SPECIFIC INHIBITION BY ETHIDIUM BROMIDE OF MITOCHONDRIAL DNA SYNTHESIS IN *PHYSARUM POLYCEPHALUM*

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INTRODUCTION

Eucaryotic cells contain two complete genetic systems, one based on nuclear DNA and the other on cytoplasmic DNA. The study of these systems has been greatly facilitated by the existence of specific inhibitors. One such inhibitor is the trypanocidal drug, ethidium bromide (Short, Peak, and Watkins, 1953). Application of the drug to trypanosomes in vitro causes alterations in the structure of their kinetoplast (mitochondrial) DNA (Riou and Delain, 1969). In HeLa cells, ethidium bromide (EtBr)[1] inhibits the synthesis of mitochondrial DNA (Leibowitz, 1970), of mitochondrial RNA (Zylber, Vesco, and Penman, 1969; Zylber and Penman, 1969), and of proteins synthesized on a ribosome-like structure associated with mitochondria (Perlman and Penman, 1970 *a* and 1970 *b*); it does not inhibit nuclear DNA synthesis, nuclear RNA synthesis, or cytoplasmic protein synthesis. Recent work on yeast (Goldring et al., 1970) shows specific inhibition of mDNA synthesis by EtBr. In this case, not only is mDNA synthesis inhibited, but preexisting mDNA is completely degraded over a period of about 14 hr.

We report here that EtBr produces a specific inhibition of mDNA synthesis in plasmodia of *Physarum polycephalum*, an acellular slime mold which is an obligate aerobe (Daniel, Kelley, and

[1] *Abbreviations used in this paper*: ATP, adenosine triphosphate; DNP, 2,4-dinitrophenol; EtBr, ethidium bromide; SSC, standard saline citrate; TCA, trichloroacetic acid; TTP, thymidine triphosphate.

Rusch, 1962). The mDNA breakdown seen in yeast is not observed in *Physarum*. Growth continues for three doublings in EtBr and this growth is inhibited by dinitrophenol or sodium azide. Thus, any possible disruption of mitochondrial structure by EtBr (Perlman and Penman, 1970 *b*) does not seem to extend to the respiratory chain.

Inhibition of mDNA synthesis can be measured readily in *Physarum* because mDNA is the main DNA species synthesized during the G_2 period (Evans, 1966; Guttes, Hanawalt, and Guttes, 1967; Holt and Gurney, 1969; Braun and Evans, 1969). Surface plasmodia of *Physarum* exhibit spontaneously synchronous nuclear division (Guttes, Guttes, and Rusch, 1961) and provide convenient amounts of growing material in G_2 phase. The ease of detection of mDNA synthesis in *Physarum* is further enhanced by the facts that mDNA represents a relatively large fraction (about 10%) of the total DNA and that the buoyant density of mDNA (1.686 g/cc) differs from that of nuclear DNA (1.700 g/cc). *Physarum* contains another satellite DNA (*circa* 1.706 g/cc) synthesized during G_2 but of nuclear origin (Braun et al., 1965; Holt and Gurney, 1969; Braun and Evans, 1969). As is shown below, synthesis of the nuclear satellite is unaffected by EtBr.

MATERIALS AND METHODS

Culture Methods and Labeling

All experiments were carried out with *Physarum polycephalum*, strain a + i, obtained from Dr. Jennifer Dee. The strain was prepared by fusion of two amebal clones and may be more homogeneous than strains which have not been cloned. Culture methods are as described by Holt and Gurney (1969), except that all cultures were grown at 26°C, the tryptone was replaced by N-Z Case (Sheffield Chemical Co., Norwich, N. Y.), and the final hematin concentration was 0.0005%. Thymidine-methyl-^{3}H (16 mCi/μmole) and thymidine-2-^{14}C (59 μCi/μmole) were obtained from New England Nuclear Corp., Boston, Mass. Labeling was carried out as described earlier (Holt and Gurney, 1969). In the experiments on the stability of mDNA, cultures were incubated in EtBr in the following manner. The labeled culture was cut into sections, leaving each part of the plasmodium on its underlying paper. Special Petri dishes, containing glass beads, medium, and filter paper (100 mm), had already been prepared by cutting holes in the filter paper. The plasmodia were placed over these holes, which were of the same shape as and slightly smaller than the pieces underlying the plasmodia, and allowed to grow for the duration of the experiment. This procedure ensures, first, that the expanding plasmodium has filter paper on which to grow, and second, that the center section of the plasmodium is separated from the medium by only a single layer of filter paper. Ethidium bromide was a gift of Boots Pure Drug Co., Ltd., Nottingham, England.

