion of cytochemical substances:

Feulgen. Positive Substance (fig. 2). In the trophozoites DNA is restricted to nuclei; the cytoplasm and the outer membrane show negative reaction. Feulgen-positive granules are distributed throughout the nucleoplasm. No differentiation could be made between vegetative and generative nuclei.

In the sproroblast and earlier stages of monosporoblast only the nuclei show a positive reaction.

In maturing spore DNA gradually begins to appear in extranuclear regions. In the nature spore one or two generative nuclei lying dorsally are strongly positive,

while the two capsular nuclei lying just above the capsules are pycnotic. The shell valves show fairly positive reaction for DNA, especially on the dorsal side. Polar filaments also show positive reaction for DNA. Polar capsules are seen to be ringed by Feulgen-positive granules.

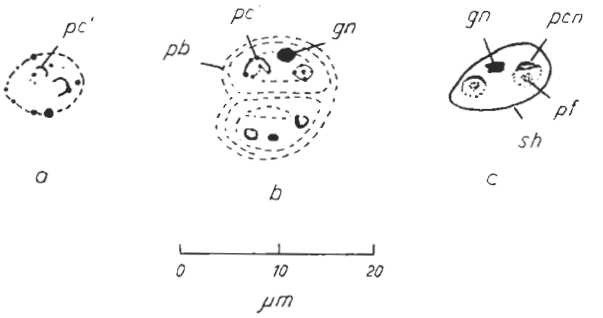
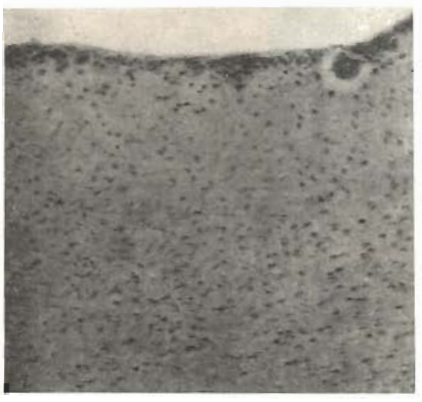


Fig. 2a, b and c. Localisation of DNA by Feulgen reaction in later stages of spore development. DNA represented by solid and fine dotted lines. (Camera lucida drawings.) *gn* — germinal nuclei; *pb* — pansporoblastic membranes; *pc'* — developind polar capsules; *pcn* — capsular nucleus; *sh* — shell valves.

Periodic Acid Schiff (PAS) Positive Substances (figs. 3 and 4). The cytoplasm of the trophozoite shows a violet colour indicating the presence of polysaccharides. The positive granules are very fine as well as coarse, and give the cyotplasm a reticular appearance. At some sites coarse granules are more densely localised. The outer membrane of the trophozoite is very faintly positive.



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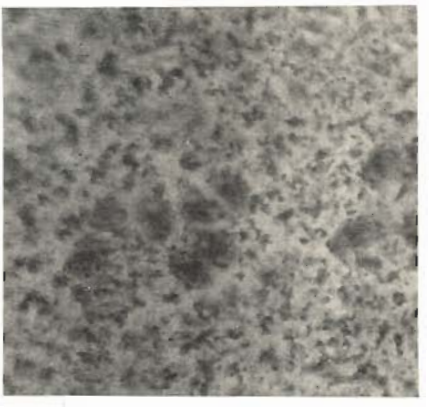

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Fig. 3. Showing localisation of PAS-positive substances in trophozoite and endogamous bud (×70).

Fig. 4. Showing localisation of PAS-positive substances in trophozoite and spores (×800).

In the pansporoblast the intensity of reaction is more and this is heaviest in mature spore. Some coarse and fine granules are present throughout the pansporoblastic membrane.

In the immature spore the polar capsules are lined with positive granules and the polar filaments appear distinctly in some cases. The sporoplasm is positive in all the stages.

In mature spores the shell valves are strongly positive. The nuclei are distinctly negative for PAS-positive substances.

In the endogamous buds the PAS-positive granules are very densely packed in a reticular fashion. The outer membrane of the bud also shows positive reaction.

