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THE RELATION OF MICROTECHNIQUE TO THE MORPHOLOGY OF SOME PROTOZOAN PARASITES[1]

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To a considerable extent the diagnosis of parasitic protozoa, and certainly the study of their detailed morphology, is dependent upon the application of technical processes of fixing and staining and the necessary accessory procedures. The taxonomy of many protozoan parasites is based upon morphological characters determined by a study of fixed and stained specimens. The diagnosis of intestinal protozoa is greatly aided by resorting to properly prepared fixed and stained slides, and in the experience of my associates and myself in making protozoological surveys, both the number of positives and the accuracy of their recognition are greatly enhanced by the study of permanent slides. The tendency to conduct surveys without the use of such preparations is to be deplored.

The following presentation will be largely concerned with intestinal protozoa, to which I have devoted considerable attention for more than twenty years. More especially the intestinal amoebae of man will be considered. During these years several thousand slides have been made and examined; over 130 different chemical substances or different dilutions or combinations of them have been tried out as fixing agents; but not so much has been attempted with different

[1] Condensed with slight revision from the presidential address delivered before the American Society of Parasitologists, December 31, 1940. The complete article with illustrations is published in the *Journal of Parasitology*, 27: 1, February, 1941.

stains. It is inappropriate and impractical to do more than refer to some of the more important results obtained.

In connection with these studies I am indebted to many sources and many individuals for assistance. For financial assistance I am indebted to the Special Research Fund of the University of Pennsylvania, the Penrose Fund of the American Philosophical Society and a special research fund contributed by John Wyeth and Brother of Philadelphia. For technical assistance at various times my thanks are due to Dr. Q. M. Geiman, Dr. R. M. Stabler and especially to Dr. Sarah H. Stabler. For help in securing source material I am indebted to many individuals, but more especially to Drs. A. D. Waltz, R. M. Stabler, John H. Arnett, H. A. Shelanski, J. H. Clark, A. L. Luchi and M. M. Rothman and Mr. R. L. Brown.

Before taking up the results of the study of particular species of parasites, I wish to make some comments on certain technical processes.

My experiments show that time is a relatively unimportant factor in the fixing process for these parasites. Fixation of smears of intestinal protozoa for one minute with the fixing agents commonly employed gives results as good as or better than those obtained by fixation for longer periods of time.

Heidenhain's iron alum hematoxylin is probably the best and the most commonly used stain for the smaller intestinal protozoa. Larger kinds, such as many of the ciliates and gregarines, do not stain so well with this stain unless they are sectioned. For these larger animals destaining with a saturated aqueous solution of picric acid will often give more satisfactory results than destaining with iron alum. This method[2] may be used to advantage for intestinal amoebae but does not do so well for flagellates. The Heidenhain method as described in many books requires a number of hours to carry through. However, as recommended by Craig and Faust,[3] if the mordant and stain are heated to 35 or 40 degrees C., the time in each can be cut to two to five minutes and results secured which can be used in diagnosis.

Mayer's hemalum is a quick and useful stain but does not stain cytoplasmic structures well and hence is not so appropriate for flagellates. It may also fail to stain the endosomes of species of *Entamoeba*. It has the advantage of being rapid and of staining all parts of a smear with approximately equal intensity. With proper precautions in interpretation, it becomes a useful diagnostic stain.

Feulgen's nucleal reaction is a valuable technique for recognizing certain details of nuclear structure, but results with its use are sometimes disconcerting. For example, it has often been surprising to find that a standard Feulgen procedure which produces typical coloring of nuclear chromatin in metazoan nuclei, may show a weak coloring or none at all in the nuclei of protozoa on the same slides. Nuclei at one stage in the life history of a protozoan may show the proper color reaction, while those of some other stage do not. On smears of the seminal vesicle of earthworms containing monocystid gregarines, the nuclei of trophozoites appeared to have no color reaction, while nuclei of the spores were well stained. Specimens of *Cryptobia* (*Trypanoplasma*) from fresh-water fishes showed only a faint reaction in the nucleus, while the kinetoplast (parabasal) was intensely colored. As pointed out by Margolena[4] and others, various plant materials containing aldehydes, such as lignin, etc., may give a positive Feulgen reaction. It is obvious, therefore, that nuclear chromatin does not always give a positive Feulgen reaction, and not everything that stains by this method is necessarily chromatin.