Analytical Procedures

Cultures were lysed, fractionated on CsCl, and counted as described previously (Holt and Gurney, 1969; Sonenshein and Holt, 1968), with the following exceptions. Each tube for CsCl analysis contained 4.0 ml 64.8% w/w CsCl in water, 0.05 ml 1 M Tris-HCl (pH 8), 1 ml standard saline citrate (SSC) (0.15 M NaCl, 0.015 M sodium citrate), 0.1–0.3 ml lysate in lysis medium, and ^{14}C-marker DNA. The refractive index (25°C; Abbé refractometer) was adjusted to 1.3981–1.3989 by the addition of CsCl or SSC. The tubes were filled with mineral oil (Nujol; Plough, Inc., New York) and centrifuged at 33,000 rpm in a 50 rotor in the Beckman Model L ultracentrifuge for 58 hr at 20°C. Three- or four-drop fractions were collected through the bottom of the tube onto filter paper discs (21 mm, No. 895E Schleicher and Schuell, Inc., Keene, N.H.). The discs were acid-washed and counted as described previously (Holt and Gurney, 1969).

Since sample size from a surface culture cannot be controlled precisely, sample size was determined by measurement of protein in lysates. Lysate aliquots were acid-washed in 5% trichloroacetic acid (TCA), suspended in 0.5 N NaOH, and assayed by the method of Lowry et al. (1951). For the measurement of growth (Figs. 4 and 5), samples of 1–5 ml volume were taken rapidly from a well-mixed culture and centrifuged at 1000 *g*. The supernatant was discarded and 5 ml of TCA-acetone (4% TCA, 48% H_2O, 48% acetone (w/v/v); Sachsenmaier and Rusch, 1964) was added. The precipitate was washed twice with 5% TCA, resuspended in water, and assayed by the biuret method (Gornall et al., 1949).

RESULTS

In order to determine whether ethidium bromide inhibits mitochondrial DNA synthesis in *Physarum*, surface cultures in G_2 phase were labeled with thymidine-^{3}H in the presence and absence of the drug. The cultures were lysed with sodium dodecyl sulfate and sodium deoxycholate and fractionated on CsCl equilibrium density gradients. The acid-insoluble radioactivity of the gradient fractions was measured. The profile of the control culture shows peaks due to mDNA and nuclear satellite DNA as well as a peak in the position of the prin-

cipal nuclear DNA (Fig. 1 *a*). The last peak probably reflects a small number of nuclei out of phase with the remainder (Guttes and Guttes, 1969). When the labeling was carried out in the presence of 1 μg/ml EtBr, incorporation of thymidine into mDNA was reduced by about 50% (Fig. 1 *b*). At 10 μg/ml, only a small peak of radioactivity was present at the mDNA position (Fig. 1 *c*). At 20 μg/ml, there was no peak evident, although a low level of radioactivity remained (Fig. 1 *d*). The observed inhibition did not result from failure to recover the labeled mDNA in the presence of EtBr. The crude lysates were fractionated on CsCl without prior purification, and 76–83% of the acid-insoluble radioactivity applied to the gradients was recovered in the fractions. Incorporation of thymidine into the nuclear satellite was unchanged by ethidium bromide at all concentrations studied (Fig. 1). Although EtBr seemed to reduce the synthesis of principal nuclear DNA in G_2 phase (Fig. 1), the effect was not reproducible.