PAS. Positive Substances after digestion with Saliva (fig. 5). After digestion with saliva for two hours at 37 °C the intensity of reaction decreases in all the stages. Only the fine granules remain showing that these are saliva-resistant, the pattern of distribution of these fine granules being the same as in the previous reaction. Saliva — digested substances are considered as glycogen.

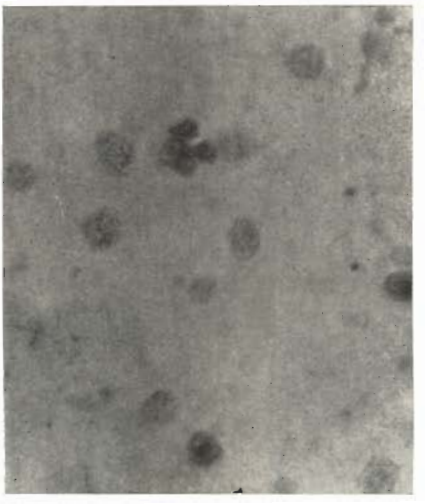


Fig. 5. Showing localisation of PAS-positive substances after saliva digestion (×800).

Toluidine Blue Metachromatic Substances (fig. 6). All stages of the parasite stain orthochromatically with Toluidine Blue except the spores, which exhibit metachromasia. In the monosporoblast the cytoplasm stains orthochromatic while the nuclei are not clear. Polar areas are with rounded orthochromatic bodies surrounded by a clear space. Metachromasia first appears as a narrow pink ring round these orthochromatic bodies. Gradually acid muco-polysaccharides also become deposited in the periphery so that these regions also stain metachromatically with Toluidine Blue.

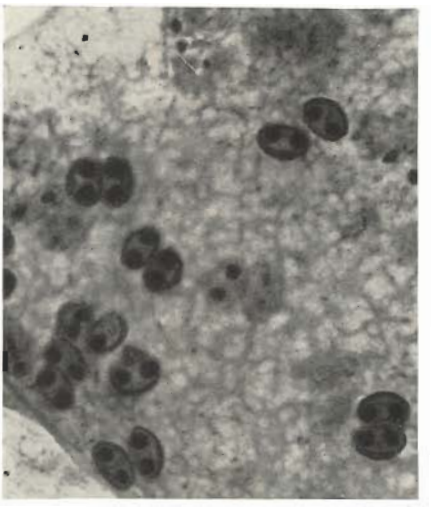


Fig. 6. Showing localisation of Toluidine Blue metachromatic substances in trophozoite and spores ($\times 800$).

In mature spores the polar capsules show purple colour. The polar filaments are faintly metachromatic, while the nuclei do not take any stain. The shell valves are sligthly metachromatic.

Alcian Blue Positive Substances (fig. 7). The outer membrane of the trophozoite is fairly positive. Scattered throughout the cytoplasm are very fine positive granules

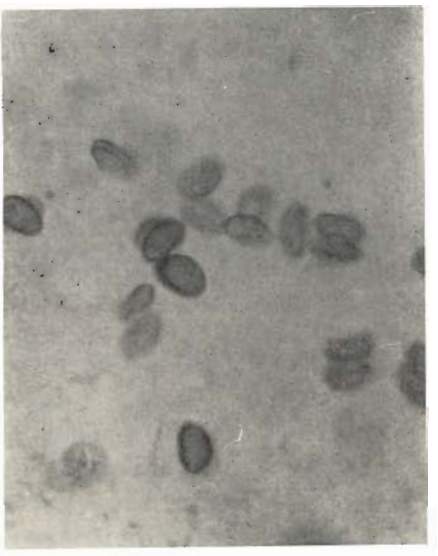


Fig. 7. Showing localisation of Alcian Blue positive substances in trophozoite and spores ($\times 800$).

indicating the presence of acid muco-polysaccharides. Early stages of pansporoblast are not differentiable. Monosporoblast shows slightly stronger reaction than the general cytoplasm, the polar filaments and the polar capsules are positive.