In metazoan nuclei the chromatic reticulum usually colors by the Feulgen method, while the nucleoli do not. There are cases, like some of the large oocytes, when even the reticulum does not show a typical reaction. Failure of nuclei to show this reaction may be due to the finely divided state of the reacting material. For staining reactions in general, the dispersion factor is important. If a stainable material is divided into fine granules and scattered amongst non-staining substances, the stain, if any, in these small particles, may be overlooked or masked by the associated substances. As a matter of fact, a close re-examination of the nuclei of the trophozoites of the gregarines referred to above revealed that there were fine granules of Feulgen reacting material in the reticulum of these nuclei, although absent in the endosomes (nucleoli). When this material is condensed to form chromosomes at the time of nuclear division, the coloring is much more readily recognizable as Meglitsch[5] noted for *Endamoeba blattae*. The Feulgen method is an important technical procedure, but here, as always, interpretation needs to be made with due caution.

A few results of experiments with different fixing agents will be referred to. One of the methods of experiment has been to study the effects of the constituents separately and then put them together again in different ways. This has been done for some of the commonly used fixing solutions. Various dilutions of mercuric chloride, alcohol, acetic acid, picric acid, chromic acid, etc., have been tried and approximately one hundred new combinations of them—with various other substances added—have been tested. None of

[2] H. C. Tuan, *Stain Tech.*, 5: 135–138, 1930.

[3] "Clinical Parasitology," Lea and Febiger, Philadelphia, 1937.

[4] *Stain Tech.*, 7: 9–16, 1932.

[5] *Jour. Parasitol.*, 25: 441–442, 1939.

these new combinations has appeared to have any great superiority over those in common use.

A study of the effects of different concentrations of some of these commonly used ingredients showed that weaker solutions, such as 25 per cent. to 50 per cent. of saturated mercuric chloride; 1 per cent. to 5 per cent. acetic acid or 50 per cent. alcohol are more satisfactory fixing agents for intestinal protozoa than stronger concentrations. It was shown by Dr. Geiman and myself[6] that one-half-strength Schaudinn's fluid is a satisfactory fixing agent for these organisms, and I have further found that even one-fourth-strength does very well for the same species. It is suggested that new fixing solutions which are much weaker and therefore more economical than those commonly employed may well be devised.

Schaudinn's sublimate alcohol, with a little acetic acid added, is probably more commonly used as a fixing agent for intestinal protozoa than any other single fixative. This is desirable, since it is a great advantage to have different workers use some one method so that comparison of results will be more valid. However, this fixing solution does not always give the same results, as explained beyond, and it is not the best fixative for many of the larger protozoa, such as the larger ciliates and gregarines, since it causes too much shrinkage and distortion. It is always advisable to try a number of methods with each different species to be investigated, in order that more worth-while results may be obtained.

As might be expected, many protozoa present different appearances when treated with different techniques. However, the same technique does not always produce the same result with the same species of parasite, because of the variability of the organisms themselves or of the environments in which they are found; and the different species often give different results with the same technique because of inherent specific differences. It is obvious, therefore, that a number of techniques needs to be employed to secure anything like a full picture of the morphology of any one species, and a single method is usually not applicable to any considerable number of species.

To show the effects of certain reagents and to illustrate the application of the general statements made above, it will be convenient to refer to a few particular species of protozoan parasites.

Various workers have shown that the parabasal body of trichomonad flagellates does not ordinarily stain with iron hematoxylin when the flagellates are fixed with Schaudinn's fluid with a small amount of acetic acid added, but does stain when they are fixed with Flemming's fluid or other chrom-osmic combinations. Grassé[7] and his associates have stated that acetic acid is especially destructive of the parabasal body in these organisms. Kirby[8] has shown that even though the parabasal will not stain with Heidenhain's after Schaudinn's fluid plus acetic, it will stain with Delafield's hematoxylin.