The influence of EtBr on S-period nuclear DNA

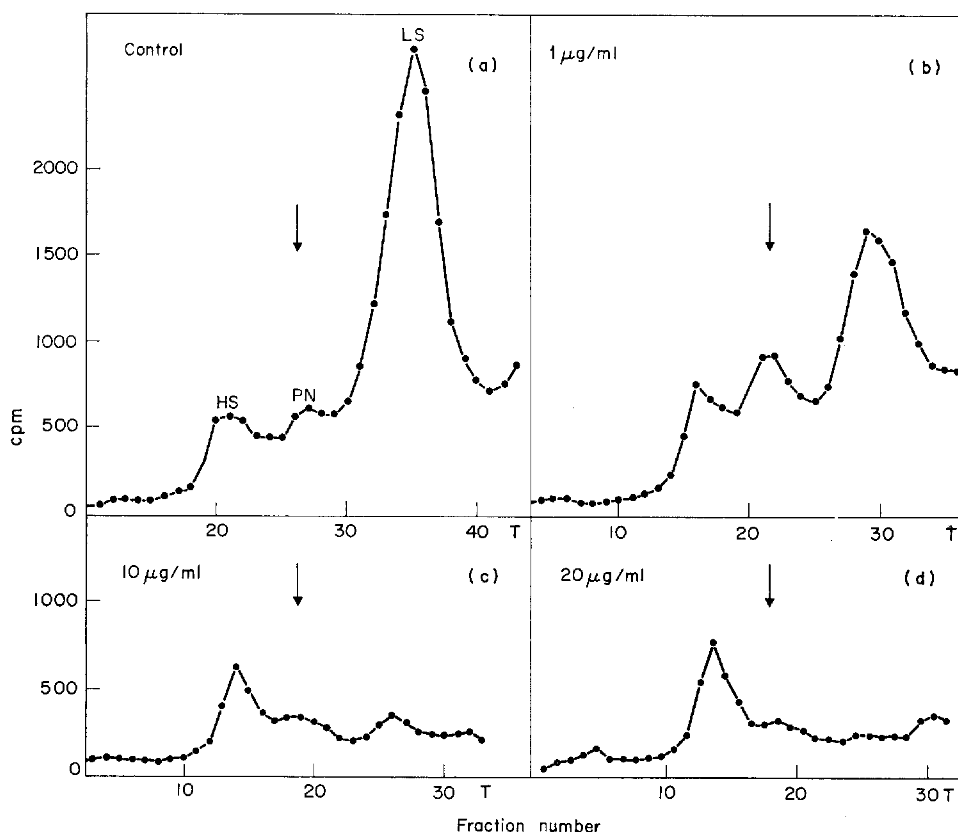

FIGURE 1 Effect of EtBr on replication of mitochondrial DNA and nuclear satellite DNA. A surface culture in G_2 phase (6 hr after the second postfusion mitosis) was cut into four parts; each part was incubated separately in growth medium containing either 0, 1, 10, or 20 μg/ml of EtBr. 1 hr later the cultures were placed on medium containing the above amounts of EtBr as well as thymidine-^{3}H (50 μCi/ml). All the cultures were harvested after 2 hr in thymidine-^{3}H and were analyzed by CsCl gradient centrifugation as described in Materials and Methods. The levels of EtBr are indicated in the figure. The arrows indicate the position of the peak of ^{14}C-principal nuclear DNA included in the gradients. Abbreviations: *HS*, high-density (nuclear) satellite DNA; *PN*, principal nuclear DNA; *LS*, light (cytoplasmic) satellite DNA; *T*, top fraction of gradient. Data plotted in *a* are measured radioactivity from a CsCl gradient of an amount of culture corresponding to 900 μg of protein. The data in *b*, *c*, and *d* are measured radioactivity divided by 1.076, 0.931, and 0.734, respectively, to normalize to 900 μg of protein (see Materials and Methods).

synthesis was studied in surface plasmodia and in shaker cultures of microplasmodia. In the latter case, nuclear division within a given microplasmodium is synchronous but the culture as a whole is asynchronous. In the experiment, the results of which are shown in Fig. 2, shaker cultures were labeled with thymidine-^{3}H in the absence of EtBr and in the presence of EtBr at 10 μg/ml. The cultures were lysed and fractionated on CsCl gradients. The CsCl profiles of the two cultures approximately coincide, showing that EtBr at this level has little or no effect on principal nuclear DNA synthesis. The EtBr curve lacks the mitochondrial DNA shoulder of the control, as expected. A normal rate of DNA synthesis was also observed in S-period surface cultures in the presence of 10 μg/ml EtBr. In other experiments with shaker cultures, EtBr at 100 μg/ml reduced the

