

CELL DIVISION AND DNA SYNTHESIS
IN *TETRAHYMENA PYRIFORMIS*
DEPRIVED OF ESSENTIAL AMINO ACIDS

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ABSTRACT

The question of amino acid requirements for DNA synthesis and cell division has been studied in *Tetrahymena pyriformis* by depriving cells of histidine and tryptophan at defined stages in the interdivision interval. Deprivation any time before DNA synthesis does not prevent the initiation of such synthesis but completely inhibits the following division and limits the increase in DNA, as measured microspectrophotometrically, to 20 per cent. H³-thymidine added to the medium is not incorporated during the 20 per cent increase. Deprivation *after* DNA synthesis is initiated does not prevent the continuation (to completion) of DNA synthesis, and cell division ensues. H³-thymidine added to the medium under these conditions is incorporated into macronuclear DNA. The data indicate that some amino acid-dependent event occurs, about the time of the beginning of the DNA synthesis period, which is not essential for initiation of DNA synthesis but which is essential for the maintenance of synthesis once it has begun. These results are further discussed in terms of enzymes required to convert thymidine (and possibly the other three deoxyribonucleosides) to the immediate precursor of DNA synthesis.

INTRODUCTION

The initiation of DNA synthesis is currently the earliest recognizable, definitive step in preparations leading to cell division. The control of synthesis has sometimes been discussed in terms of nucleotide pools, conversion of DNA to a primer condition, and the induction of DNA polymerase activity, but the biological basis for control of the initiation of DNA synthesis remains to be determined. The possibility that the synthesis of some new proteins might be required in each cycle for the initiation and/or maintenance of DNA synthesis has been investigated in *Escherichia coli*, but the experiments have not given completely satisfactory answers. In two experiments (3, 8), protein synthesis was found necessary for

initiating DNA synthesis but not for the maintenance of synthesis already in progress; in another experiment (6), the inhibition of protein synthesis by deprivation of an essential amino acid stopped DNA synthesis already in progress. In the latter case the effect of amino acid deprivation on DNA synthesis could be reversed by chloramphenicol, and this puzzling situation cannot yet be explained.

We have taken up the question of amino acid requirements for DNA synthesis and cell division in *Tetrahymena*. This cell type is large enough to be handled individually, synthesis of DNA in single cells can be detected by autoradiography or microspectrophotometry, and the position of each individual cell within the cell life cycle can be estimated with reasonable certainty.

MATERIALS AND METHODS

Stock cultures of *Tetrahymena pyriformis*, strain HSM, were maintained at 29°C and subcultured daily. Three different media were used in the experiments and are defined as follows: (a) synthetic medium (2) enriched with 0.04 per cent w/v proteose peptone (stock cultures were maintained in this medium); (b) synthetic medium containing 0.05 per cent v/v Tween 80 (the addition of Tween 80 eliminated lysing of cells when transferred from enriched medium to synthetic medium); and (c) synthetic medium lacking histidine and tryptophan.

Groups of 20 to 25 cells in an early stage of fission were selected with a braking pipette and injected

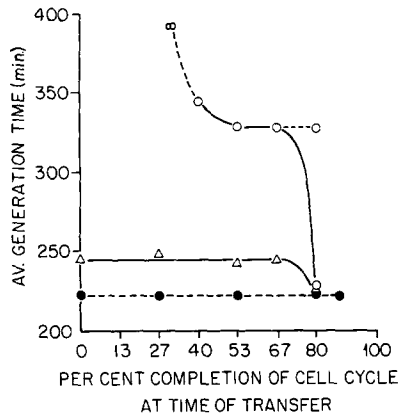


FIGURE 1 Generation time of cells transferred to different media at various stages of cell cycle. Open circles, cells transferred to medium lacking histidine and tryptophan; open triangles, cells transferred to complete synthetic medium; solid circles, cells transferred to enriched synthetic medium. All cells had been incubated in enriched synthetic medium up to time of transfer.

into capillary culture pipettes according to the methods described for handling single *Tetrahymena* (14). The use of capillary culture pipettes prevents contamination or evaporation of the medium during the experiment and allows individual cells to be observed with a dissecting microscope. The selection of a group of dividing cells took 3 to 5 minutes. For marking the age of each group of cells in the cell cycle, zero time was considered as the point at which one-half of the cells in the group had completed cytoplasmic fission.