When *Trichomonas muris* is fixed in Schaudinn's plus 5 per cent. of acetic and then some of the slides stained with Heidenhain's and some with Delafield's, the difference in appearance of the flagellates is very striking. After Heidenhain's the parabasal body fails to stain but all the other organelles, including the large number of cytoplasmic granules, usually stain intensely. After Delafield's the parabasal stains but the cytoplasmic granules are stainless and practically invisible. Since such granules are considered diagnostic for certain species, the differences here might readily be misinterpreted if they were not known to be the result of different stains. That acetic acid does not destroy the parabasal body of *T. muris* is shown by the fact that this structure will stain with Delafield's even when the flagellates are fixed in 20 per cent. acetic acid used alone. When such smears are treated with Heidenhain's none of the cytoplasmic organelles stain.

Cysts of *Girardia lamblia,* from the small intestine of man, tend to show a remarkably constant staining reaction with iron hematoxylin when a variety of fixing agents are employed, although the amount of shrinkage within the cyst membrane may vary considerably. The endosomes of the nuclei and the various parts of the fibrillar apparatus commonly show an intense coloration with this stain. There are some interesting exceptions, however. In one case fixation with chrom-acetic with a little copper oxide added produced practically no shrinkage inside the cyst wall but none of the cell structures was stained. Phosphotungstic acid hematoxylin was found to stain the peripheral layer of the nucleus in *Giardia* cysts when Heidenhain's seldom does, and this feature of nuclear organization is commonly overlooked. While the staining reactions within the cysts of *Giardia* are usually fairly uniform, sometimes they are surprisingly variable. Even on the same slide one may find variations from practically no stain to strong staining of organelles. Such individual variations are difficult to explain in terms of variation in the environment.

Since the genera of endozoic amoebae are differentiated to a large extent by the differences in their nuclear structure, the effects of different reagents on these nuclei were studied.

Attention had previously been called to the fact that higher percentages of acetic acid added to Schaudinn's fluid tended to reduce the stainability of the endosome in the nuclei of the cysts of *Iodamoeba*

[6] *Stain Tech.*, 8: 158, 1933.

[7] *Arch. Zool. Exper. et Gen.*, 65: 345–602, 1926.

[8] *Tr. Am. Micr. Soc.*, 50: 189–195, 1931.

bütschlii.[9] There is much variation in these reactions, but when various strengths of acetic acid were used alone as fixing agents, 20 per cent. or higher caused the nucleus to lose its stainability in iron hematoxylin. When one half of a saturated solution of mercuric chloride was used as a fixing agent, the nuclei in *Iodamoeba* tended to stain homogeneously with Heidenhain's, but with hemalum the entire nucleus stained lightly except a small granule in the endosome. It is possible that this granule represents a centriole.

For *Endolimax nana* Stabler[10] showed that when the content of acetic acid in Schaudinn's fluid was increased to 15 or 20 per cent., the endosome of the nucleus stained less readily with iron hematoxylin. This result seems to be characteristic for this species but there are many variations. One sometimes finds nuclei with unstained endosomes even when no acetic has been added to the Schaudinn's fluid used as the fixative, and when 20 per cent. of acetic is added, one may find that stainability of the endosomes has been retained by some of the nuclei. Acetic acid used alone as a fixing agent may reduce the subsequent stainability of the endosomes even in concentrations as low as 2 per cent. and when 20 per cent. is used alone the nuclei fail to stain. In combination with other substances the effect of acetic acid varies. Adding 5 per cent. to saturated mercuric chloride solution increases the stainability of the endosome, but when the same strength is added to one-half saturated solution, the stainability of the endosome is decreased. Adding 5 per cent. of acetic to 50 per cent. alcohol decreases the stainability of the endosome, but does not do so when added to 95 per cent. alcohol. It would seem that the stronger reagents prevent the action of the acetic toward diminishing the stainability of the endosome. These experiments were carried out on the trophic stages.