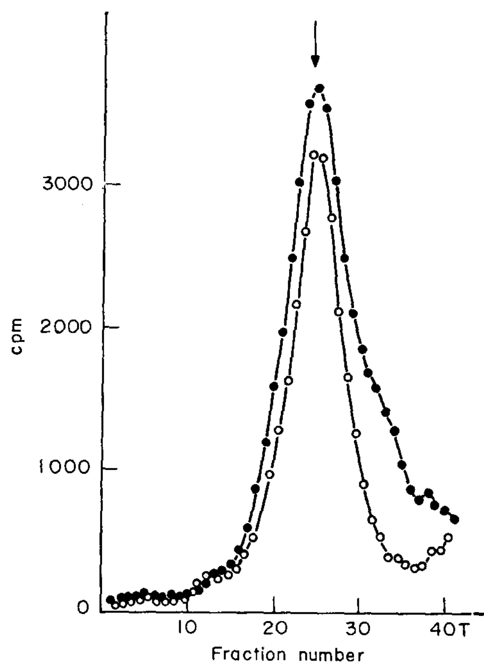

FIGURE 2 Effect of EtBr on the replication of nuclear DNA. A shaker culture was divided into two 1 ml parts. One was used as a control, and to the other was added 10 μg of EtBr. 1 hr later, 50 μCi of thymidine-^{3}H were added to each of the cultures. Growth continued for another 2 hr and 40 min, at which time the cultures were harvested and analyzed on CsCl gradients. The positions of the peaks of ^{14}C-marker DNA (arrow) coincided. The two curves correspond to equal amounts of culture. Closed circles, control; open circles, cells grown in 10 μg/ml EtBr.

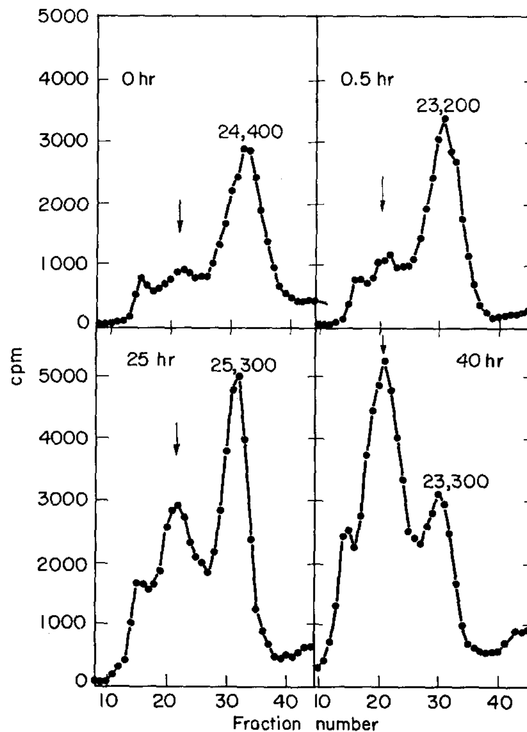

FIGURE 3 Effect of EtBr on prelabeled mitochondrial DNA. A surface culture in G_2 phase was placed in 1 ml of medium containing 200 μCi of thymidine-^{3}H. 3 hr later the culture was blotted on the underside, rinsed, blotted again, divided into sectors, and incubated in 10 μg/ml EtBr in growth medium for the times indicated. Arrows mark the peaks of ^{14}C-principal nuclear DNA. Total radioactivity in mDNA, given in each panel, equals two times the following sum: ½ the cpm in the peak mDNA fraction plus the cpm in the seven fractions to the light side of the peak fraction.

rate of incorporation of thymidine-^{3}H into an acid-insoluble form by about 50%. The reduction, apparent after 1 hr of incubation in EtBr, is probably due to inhibition of nuclear DNA synthesis.