Cells were deprived of amino acids by washing through a medium lacking histidine and tryptophan immediately before injection into capillary culture pipettes or by removal from a capillary pipette, washing in amino acid-deficient medium, and

re-injection into a capillary pipette. In either case, the dilution of histidine and tryptophan was 5,000- to 10,000-fold.

To detect protein and DNA syntheses, cells were incubated in medium containing 10 $\mu\text{C}/\text{ml}$ H^3 -leucine (New England Nuclear Corp., Boston, 5.0 c/mm) or 10 $\mu\text{C}/\text{ml}$ H^3 -thymidine (Schwarz Bio-Research, Inc., Mount Vernon, New York, specific activity, 6.0 c/mm), washed free of excess isotope, and air dried on subbed slides. Cells labeled with H^3 -leucine were fixed in 3:1 alcohol:acetic acid, extracted in hot 5 per cent trichloroacetic acid for 5 minutes, thoroughly rinsed in water, and air dried. Cells labeled with H^3 -thymidine were fixed for 30 minutes in three changes of 3:1 fixative, left in 70 per cent alcohol for several hours, and air dried. Autoradiography was done with NTB2 or NTB3 liquid emulsions (Kodak, Rochester) according to the procedures described by Prescott (10).

The total Feulgen-positive material was determined microspectrophotometrically with a Canalco microspectrophotometer using the two-wavelength method of Patau (9).

RESULTS

1. Effects of Amino Acid Deprivation on Cell Division

Fig. 1 describes the effects on the cell cycle of three types of medium transfer: from enriched synthetic medium, (a) to a fresh aliquot of the same medium, (b) to synthetic medium, and (c) to synthetic medium lacking the two amino acids, histidine and tryptophan. A transfer of type (a) did not cause a change in the generation time and demonstrates, therefore, that the manipulations involved in transfer are without detectable consequence. Transfer from enriched synthetic medium to type (b) resulted in a 10 per cent increase in generation time, although such a transfer during the last 20 per cent of the inter-division interval caused no increase. This type of medium transfer serves as the specific control against which the effects of amino acid deprivation are to be evaluated. Cells which had completed 0, 13, 27, 40, 53, 67, or 80 per cent of the inter-division interval were transferred from enriched synthetic medium to type (c) and observed for cell divisions over the subsequent 24 hours. Deprivation of the amino acids at 0, 13, or 27 per cent completion of the cycle resulted in complete inhibition of the next cell division. Deprivation at 40, 53, and 67 per cent of the cycle produced division delay, but there was no sig-

nificant difference in the amount of delay among the three groups (the generation time was increased about 75 minutes over the generation time for cells transferred from enriched synthetic medium to synthetic medium). Cells deprived of histidine and tryptophan at 80 per cent completion of the cycle fell into two groups: those that divided with 75-minute delay, *i.e.*, identical to the previous three groups, and those that divided without any delay. In all cases, the effects of transfer were evaluated against the generation time of cells subjected to the same type of medium shift but without amino acid deprivation.

the cell cycle under our experimental conditions. Therefore, groups of 30 to 40 cells were introduced into medium containing H^3 -thymidine at 15-minute intervals after division, incubated for 30 minutes, washed, air dried, and processed for autoradiography. This procedure permitted definition of the initiation and termination of DNA synthesis with an accuracy of ± 6 or 7 minutes. The results are given in Fig. 2. About 90 per cent of the cells begin incorporating H^3 -thymidine between 27 and 31 per cent completion of the cycle and continue to incorporate radioactivity until 60 to 67 per cent completion of the cycle.

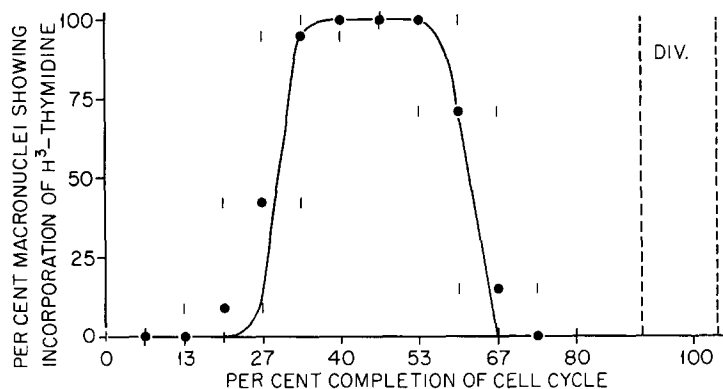


FIGURE 2 Per cent cells incorporating H^3 -thymidine into macronucleus after a 30-minute labeling time at various intervals in the cell cycle. The vertical marks represent the beginning and end of exposure to the isotope.