Segal[11] has called attention to the fact that "buds" were produced on the cysts of *Endolimax nana* more frequently when they were fixed in picromercuric than when fixed with Schaudinn's and several other fixatives. She also noted that different races of this species had different tendencies in regard to the formation of "buds." The present study has confirmed the work of Segal and has shown further that "buds" can be produced by 25 to 50 per cent. acetic acid.

Both hemalum and phosphotungstic acid hematoxylin, after most fixatives used, had a greater affinity for the peripheral layer of the nucleus of *E. nana* than did iron hematoxylin.

Dientamoeba fragilis in its reaction to technical agents shows many contrasts to the other amoebae living in man. It is usually necessary to add from 10 to 20 per cent. of acetic acid to Schaudinn's fluid to get the nuclei to stain with Heidenhain's. But there are interesting exceptions. In some cases, when nuclei failed to stain after Schaudinn's plus 5 per cent. of acetic, they stained very well when the same material was fixed in one-half-strength of the same fixing solution. In certain cases stainability of the nuclei was greater when only 2 per cent. of acetic was added to Schaudinn's than when 5 per cent. was added, as it was also when 20 per cent. was added. In some other cases, the nuclei have stained readily after Schaudinn's without any acetic added. In one such case the feces were strongly acid to litmus so that it is supposed that organic acids in feces would affect the stainability of the nuclei upon fixation in sublimate alcohol. It is obvious, therefore, that the environmental conditions may markedly affect the results of such technical processes.

Experiments show that acetic acid will increase the stainability of the nuclei of this species when added to mercuric chloride, alcohol and other single substances. When used alone in any strength from 2 to 100 per cent., the nuclei of *Dientamoeba* are rendered stainable with iron hematoxylin. Bouin's fluid is an excellent fixative and hemalum an excellent stain for this species.

Entamoeba histolytica shares with *Endolimax nana* and *Iodamoeba bütschlii* the tendency for the endosome of the nucleus to lose stainability with Heidenhain's when increasing strength of acetic acid are added to Schaudinn's fixing solution. The stainability of the endosome may be lost when 5 per cent. acetic is used alone, and when 20 per cent. is used the entire nucleus remains unstained. When 50 per cent. is used as the fixative, some stainability may remain in the peripheral layer of the nucleus. After 50 per cent. acetic a wide shrinkage space appeared between the cytoplasm of cysts and the cyst membrane, or else extensive extrusions of cytoplasm were produced. These relatively enormous "buds" lacked a definite boundary at their outer surfaces, in contrast to "buds" produced by other agents. Fixation with Bouin's fluid may cause shrinkage spaces to appear either inside or just outside the nucleus of trophic stages, or, in some cases, may cause the endosome to fail to stain with iron hematoxylin. Alcoholic Bouin's may produce "buds" on the cysts of *E. histolytica*.

The cysts of *Entamoeba coli* often show much more variation in stainability than other species when fixed with Schaudinn's fluid and stained with Heidenhain's. Hemalum stains these cysts much more uniformly. Higher percentages of acetic acid added to Schaudinn's fluid have less tendency to diminish the stainability of the nuclei than in the case of *E. histolytica*. When 50 to 100 per cent. of acetic acid is used alone as a fixa-

[9] D. H. Wenrich, *Proc. Am. Phil. Soc.*, 77: 183–205, 1937.
[10] *Jour. Parasitol.*, 18: 278–281, 1932.
[11] *Am. Jour. Hyg.*, 15: 741–750, 1932.

tive, there usually appear in the cysts of *E. coli* extensive shrinkage areas between the cytoplasm and the cyst membrane or there may be large extrusions or "buds," as in *E. histolytica*. These "buds" appear to have been produced explosively and lack a definite boundary at the outer periphery.

