oxygen tension. The function of oxygen in this process can best be explained by the assumption that the enzyme contains a reversibly oxidizable active group, which in its reduced state favors lysis, and in its oxidized state synthesis of protein. The state of oxidation of this active group may be determined by the apparent oxidation-reduction potential of the system, in the regulation of which oxygen, catalytic heavy metals and organic sulphur compounds appear to play an important rôle.

Analysis of the action of glutathione upon *Amoeba proteus* indicates that this cell component, under the experimental conditions used, accelerates nuclear growth and thus promotes mitosis. No evidence was obtained which would indicate that glutathione is a mitotic stimulus in the strict sense. Minute amounts of copper salts inhibit nuclear growth. Within certain limits the effect of copper can be antagonized by glutathione.

Work on dividing amoebae indicates that an increase in the external hydrogen ion concentration by HCl does not influence cell division; lactic and pyruvic acids inhibit cytoplasmic division; CO_2 has a tendency to inhibit mitosis. Inhibition of mitosis results from exposure to H_2S, HCN and ether. The inhibition observed with H_2S and HCN is reversible. The implications of these results are discussed with regard to the chemical reactions underlying mitosis. There is a considerable independence of nuclear and cytoplasmic fission in *Amoeba,* depending on chemical changes of the environment.

AMOEBA PROTEUS AS MATERIAL FOR THE STUDY OF CELL DIVISION AND CELL GROWTH*

H. W. CHALKLEY

Requisite for any research is the possession of two things—a suitable material and a suitable method for the study of this material. In the fundamental problem of growth presented by cell division, the material used has been in the main the metazoan egg, the metazoan cell, and plant cells. With the exception of the unicellular plant cells, these forms have as material certain faults which complicate the analysis of the problem. In the egg there is the presence of a surplus of formative material sufficient not for one but for many cell divisions. In the metazoan tissue cell there is the need for a complex, poorly controllable, environmental medium, and in both the last and the higher plant cell there is an inability to culture isolated cells easily. Even in many of the unicellular plants photosensitivity appears as a complicating factor when it is considered that microscopic observation will ordinarily involve variation of illumination. It would appear from this that a search for at least additional material is worth while, and I venture to suggest that *Amoeba proteus* is in many ways well suited for the study of cell division, and to present methods which have been found satisfactory for such study. It is necessary that in so doing I present also a certain amount of biological data with respect to *Amoeba proteus* since it is, of course, upon such a basis that methods depend for their validity. It is obviously essential in any experimental procedure to establish the normal course of events in order that valid controls may be set up for experimental results.

*** A general discussion of the material and methods used in the work described by Dr. Voegtlin.**

To show the relation between the methods and their supporting data, it seems most simple to recount briefly their development during the series of researches on cell division in *Amoeba* as carried out at the National Institute of Health at Washington, D. C., from 1930 to the present time.

In selecting *Amoeba* as material for investigation of the division process, certain advantages and disadvantages were manifest. The most obvious advantages are the ease of culture, which makes it possible to carry on research continuously throughout the year; its relatively large size, which makes it easy to deal with individual cells; the absence of *reserve* food material above that small amount normally present as recently ingested nutrient, which insures a minimum complication consequent upon the confusion of growth and division; the slow movement, which facilitates observation; and the fact that the multiplication is vegetative and hence uncomplicated by sexual phenomena, such as fertilization.

The outstanding disadvantage when work was begun was that apparently little was definitely known about the phenomena of fission, or the growth of the cell. It is also a disadvantage that a synthetic medium for growth is not known, but in this respect *Amoeba* is neither better nor worse than cells in tissue culture, or egg cells. During the progress of the research, the life history of *Amoeba* has been carefully studied, and the phenomena of fission have been worked out in detail. As a result, methods of extreme simplicity have been devised which make it possible to investigate the effect of reagents upon division to the point that it is possible to determine which phase of mitosis is most strongly affected.

In the first series of experiments undertaken, that of Voegtlin and Chalkley (1930), as to the effect of glutathione upon fission, the amoebae were at first selected at random from culture, washed and isolated, each in a small beaker containing a solution of the reagent. The results of the reagent do not here concern us, but the fact that these results were found to be dependent upon the volume of the cells used, immediately indicated the necessity of considering cell volume as a factor in securing reliable data. It also raised the question as to the degree of correlation of cell volume with stage of the life of the cell.

An investigation (Chalkley, 1931) showed that cell volume was, as might be expected, a close correlate of age, and also that it was closely correlated with the volume of the nucleus and the number of nuclei in a cell. All of this, insofar as method is concerned, simply emphasizes the necessity of selecting cells of comparable volume if comparable and uniform results are to be obtained. The effectiveness of so doing is brought out in the results obtained by Chalkley and Voegtlin (1932) on the effect of copper and glutathione.