2. Effects of Amino Acid Deprivation on Protein Synthesis

To obtain an indication of the effects of deprivation on general protein synthesis, dividing cells were transferred to synthetic medium or to synthetic medium lacking the two amino acids. At various times after transfer, cells from each situation were incubated for 10 minutes in H^3 -leucine, washed, and dried on slides. Autoradiographs showed that, within the first 15 minutes of deprivation, H^3 -leucine incorporation had dropped by 86 per cent. With 45 minutes of deprivation, incorporation of H^3 -leucine was no longer detectable.

3. Effects of Amino Acid Deprivation on DNA Synthesis

Determination of this effect required knowledge of the position of the DNA synthesis period within

To determine the effect of amino acid deprivation on DNA synthesis, cells were transferred immediately after division and at 40 per cent completion of the cycle to medium lacking the two amino acids. Samples of cells deprived at 0 time were then incubated for 1 hour in H^3 -thymidine every hour for 24 hours. Immediately following exposure to H^3 -thymidine, the cells were air dried on slides, and prepared for autoradiography. Autoradiographs showed substantial incorporation of radioactivity into the cytoplasm (see Figs. 3, 4) during the first 2 to 3 hours of deprivation but no incorporation that could be clearly ascribed to the macronucleus. The cytoplasmic radioactivity was not extracted by acid or ribonuclease but could be removed by DNase treatment, a somewhat surprising finding for which no explanation is available. Beyond 3 hours of deprivation, no H^3 -thymidine was incorporated into either the nucleus or cytoplasm.

Samples of cells deprived at 40 per cent completion of the cell cycle were tested for incorporation of H^3 -thymidine (30-minute incubations at various intervals up until cell division). All samples incorporated radioactivity into macronuclear DNA. The extension of the DNA synthesis period up to cell division is not an effect of amino acid deprivation because it also occurs normally in complete synthetic medium, although

per cent completion of the interdivision interval (just before DNA synthesis) and incubated for 24 hours, and (d) cells deprived at 40 per cent completion of the interdivision interval (during the early part of DNA synthesis) and allowed to continue through the next division. The data are summarized in Fig. 5 with 95 per cent confidence limits of mean of the relative amounts of Feulgen-positive material in the macronucleus