That different species of intestinal amoebae show divergent reactions to the same technique has been indicated in the preceding accounts. These differences probably represent chemical peculiarities in the composition of the nuclei of these several taxonomic categories. Such differences were revealed by fixing the five species of amoebae found in the intestine of man with the same fixing agent, namely, Schaudinn's fluid plus 5 per cent. of acetic acid, and then staining them by three different methods: Heidenhain's hematoxylin, Mayer's hemalum and the Feulgen reaction. If our attention is confined to the nuclei it may be noted that the picture that has been considered characteristic for each species is given by the Heidenhain's stain. For *Entamoeba histolytica* and *E. coli* the endosome and peripheral layer stain well and a certain amount of periendosomal material also. This periendosomal material is usually more voluminous in *E. coli*. For *Iodamoeba bütschlii* there is no peripheral layer but the endosome and periendosomal layer stain deeply. For *Endolimax nana* the endosome stains deeply but the peripheral layer does not stain so well. With *Dientamoeba fragilis* no peripheral layer is seen, but the central nuclear mass stains well.

With hemalum the peripheral and periendosomal layers of *E. coli* and *E. histolytica* stain well, but the endosome tends to remain unstained, especially in *E. histolytica*. In *Iodamoeba* the endosome tends to stain more lightly than the peripheral layer, especially in the cysts, while in *Endolimax* both the endosome and the peripheral layer stain well. In *Dientamoeba* the stain is about the same as with Heidenhain's.

After Feulgen's technique only the periendosomal material of *E. histolytica* and *E. coli* show a coloration which is weak, but usually more pronounced for *E. coli*. In *Iodamoeba* only the periendosomal layer stains but the reaction is strong. In *Endolimax* only the peripheral layer stains but the reaction is weak. In *Dientamoeba* the reacting material is apparently identical to that which stains with Heidenhain's and hemalum. These evidences of chemical differences in the constitution of the nuclei of different kinds of intestinal amoebae offer important confirmation to the validity of the taxonomic groupings that have been established.

It may be well to repeat that different techniques may produce decidedly divergent appearances in the same species of parasite; on the other hand, there may be much variation in the appearances of various races or strains or individuals of the same species when treated with the same technique due to peculiarities of the organisms themselves or to differences in the environment at the time of fixation; various species give divergent reactions to the same technique because of inherent differences in their chemical constitution.

SCIENTIFIC EVENTS

MEDICAL FELLOWSHIPS OF THE NATIONAL RESEARCH COUNCIL

FOURTEEN fellowships in the medical sciences, including four renewals, were awarded at the recent meeting of the Medical Fellowship Board of the National Research Council, Washington, D. C., of which Dr. Francis G. Blake, Sterling professor of medicine at Yale University, is the chairman. A list of the successful candidates and institutions where they are to work follows:

Reginald M. Archibald (renewal), Hospital of the Rockefeller Institute.
Lindsay E. Beaton (renewal), Northwestern University.
Lauritz R. Christensen, New York University.
Albert H. Coons (renewal), Harvard Medical School.
William J. Darby, Jr., Columbia University.
J. Russell Elkinton (renewal), Yale University.
Frank L. Engel, Yale University.
Nathan B. Friedman, Yale University.
Robert Hodes, University of Pennsylvania.
Max N. Huffman, Columbia University.
Joseph L. Irvin, Columbia University.
Harry Lusk, University of Michigan.
Maclyn McCarty, Hospital of the Rockefeller Institute.
Francis D. Moore, Harvard Medical School.
Richard J. Porter, Harvard Medical School.
Douglas S. Riggs, Yale University.
Richard B. Singer, Harvard Medical School.
Stewart G. Wolf, Cornell University Medical College.

In addition to the above, in the new series of fellowships announced last fall for work in the filtrable viruses and orthopedic surgery, made possible through a grant from the National Foundation for Infantile Paralysis, Inc., seven fellowships were awarded, as follows:

Seymour S. Cohen, Rockefeller Institute for Medical Research, Princeton.
Charles E. Evans, University of Rochester.
Robert H. Green, Hospital of the Rockefeller Institute.
Walter P. Havens, Jr., Hospital of the Rockefeller Institute.
Joseph L. Melnick, Yale University.
Eleanora Molloy, Columbia University.
Addison B. Scoville, Vanderbilt University.