In the mature spore the intensity of reaction is very high. The shell valves take bright blue green colour and display the striations very prominently. Polar filaments and polar capsules are positive. Sporoplasm shows weak reaction.

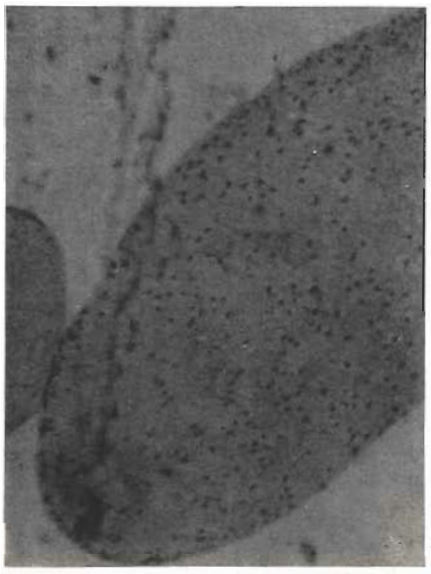
Bromophenol Blue Positive Substances (fig. 8). In the mature trophozoite both nuclei and cytoplasm take strong blue colour with Bromophenol Blue indicating the presence of protein. The outer membrane is fairly positive. Cytoplasm shows a reticulated appearance.

A gradual increase in the intensity of reaction for positive substances is observed during various stages in the formation of spores, the reaction reaching its maximum in the mature spore. In the developing and the mature spores all the structures show strong localisation of protein.

Fig. 8. Showing localisation of Bromophenol Blue positive substances in trophozoite (×70).

Acetone Sudan Black B Positive Substances (figs. 9 and 10). The cytoplasm of mature trophozoite with its outer membrane shows a weak reaction exhibiting a reticulated structure. The nuclei lying scattered in the cytoplasm also show a weak reaction.

Both cytoplasm and nucleus of the pansporoblast reveal a stronger reaction. In mature spores the shell valves show positive reaction with faintly visible striations. The reaction in the capsule coat and filaments decreases while sporoplasm appear negative. The nucleus becomes slightly distinct at this stage.



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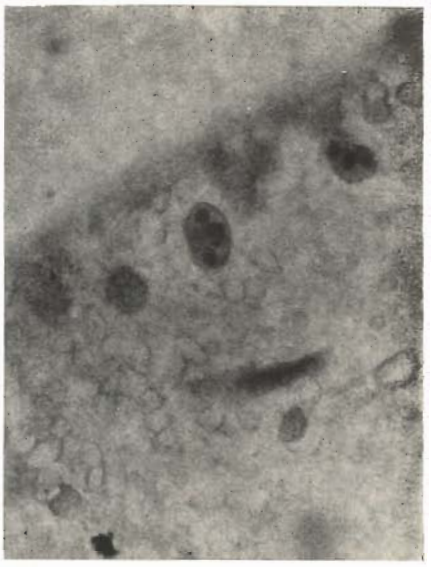

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Fig. 9. Showing localisation of Acetone Sudan Black B positive substances in trophozoite (×70).

Fig. 10. Showing localisation of Acetone Sudan Black B positive substances in trophozoite and spores (×800).

Discussion

The Feulgen reaction is specific for deoxyribonucleic acid (DNA). DNA is now well known to be the hereditary material and as such is found chiefly in the nucleus being associated with chromosomes. In the mature spores usually two nuclei are present while they are still in the parent trophozoite cytoplasm, but in the liberated mature spores there is usually only one nucleus, and this suggests that some kind of nuclear fusion occurs in the mature spore before it infects a new host. This zygote nucleus transmits the genetic information to the next generation. The function of the pycnotic capsular nuclei is not clear. They may have a controlling function on the extrusion of the polar filaments, but there is no direct evidence for this.