The volume of a cell can be ascertained roughly by stimulating the cell mechanically, which results in its assuming a spheroidal form, measuring the diameter with an ocular micrometer and calculating the volume on the assumption that the cell is spherical. If more accurate measurement is needed the cell may be drawn into a capillary of known diameter, its length measured and the volume calculated assuming the form to be a cylinder of the diameter of the capillary with hemispherical ends having radii equal to the radius of the capillary. The volumescope devised by Chalkley (1929) is suitable for this. During the progress of this research some cells divided while under examination and their characteristics were noted so that it became possible to select dividing forms from a culture. Chalkley and Daniel (1933) made a detailed study of the fission process and ascertained the correlation between external form and the nuclear phases of mitosis.

As fission progresses there is noted especially the initial assumption of a pseudopodia-studded spheroidal shape coupled with the appearance of a hyaline spot in the center of the cell, then a progressive change in the pseudopodia which become wider and longer, then the disappearance of the hyaline spot followed shortly by elongation of the cell, cleft formation and final fission.

This can be presented diagrammatically with its correlation with time, the nuclear phases of mitosis, as ascertained from stained preparations, and measurements of the changes in width of the pseudopodia. As a result of these measurements and observations it is now possible to select amoebae with an accuracy of 90% to 100% in prophase, in anaphase, or in the later stages of mitosis, thus permitting us to subject cells at any desired stage to an experimental procedure.

Finally, during the last few weeks, it has been found possible to watch the entire nuclear process of fission in the living cell. This is difficult because the cell is extremely granular and the nucleus in mitosis extremely transparent and of very nearly the same refractive index as the cytoplasm. Attempts to flatten the cell in compressoria, and thus render the nucleus visible by reducing the depth of cytoplasm between it and the lens of the microscope, resulted in damage to the cell. It was found, however, that if a couple of drops of .65% agar are placed on a slide, the amoeba placed on the hardened agar in a drop of saline and a cover glass (15 mm. square #1's are satisfactory) dropped gently upon it, the cell is flattened but not damaged, owing to the elasticity of the agar. Then by using an oil immersion objective and a good light source the nuclear changes can be observed. Photographs have not yet been made, but it is hoped that they can be, although at actual fission the nucleus is certain to be an extremely difficult object to photograph.

At present, camera lucida drawings at a magnification of 325 diameters are the best that can be presented. From these it is seen that in the prophase the chromatin wanders in from the cell membrane to form the metaphase plate, that the nuclear membrane disappears when the plate splits and that the figure is anastral with well developed polar caps and spindle. In the living cell the fibres and polar caps are represented merely by hyaline areas.

Data and techniques* are available which permit the direct observation of all phases of fission (cytoplasmic and nuclear) in this organism. Also, there are technique and data in respect to volume which, within limits, permit the selection of cells at any desired stage of the interkinetic period. It might be pointed out in this respect that it is possible to get cells for experimental purposes of exactly known age by selecting dividing cells, noting the time of division, and making use of them at the expiration of a determined interval.

As always in exact studies based on biological material, great care must be exercised to avoid impurities of all kinds, and to procure sufficient data to permit statistical treatment.

The experience at the National Institute of Health indicates that results based on observations of less than five individuals are doubtful.

*** For the best staining procedure, for description of the culture medium used, and for figures and more details concerning fission, see Chalkley and Daniel, 1933.**

Ten, at least, should be used for quantitative results, and more if the results are not markedly consistent.

The fission process is affected by temperature (Daniel and Chalkley, 1932). In all experiments, therefore, temperature changes should be prevented, or recorded. A water bath controlled within limits of one degree is usually sufficient control. The best temperature for growth is about 24-27° C. which is close to the optimum temperature (30°) for fission.

One peculiarity of *Amoeba* is the apparently normal occurrence in culture of polynucleate cells. These constitute on an average approximately 8% of the population of a culture. Such cells would seem to furnish material for investigation of the effect of such polynucleation upon division. They have been observed to occur by failure of cytoplasmic fission in apparently normal cells, and a statistical study of their occurrence and size relations (Chalkley, 1933) leads to the conclusion that this is the usual, if not the only, way in which they are formed.

The techniques outlined, it will be noted, provide methods not only for the study of fission, but, with the exception of complete control of nutrition, for the study of cell growth. The criteria for choosing dividing forms permit the exact time of fission to be noted so that it is possible to start with the "birth" of the cell. By use of the volumescope the growth of the cell can be accurately measured (the average deviation of such measurements is about 5%), and flattening the cell on agar permits accurate measurement of the nucleus. A series of measurements thus carried out has not yet been made but it is hoped that the opportunity will arise.

In conclusion, certain advantages of this organism may be emphasized.