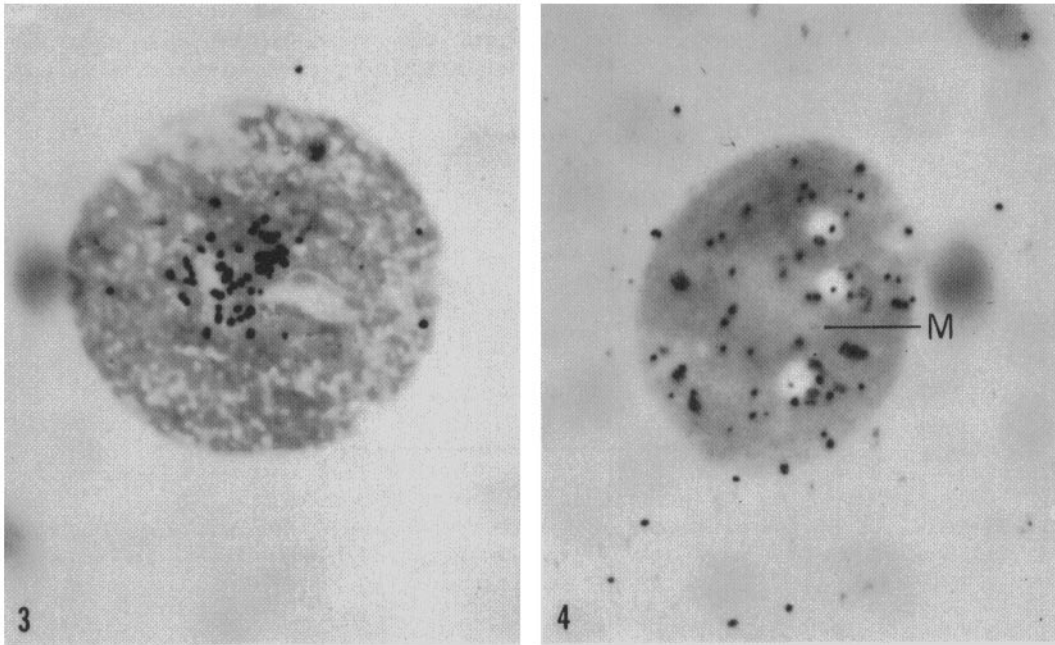


FIGURE 3 Cells incubated with H^3 -thymidine in enriched synthetic medium for 30 minutes. Note the radioactivity confined to the nucleus. Approx. $\times 1200$.

FIGURE 4 Cells incubated with H^3 -thymidine under deprived conditions. Note the radioactivity over the cytoplasm and lack of concentration over the nucleus (M). Approx. $\times 1000$.

the total DNA synthesis time in deprived cells is, in fact, 75 minutes longer since this is the amount by which division is delayed. In enriched synthetic medium, DNA synthesis is completed by 60 to 67 per cent completion of the cycle.

The relation of amino acid deprivation to DNA synthesis was also studied by spectrophotometric measurement of Feulgen-positive material in the macronuclei of (a) cells which had just completed division in enriched synthetic medium, (b) cells deprived of the two amino acids immediately after division and incubated for 24 hours, (c) cells deprived of amino acids after 22

plus micronucleus. Cells deprived at 0 time or at 22 per cent completion of the cycle and incubated for 24 hours are not significantly different from each other in DNA values but contain about 20 per cent more DNA than normal cells measured immediately after division. This means that, under conditions of amino acid deprivation, these cells were able to initiate DNA synthesis but were not able to carry synthesis beyond more than a 20 per cent increase. Cells deprived of the two amino acids at 40 per cent completion of the cycle are capable of division, and Feulgen measurements on the two daughter cells from such a division

show that DNA had doubled in amount, *i.e.*, DNA synthesis had proceeded to completion in the absence of two essential amino acids.

DISCUSSION

The inhibition of cell division by deprivation of histidine and tryptophan appears to be mediated through effects on DNA synthesis. In this respect,

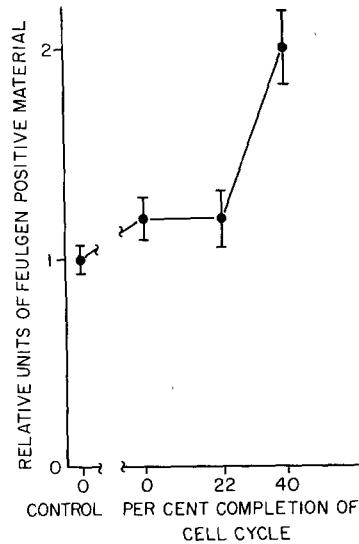


FIGURE 5 Relative units of Feulgen-positive material of: cells measured immediately after division (control); cells deprived at 0 time and incubated for 24 hours, and then measured; cells deprived at 22 per cent completion of the cell cycle (just prior to DNA synthesis) and incubated for 24 hours, and then measured; and cells deprived at 40 per cent completion of cell cycle (after DNA synthesis has been initiated) and allowed to continue through division. The values for the daughter cells were doubled, as were the 95 per cent confidence limits, in order to illustrate directly that cells deprived at 40 per cent completion did double their DNA.

the period around the beginning of the DNA synthesis is critical. Amino acid deprivation *after* this critical time does not prevent the continuation (to completion) of DNA synthesis, and cell division ensues. Amino acid deprivation *before* this critical time of the cycle does not inhibit the *initiation* of DNA synthesis, but it does prevent a doubling in DNA (limited to a 20 per cent increase), and it does prevent cell division. The data indicate that some event occurs about the time of the beginning of the DNA synthesis period which is not essential

for initiation of DNA synthesis but which is essential for the maintenance of synthesis once it has begun.