DNA has also been observed in extra-nuclear regions in the present study, especially in the spore. It has been found to be located in the shell valves, polar capsules and filaments. In Myxosporidia, similar localisation of DNA has been reported by KUDO (1921), BOND (1937), CHAKRAVARTY, MAITY and RAY (1962) and MAITI, CHAKRAVARTY and RAY (1964). The occurrence of extra-nuclear DNA in myxosporidian spore in due to the fact that the valvular nuclei completely degenerate and fragment during formation of spores. BOND (1937) reported, and the present study also shows, that the extra-nuclear DNA is absent from pansporoblastic and monosporoblastic membrane. It is during the later stages of the spore development that the valvular and capsular nuclei break down into small particles that take intense stain with haematoxylin, and these are finally incorporated into the shell valves and polar capsules. Thus it can be assumed that the nuclear DNA is incorporated in the cytochemical make up of the shell valves. As was reported by KUDO (1921), the polar filaments are formed partly by the mixture of nuclei and partly by some substance differentiated in the capsulogenous cell; that is why the polar filaments also show presence of DNA.

What is the role of extra-nuclear DNA in myxosporidian spore? The answer is not clear. It must have some role in the vital activities of the spore. It may carry genetic information in a diffused manner or it may be associated with some other chemical compounds like protein or polysaccharides etc., for some specific function. However, these are only speculations, nothing definitely can be said as yet.

The various PAS-positive substances include, among others, glycogen, starch, cellulose, muco- or glyco-protein and glycolipids. The acid muco-polysaccharides are said to be negative for PA-Schiff technique (Pearse 1961). In the present case the PAS-positive substances have been found chiefly in the form of coarse and fine granules in various developmental stages. After digestion with saliva the coarse granules disappear. These coarse granules are then nothing but glycogen, which is a ready source of energy. It has been suggested that glycogen may also be utilised for the synthesis of muco-polysaccharides (Gill and Ray 1954), and these may well explain the high concentration of glycogen granules in the developing spores.

Amylase-fast PAS-positive substances may be neutral muco-polysaccharides, or protein or lipid derivatives thereof. In the cytoplasm of the trophozoite amylase-fast PAS-positive granules are scanty but the spore shows a moderate reaction. In the spore, the shell valves and the capsules show positive reaction for amylase-fast PAS-positive substances, proteins and bound lipids. This suggests that the polysaccharides present here may be in the form of mucoprotein and glycolipids.

Acid muco-polysaccharides, as indicated by the gamma metachromasia with Toluidine Blue, are localised in the shell valves, capsule coat and also slightly in the polar filaments; all other stages show orthochromasia. Alcian Blue positive acid muco-polysaccharides are also present in high intensity in mature spore, but with lesser concentration in the trophozoite cytoplasm. It seems, therefore, that the muco-polysaccharides play an important role in the structure and function of these parasites. Lom (1964) reported that the shell valves of spores of several genera of Myoxsporidia including *Zschokkella* give a negative reaction with histochemical tests for polysaccharides and chitin, while they are positive for general protein. However, in the present case the shell valves are definitely positive for various polysaccharides. Kudo (1921) concluded that the chemical reaction of myxosporidian spore membrane are less similar to those of chitin compared with microsporidian spore membrane. The present findings readily explain this. Chitin is a polyhexosamine, a simple neutral muco-polysaccharide, whereas in this myxosporidian the polysaccharides are presumably of a complex nature.

The capsules formed by many micro-organisms also contain extra-cellular mucostubstances and these may be regarded as primitive cytoskeletal structures. They real significance of these amino-sugars on the spore coat is not clear. The muco-polysaccharides are known to form antibodies and thus in this case the muco-polysaccharides may protect the spore from various bacterial attacks during its transport from one host to another. Lom and Vavra (1961) have suggested that the mucosubstances by their hygroscopic properties help the spore to float on water surface and thus assist in spore dispersal. The amino-sugars may also form surface layers which are resistant to many hydrolytic enzyme systems and which may regulate ionic and water transport (Kent and Whitehouse 1955).

Protein is present in all the stages of *Z. auerbachi*. Proteins form the structural basis of the organism and also, as is well known, all the enzymes are protein in nature. Concentration of protein reaches its maximum in mature spores and this is quite apparent in view of the fact that the spores have to pass through the most crucial part of the life cycle.