1. It is available throughout the year and easy to culture.

2. It is large, can be handled rapidly and easily, and each cell can be observed individually.

3. All phases of fission and development are easily selected directly from cultures.

4. The cytoplasmic phenomena and nuclear phenomena of fission are normally almost separate in time, and therefore can be studied separately.

5. The cells are not carrying a reserve of material for the production of an entire organism as in eggs, and hence growth and fission phenomena are less likely to be confused.

6. The fission is asexual and not the result of a specialized sexual process linked to it.

BIBLIOGRAPHY

Chalkley, H. W., 1929. Changes in water content in Amoeba in relation to changes in its protoplasmic structure. Physiol. Zool., 4, 535-574.

Chalkley, H. W., 1931. II. The relation between cell growth and division in Amoeba proteus. Pub. Health Reps., 46, 1736-1754.

Chalkley, H. W., and Carl Voegtlin, 1932. III. Inhibition of cell division of Amoeba proteus by high dilutions of copper salts, antagonism of copper and glutathione. Pub. Health Reps., 47, 535-560.

Chalkley, H. W., and George E. Daniel, 1933. The relation between the form of the living cell and the nuclear phases of division in Amoeba proteus. Physiol. Zool., 6, 592, 619.

Chalkley, H. W., 1934. The observation of mitosis in the living cell in Amoeba proteus. Science (in press).

Daniel, George E. and Chalkley, H. W., 1932. The influence of temperature upon the process of division in Amoeba proteus. Jour. Cell. and Comp. Physiol., 2, 311-327.

Voegtlin, Carl and Chalkley, H. W., 1930. I. The effect of glutathione on cell division in Amoeba proteus. Pub. Health Reps., 45, 3041-3063.

DISCUSSION

Dr. Gudernatsch: Did I understand that no cell division occurs even under glutathione stimulation unless both cell and nucleus have reached the proper size? We all have been trained under the dogma of the nucleus-plasma relation. This is a very important point because, under glutathione stimulation, nuclear division occurs without ensuing cell division, as Dr. Chalkley has pointed out. Nuclear division alone may occur, as we know, in some cells; particularly under pathological conditions (e.g. tuberculosis) giant cells are formed—sometimes with as many as forty or fifty nuclei.

Dr. Voegtlin: In amoebae exposed to glutathione, nuclear fission occurs when the nucleus of control cells divides. Nuclear division may or may not be followed by cytoplasmic division, indicating a certain degree of independence of the two processes.

Dr. Gudernatsch: Glutathione contains glycine, glutamic acid and cystine. Have you tried the three acids or any two of them in mixture?

Dr. Voegtlin: As one control solution we used alanine (in place of cystine) plus glycine and glutamic acid. We have never tried glycine, glutamic acid or cystine separately.

Dr. Gudernatsch: I asked this question about mixtures of acids because, as I mention in my paper, we have used various two and three acid mixtures. Which of the three acids do you consider the important one in the molecule?

Dr. Voegtlin: Cystine or cysteine. Apparently glycine is inactive.

Dr. Gudernatsch: Have you tried cysteine?

Dr. Voegtlin: No, we have never worked with cysteine for the reason that cysteine is not satisfactory for working in the physiological pH range. Cystine is rather insoluble.

Dr. Gudernatsch: Cysteine is much more soluble than cystine, but if you use cystine, how much of it remains in the aqueous medium?

Are you satisfied that adequate proof of temperature effect has been submitted?

Dr. Voegtlin: I place very great importance on the temperature factor in studies on mitosis. However, I question whether the temperature coefficient of mitosis alone is sufficient proof for the assumption that mitosis is a chemical process.

Dr. Harris: Do you consider the size of the cell to be significant, merely as indicating the size of the nucleus?

Dr. Chalkley: Under given conditions for a certain cell size, there is a certain nuclear size. It tends to be only very slightly variable. In other words, I wouldn't say it is a fixed thing, but characteristically, without a doubt, you have associated with a volume of cytoplasm a certain volume of nuclei. That holds not only for the mono-nucleate, but for the poly-nucleate cells. If you have a cell with three nuclei in it, it will be approximately three times the volume of a cell with one nucleus.

Dr. Harris: Do you consider that cell division or nuclear division is dependent wholly upon the size of the nucleus?

Dr. Chalkley: In this sense, the size of the nucleus is merely an index of what Dr. Voegtlin referred to as a general chemical condition for the nucleus. I think you will find that under any normal growth conditions the nucleus must grow to a certain size before the cell divides. It is an important index of the physiological state of the nucleus. You might assume it to be an index of the quantity of chromatin, or more likely, an indicator of the osmotically active material within the membrane or an index of the amount of water in the nuclear protoplasm. We have some evidence that hydration of the protoplasm is one of the important factors influencing cell division. There is an intake of water into the cytoplasm. The nucleus swells remarkably during the prophase.