In the same context, the criticalness of the time at which DNA synthesis begins is also demonstrated by the change in the utilization of H^3 -thymidine of the medium for macronuclear DNA synthesis. Amino acid deprivation before DNA synthesis begins prevents the subsequent uptake of H^3 -thymidine into macronuclear DNA although macronuclear DNA increases about 20 per cent. Deprivation of amino acids after synthesis has begun has no inhibitory effect on the utilization of exogenous H^3 -thymidine of the medium for macronuclear DNA synthesis. The critical transition point as defined by these experiments takes place between 27 and 40 per cent completion of the cycle; DNA synthesis begins between 27 and 31 per cent completion of the cycle.

These data on amino acid deprivation in relation to the initiation and maintenance of DNA synthesis and the utilization of H^3 -thymidine of the medium are explicable in terms of enzymes required to convert thymidine (and possibly the other three deoxyribonucleosides) to the immediate precursor of DNA synthesis. In view of the work of Hotta and Stern (4), we are inclined to believe that the limiting enzyme is thymidine kinase (and possibly the other deoxyribonucleoside kinases). On this basis, the limited amount of DNA synthesis that can be initiated after amino acid deprivation would be independent of thymidine kinase and occur at the expense of pre-existing nucleotide pools. It should be noted that there is about a 20 per cent increase in DNA whether the cells are deprived at 0 time or at 22 per cent completion of the cycle. This is consistent with some additional findings that the pools of thymidine derivatives are present and unchanging through the full cell cycle but turn over only during DNA synthesis (G. E. Stone and O. L. Miller, Jr., unpublished results). The nucleotide pools presumably would not be refilled because of the absence of the appropriate kinases, and DNA synthesis (20 per cent increase) would come to a stop when the pools were exhausted. Separate studies (5; Stone and Miller, unpublished results) suggest that the thymidine monophosphate, diphosphate, and triphosphate pools are large enough to support this amount of DNA synthesis.

The explanation requires (a) that thymidine

kinase be absent from *Tetrahymena* at least between cell division and the beginning of DNA synthesis, (b) that new kinase be synthesized at the beginning of each DNA synthesis period, and (c) the kinase be destroyed when DNA synthesis is over. These assumptions are consistent with earlier findings that show H³-thymidine is not taken up into the nucleotide pools of *Tetrahymena* during non-DNA synthesis parts of the cycle (Stone and Miller, unpublished results), and that uptake normally begins close to the time of initiation of DNA synthesis. Thymidine kinase may, in fact, be formed shortly *after* DNA synthesis has begun. Induction of this enzyme may be related to the beginning of depletion of the thymidylic acid pool. In any case, amino acid deprivation at 27 per cent completion of the cycle is able to block H³-thymidine utilization although DNA synthesis normally begins at 27 to 31 per cent completion of the cycle. Once DNA synthesis has begun, amino acid deprivation becomes ineffective, presumably because there is sufficient kinase present to carry the cell through the full DNA synthesis.

The immediate effect of amino acid deprivation in these experiments is not surprising because of the absence of free histidine or tryptophan in *Tetrahymena* (7, 15, 16).

In the absence of histidine and tryptophan, the incorporation of H³-leucine by *Tetrahymena* declines to an undetectable level within 45 minutes of deprivation. We interpret this to mean that DNA synthesis can be *initiated* without protein synthesis, and conclude tentatively that the initiation of DNA synthesis is not dependent upon the formation of any new enzymes. This does not tell us what brings about DNA synthesis but suggests that we look to other events besides enzyme formation. The finding that inhibition of DNA synthesis by amino acid deprivation in *E. coli* can be reversed by chloramphenicol perhaps points in the same direction. Finally, our results differ from the two investigations on *E. coli* (3, 6, 8) to the extent that amino acid deprivation in *Tetrahymena* does not block the *initiation* of

DNA synthesis and, if imposed after initiation, does not prevent *continuance*.

Once the critical period around the time of the beginning of DNA synthesis has been passed, *Tetrahymena* are apparently capable of finishing preparations (60 per cent of the cycle) for the next cell division without net synthesis of protein. We have not tested for H³-leucine incorporation (indicating protein turnover) at later parts of the interdivision interval. We have noted a transition point late in the interdivision interval (80 per cent of the cycle) after which division delay is not inducible by amino acid deprivation or step-down in medium (enriched synthetic to complete synthetic). Rasmussen and Zeuthen (11) have noted a qualitatively similar transition point in *Tetrahymena* using various inhibitors of protein synthesis, and the reader is referred to their paper for a full discussion of cell cycle events during this period.