The bound lipids are seen to be present in the membranes lining the trophozoite, the pansporoblast and the monosporoblast. The lipids together with the proteins form an essential part of the cell membrane. In the nucleus also lipids occur together with protein. Lipids present in the cytoplasm of the trophozoite represent stored energy. It is seen that the concentration of bound

lipids in polar capsules and filaments decreases with the maturity of spores. It seems that in the developmental stages bound lipids play an important role but with the completion of development they gradually lose it. On the other hand the intensity of bound lipids in the shell valves of mature spore increases, and here probably they occurs as lipoprotein or glyco-lipid complexes.

A brief survey of the above data shows that all the cytochemical substances analysed in the present work have their maximum concentration in the spore. This may be due to the fact that the spore has to face the hazards of transportation from one host to another and then to initiate and establish a new generation of the parasite. But the occurrence on the shell valves of substances like DNA is most surprising. It seems that the myxosporidian spore membrane is not a simple dead and protective encasement but also plays an essential role in spore's metabolism.

Zusammenfassung

Es wurden cytochemische Beobachtungen an den verschiedenen Entwicklungsstadien der *Zschokella auerbachi* (Weill) Chakravarty, 1940, eines coelozoischen Parasiten in der Gallenblase von *Bufo melanostictus* Schneider vorgenommen. Feulgenpositive Substanz (DNS) fand sich im Nukleus in allen Lebensstadien. Extranukleare DNS ist auch im Sporenmantel, den Polkapseln und den Polfilamenten vorhanden. PAS-positive Substanzen kommen in verschiedener Konzentration in allen Entwicklungsstadien vor und bleiben auch, in geringerer Intensität, sogar nach der Verdauung mit Speichel bestehen. Toluidinblau-metachromatische (gamma) Substanzen befinden sich im Sporenmantel und in den Polkapseln, Alcianblau-positive Substanzen von größter Intensität sind in allen Stadien in den Sporen lokalisiert. Bromphenolblau-positive Substanzen sind in allen Stadien mit größter Intensität in der Spore lokalisiert. Gebundene Lipoide sind in allen Stadien in unterschiedlichen Konzentrationen abgelagert.

Выводы

Проведены цитохимические исследования в различных стадиях развития Zschokkella auerbachi (Weill) Chakravarty, 1940, целозоического паразита в желчном пузыре Bufo melanostictus Schneider. Фейльген-положительное вещество (ДНК) найдено в ядре на всех этапах жизни. Внеядерная ДНК имеется и в оболочке споры, в полярных капсулах и филаментах. ПАСК-положительные вещества различной концентрации имеются во всех стадиях развития и в незначительном количестве сохраняются даже после переваривания слюной. Метахроматические (гамма) вещества толуидинового синего находятся в оболочке споры и полярных капсулах, положительные к альциановому синему вещества высшей интенсивности локализованы во всех стадиях в спорах. Положительные к бромфеноловому синему вещества высшей интенсивности имеются в споре во всех стадиях. Связанные липоиды различной концентрации найдены также во всех стадиях.

Résumé

Les auteurs ont effectué des études cytochimiques sur les différentes phases de développement du *Zschokkella auerbachi* (Weill) Chakravarty, 1940, parasite cœlozoïque dans la vésicule biliaire de *Bufo melanostictus* Schneider. Une substance Feulgen-positive(DNA) a été trouvée dans le noyau dans toutes les phases du cycle vital. Du DNA extra-nucléaire se trouve aussi dans l'enveloppe des spores, dans les capsules polaires et dans les filaments polaires. Des substances PAS-positives se trouvent en concentration variable dans toutes les phases de développement, et

elles restent décelables même après digestion par la salive, avec une intensité diminuée, c'est vrai. Des substances (gamma) métachromatiques sous l'action du bleu de toluidine ne se trouvent que dans l'enveloppe des spores et dans les capsules polaires. Des substances positives au bleu Alcian sont localisées dans toutes les phases avec l'intensité la plus forte dans les spores. Des substances positives au bleu de bromophénol sont localisées dans toutes les phases avec l'intensité la plus forte dans les spores. De dépôts de lipides liés se trouvent dans toutes les phases en concentration variable.

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