Dr. Harris: Do you consider the action of glutathione to be chiefly concerned with the intake of water in the nucleus?

Dr. Chalkley: I couldn't say, but it is certainly concerned with the transference of some material essential to the nucleus, from the cytoplasm to the nucleus. That is fairly reasonable, but is purely hypothetical. To prove this, it would be necessary to identify a given material in the cell without injuring the cell. It is more or less speculative. We haven't the equipment to do it. Until we have this we can't prove it.

Dr. Voegtlin: It is always difficult to explain mechanisms. We spent years trying to find out something about the biochemical mechanism underlying the arsenic action. We made progress but there are many points that have to be elucidated further.

Dr. Schram: Do you conclude, from your observations, that Dr. Hammett's postulates of the stimulating effect of the sulfhydryl group on mitosis is subject to revision?

Dr. Voegtlin: This is a question of definition of terms. Glutathione accelerates nuclear growth in *Amoeba* and, therefore, can be considered as a stimulus of nuclear growth.

We haven't any evidence so far to prove that glutathione in any way accelerates mitosis directly, apart from its growth accelerating action upon the nucleus.

Dr. Hammett has not worked with isolated cells. He has not differentiated between growth and division of cells, except in his experiments with plant roots. His generalization doesn't hold for *Amoeba.*

Dr. Riddle: I should like to ask whether you have made observations on the relative amount of glutathione in tumor cells as compared with normal cells.

Dr. Voegtlin: Yes, we have done what we could do. The methods for the quantitative estimation of glutathione are only reasonably accurate, because all the methods, even the latest modifications, are not specific enough for glutathione alone, because in all these methods, oxidizing agents are used. It happens that vitamin C and other reducing agents in tissues also react with these oxidizing agents. The history of blood sugar estimation shows what a tremendous amount of work had to be done to make the technique sufficiently specific. Malignant tumors have, roughly speaking, the same glutathione content as liver, or perhaps, liver has even more than the tumor, and most of that glutathione is in the reduced form. The benign tumors which we studied had an exceedingly small glutathione content, much smaller than that of normal tissues. I don't agree with Waldschmidt-Leitz. He proposed the idea that tumors grow so well in the body because of a shift in the equilibrium between the reduced and oxidized form of glutathione in malignant tissue. There is no shift in this ratio in the malignant tissues as compared with that of normal tissues.

Miss Hoffman: Wouldn't the amount of glutathione in reduced form, in contrast to the amount of the oxidized one, run parallel with the rate of oxidation in the cell?

Dr. Voegtlin: Yes. It seems to run parallel, as Hopking and Elliott showed with liver tissue. We have done similar experiments with red blood cells where we estimated the oxidized glutathione by means of a special method and found that there is a shift in the ratio of the two forms of glutathione according to the oxygenation of the blood.

Miss Hoffman: On the basis of the amount of oxidized glutathione and the limit of the error by which you could determine that glutathione, could you estimate the amount of proteolysis?

Dr. Voegtlin: That is what we did.

Dr. Abramson: Dr. Voegtlin has shown that the oxygen tension influences the quantity of amino acid nitrogen produced in a protein digest and that this action is reversible. He has, himself, pointed out his attempts to show the *resynthesis* of protein. It would be of further interest to know if:

(1) Experiments are available on crystalizable proteins with known molecular weights.

(2) There is any evidence beside the nitrogen and the cystine analyses, that a strictly reversible reaction was occurring.

Dr. Voegtlin: (1) So far, no experiments have been made with crystalline proteins of known molecular weight. We considered using crystalline ovalbumin in place of coagulated egg-white, as the former might be used for molecular weight determinations of the resynthesized protein by Svedberg's method. However, this method requires great experience.

(2) Protein was precipitated with trichloracetic acid under carefully standardized conditions. Amino acid nitrogen determinations showed a marked decrease during the oxygenation of the digests, at a time when protein synthesis was proceeding at the highest rate.

Dr. Gudernatsch: Do you happen to know if amoebae can live without all three amino acids?

Dr. Voegtlin: Not for a very long time.

Dr. Rahn: The amoeba has to feed on bacteria which probably contain a great variety of amino acids.

Dr. Chalkley: You can't tell just what is essential. You can't study the amino-acid nutrition of amoebae unless you can devise a synthetic medium.

Cold Spring Harbor Symposia on Quantitative Biology

AMOEBA PROTEUS AS MATERIAL FOR THE STUDY OF CELL DIVISION AND CELL GROWTH

H. W. Chalkley

Cold Spring Harb Symp Quant Biol 1934 2: 89-93
Access the most recent version at doi:10.1101/SQB.1934.002.01.013