It has been reported (Goldring et al., 1970) that exposure of yeast to 10 μg/ml EtBr for 14 hr causes the total degradation of mDNA. To see whether such degradation occurs in *Physarum*, a surface culture was exposed to thymidine-^{3}H for 3 hr during G_2 to specifically label mDNA. At this point, the culture was divided into sectors of approximately equal area. Each sector was lysed after incubation in 10 μg/ml EtBr for one of the times shown in Fig. 3. The labeled precursor was omitted during incubation in EtBr to reduce labeling of the principal nuclear DNA. Samples

of the lysates were analyzed on CsCl gradients. The data from each gradient were multiplied by the ratio of the total lysate volume to the volume of lysate put on the gradient, i.e., each curve shown (Fig. 3) represents total radioactivity in a sector of culture. The data were not corrected for protein content of the lysates, since protein increases in EtBr (see Fig. 4 for kinetics of increase in shaker culture). The total radioactivity in each mDNA peak was calculated from the data points on the right half of each mDNA peak (the left half includes radioactivity in principal nuclear DNA). The totals, which are given in the figure, varied by less than 10% over the 40 hr incubation in EtBr. Thus, the type of mDNA degradation seen in yeast does not occur in *Physarum*. Incorporation of radioactivity into principal nuclear DNA occurred during the 40 hr period (Fig. 3); the total radioactivity incorporated represents only about 0.1% of the radioactivity present in the culture medium during the labeling and thus could reflect incomplete washing at the start of the EtBr period. The ratio of radioactivity in mDNA to that in nuclear satellite DNA decreased during the 40 hr period, consistent with the selective effect of EtBr on the synthesis of the two species.

To study the specificity of action of EtBr further, the effect of the drug on the over-all rate of protein synthesis was measured. Protein synthesis involves, directly, a number of reactions, and, indirectly, energy metabolism, RNA synthesis, and amino acid transport and synthesis. Inhibition of any one of these reactions would stop protein synthesis. Nevertheless, protein continued to increase for over 40 hr in the presence of 4 and 10 μg/ml of EtBr (Fig. 4 *a*). Thus, this aspect of cellular metabolism is relatively unaffected during a period when the synthesis of mDNA is essentially stopped.

Growth of *Physarum* in shaker cultures is normally exponential from low densities to about 1 mg/ml of protein (equivalent to about 2 mg/ml dry weight and 0.025 ml of packed plasmodia per milliliter) (Fig. 4 *a*). In 10 μg/ml EtBr, growth continued for about three doublings and then

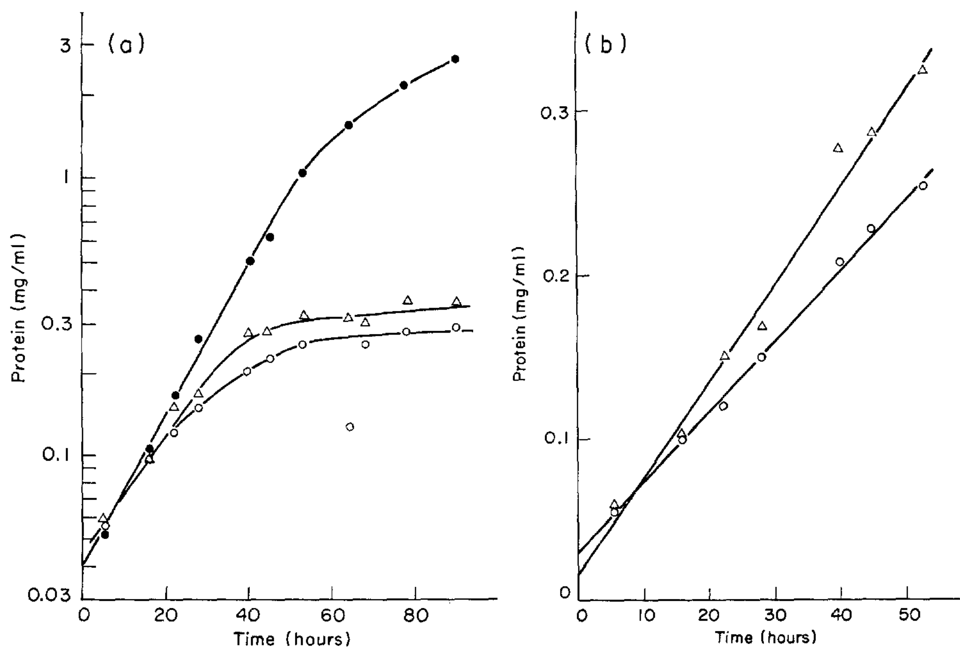

Figure 4 (*a*) Effect of EtBr on increase of protein. At $t = 0$, 50 ml of growth medium and 0.1 ml packed plasmodia were placed in each of three flasks. Samples were removed at times shown, centrifuged out of the growth medium, washed in TCA-acetone, and analyzed for protein by the biuret reaction. Values are expressed as milligram protein per milliliter of culture. EtBr at 4 or 10 μg/ml was present from $t = 0$ in two of the flasks. Part *b* is data from *a* plotted on a linear scale. Closed circles, no EtBr; open triangles, 4 μg/ml; open circles, 10 μg/ml.