Finally, we have no explanation for the peculiar finding that H³-thymidine becomes incorporated into an acid-insoluble, DNase-sensitive material in the cytoplasm of *Tetrahymena* during the first 2 to 3 hours of amino acid deprivation. With incubations in H³-thymidine of 2 hours or less, radioactivity is not normally detected in the cytoplasm although the nucleus may be extremely radioactive. Other workers have noted similar incorporation of H³-thymidine into the cytoplasm of *Tetrahymena* under other conditions (1, 12), and Seaman (13) has reported the presence of DNA in the basal bodies of the cilia. We intend to examine this further.

Much of the above interpretation of our results is influenced by the findings of Hotta and Stern (4) who demonstrated that in plant microspores thymidine kinase is synthesized about the time that DNA synthesis begins and is apparently destroyed when DNA synthesis is finished.

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REFERENCES

1. CERRONI, R. E., and ZEUTHEN, E., Asynchrony of nuclear incorporation of tritiated thymidine into *Tetrahymena* cells synchronized for division, *Compt. rend. trav. Lab. Carlsberg*, 1962, **32**, 499.
2. ELLIOTT, A. M., BROWNELL, L. E., and GROSS, J. A., The use of *Tetrahymena* to evaluate the effects of gamma radiation on essential nutrients, *J. Protozool.*, 1954, **1**, 193.
3. HANAWALT, P. C., MAALØE, O., CUMMINGS,

- D. J., and SCHAECHTER, M., The normal DNA replication cycle. II, *J. Mol. Biol.*, 1961, **3**, 156.
4. HOTTA, Y., and STERN, H., Molecular facets of mitotic regulation. I. Synthesis of thymidine kinase, *Proc. Nat. Acad. Sc.*, 1963, **49**, 648.
 5. JACOBSON, B., and PRESCOTT, D. M., The nucleotide pools for cytidine and thymidine in *Tetrahymena pyriformis*, in preparation.
 6. KELLENBERGER, E., LARK, K. G., and BOLLE, A., Amino acid dependent control of DNA synthesis in bacteria and vegetative phage, *Proc. Nat. Acad. Sc.*, 1962, **48**, 1860.
 7. LOEFER, J. B., and SCHERBAUM, O. H., Amino acid composition of protozoa. Comparative studies on *Tetrahymena*, *J. Protozool.*, 1961, **8**, 184.
 8. MAALØE, O., and HANAWALT, P. C. Thymine deficiency and the normal DNA replication cycle. I, *J. Mol. Biol.*, 1961, **3**, 144.
 9. PATAU, K., Absorption microphotometry of irregular-shaped objects, *Chromosoma*, 1952, **5**, 341.
 10. PRESCOTT, D. M., Autoradiography with liquid emulsion, in *Methods in Cell Physiology*, (D. M. Prescott, editor), New York, Academic Press, Inc., in preparation.
 11. RASMUSSEN, L., and ZEUTHEN, E., Cell division and protein synthesis in *Tetrahymena*, as studied with *p*-Fluorophenylalanine, *Compt. rend. trav. Lab. Carlsberg*, 1962, **32**, 333.
 12. SCHERBAUM, O. H., Possible sites of metabolic control during the induction of synchronous cell division. *Ann. New York Acad. Sc.*, 1960, **90**, 565.
 13. SEAMAN, G. R., Large-scale isolation of kinetosomes from the ciliated protozoan *Tetrahymena pyriformis*, *Exp. Cell Research*, 1960, **21**, 292.
 14. STONE, G. E., and CAMERON, I. L., Methods for using *Tetrahymena* in studies of the normal cell cycle, in *Methods in Cell Physiology*, (D. M. Prescott, editor), New York, Academic Press, Inc., in preparation.
 15. WELLS, C., Identification of free and bound amino acids in three strains of *Tetrahymena pyriformis* using paper chromatography, *J. Protozool.*, 1960, **7**, 7.
 16. WU, C., and HOGG, J. F., Free and nonprotein amino acids of *Tetrahymena pyriformis*, *Arch. Biochem. and Biophys.*, 1956, **62**, 70.