stopped. In addition, growth in EtBr was not exponential. When the EtBr growth curves were plotted on a linear scale (Fig. 4 *b*), it became apparent that growth in ethidium bromide is in fact linear for about 50 hr. This suggests that there is a cellular component which (*a*) produces in limiting quantities something required for growth, (*b*) functions properly in EtBr, but (*c*) does not increase in amount in EtBr. To determine whether growth in EtBr depends on respiratory chain phosphorylation, two inhibitors of the process, 2,4-dinitrophenol (Loomis and Lipmann, 1948) and sodium azide, were used. Both completely inhibited growth in EtBr, and actually caused some reduction in the level of protein (Fig. 5).

Ethidium bromide also had a marked effect on the appearance of surface plasmodia. After 16 hr in 10 μg/ml EtBr, surface plasmodia had about half the diameter of control plasmodia grown without the drug. In addition, EtBr-treated plasmodia were beaded and thick in comparison to normal cultures, which retain a smooth veinous appearance until the growth medium is exhausted. The beaded appearance is typical of starving cultures and resembles a morphological stage in sporulation.

DISCUSSION

The data presented establish that EtBr at 10 μg/ml inhibits the synthesis of mDNA to a far greater extent than the synthesis of principal nuclear DNA, nuclear satellite DNA, or protein. The observed inhibition of mDNA synthesis is probably not due to a failure of thymidine-^{3}H to label cellular pools of thymidine triphosphate (TTP) because the nuclear DNA species incorporate thymidine-^{3}H at the usual rate in the presence of EtBr. The constant, low level of radioactivity seen in the mDNA region of CsCl gradients of EtBr-treated plasmodia (Fig. 1 *d*) might possibly reflect newly synthesized mDNA molecules of low molecular weight Goldring et al., 1970). On this assumption, the extent of inhibition of mDNA synthesis at 10 μg/ml is 90%. If the constant level of radioactivity has a source other than mDNA synthesis, then the extent of inhibition at 10 μg/ml is 95%.

Protein increased ninefold in EtBr (Fig. 4). This growth is clearly based on the production of new adenosine triphosphate (ATP), since plasmodia contain, at most, 7 μmoles of ATP/g dry weight (Chin and Bernstein, 1968), and about 3500 μmoles of amino acid in protein/g dry

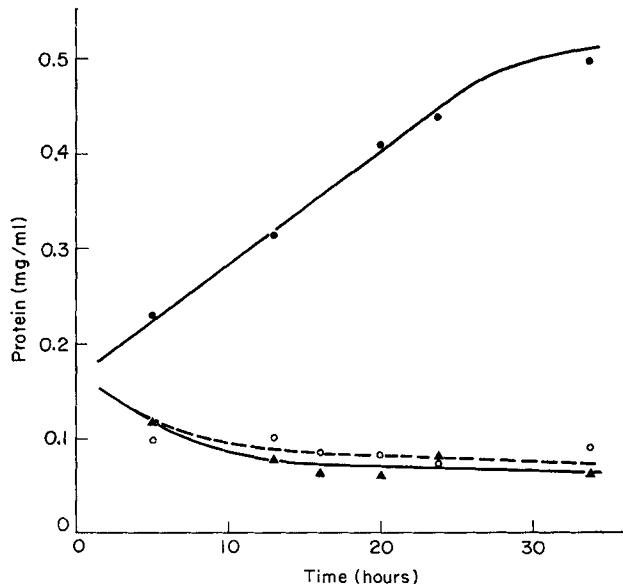

FIGURE 5 Effect of 2,4 dinitrophenol (DNP) and sodium azide on growth in EtBr. At $t = 0$, 50 ml of growth medium containing 10 μg/ml EtBr and 0.2 ml packed plasmodia were placed in each of three flasks. Other additions were as follows: closed circles, control; open circles, 10^{-4} M DNP at $t = 0$; closed triangles, 10^{-3} M sodium azide at $t = 0$. Growth was measured as in Fig. 4.

weight. Although several investigators have concluded that energy for protoplasmic streaming can be obtained anaerobically (for review, see Gray and Alexopoulos, 1968), energy for growth seems to come from oxidative phosphorylation. Plasmodia fail to grow in a nitrogen atmosphere (Daniel et al., 1962) or in a 95% N_2-5% CO_2 atmosphere (C. E. Holt, unpublished observation). In addition, growth is inhibited by cyanide or by azide (J. W. Daniel, personal communication). These results plus the direct demonstration of growth inhibition in EtBr-containing cultures by dinitrophenol or azide (Fig. 5) make it likely that the ATP for growth in EtBr comes from mitochondrial oxidative phosphorylation.

The nongrowing, functioning cellular component suggested by the linear rate of increase of protein (Fig. 4 *b*) could be the mitochondrion. Its product, ATP, would continue to be produced at a constant rate per mitochondrion throughout the linear growth period. The ultimate cessation of growth would require some additional assumption, such as imbalance between the rates of synthesis of protein and of some other cellular component.

The finding that mitochondrial protein synthesis in HeLa cells is inhibited more rapidly by EtBr than by other inhibitors of mitochondrial RNA synthesis led to the suggestion that EtBr acts by generally disrupting mitochondrial structure (Perlman and Penman, 1970 *b*). Our results make it likely that sufficient mitochondrial integrity is retained in EtBr to permit continued production of ATP through respiration. However, this may not apply to yeast and HeLa cells, even though both show growth in EtBr. Yeast is certainly capable of anaerobic growth and there are indications that limited anaerobic growth is possible in HeLa cells (Leibowitz, 1970).

The selectivity of EtBr action might arise from the structural differences between chromosomal and mitochondrial DNA's. EtBr binds differently to closed circular and linear DNA molecules, and changes the number of supercoils in the former (Radloff, Bauer, and Vinograd, 1967; Bauer and Vinograd, 1968). Chromosomal DNA is apparently linear, and at least in some systems mitochondrial DNA is circular. Nearly all of the mDNA of HeLa cells can be isolated as closed circles of 5 μ circumference (Leibowitz, 1970) and it now seems clear that yeast mDNA is in the form of 25 μ circles (Hollenberg, Borst, and Van Bruggen, 1970). *Physarum* mDNA was isolated in the form of linear molecules with an average molecular weight of 35 $\times$ 10^6 daltons, equivalent to about 18 μ (Sonenshein and Holt, 1968). However, the heterogeneity of the molecules and the variable number of single-strand breaks suggested that the isolated mDNA was the degradative product of a larger, possibly circular species. Finally, bacterial episomes, which are eliminated by EtBr (Bouanchaud, Scavizzi, and Chabbert, 1969), are probably coded by closed circular DNA. Thus, available data are consistent with the proposition that the synthesis of closed circular DNA is more sensitive to EtBr than the synthesis of linear DNA. However, the inhibition of mDNA synthesis in HeLa cells occurs at 0.1 μg/ml (Leibowitz, 1970), at which concentration one would expect EtBr to produce only a few turns in a 5 μ supercoil (Wang, 1969); whether this is a sufficient change in structure to produce an effect, and whether the external EtBr concentration is appropriate for estimating its effect inside cells, are unknown.

The finding that EtBr inhibits mitochondrial DNA polymerase more effectively than nuclear DNA polymerase (Meyers and Simpson, 1969) suggests that the specificity of inhibition may be related to the enzyme rather than the DNA. The selective inhibition was obtained with both single-stranded linear DNA as primer and native linear DNA as primer. The result must be interpreted cautiously, however, since it is not clear whether isolated DNA polymerase is a replicase or a repair enzyme (DeLucia and Cairns, 1969).

Finally, the possibility that EtBr selectivity arises from selective permeability of the nucleus and mitochondrion has not been tested. But, whatever the mechanism of action of EtBr, it is highly specific for mitochondrial macromolecular syntheses in all cells studied. It thus seems likely that the specificity arises from basic cellular features that have been preserved through evolution.

SUMMARY

At 10 μg/ml, ethidium bromide inhibited mDNA synthesis by over 90%, and had no measurable effect on the synthesis of principal nuclear DNA or nuclear satellite DNA. Prelabeled mitochondrial DNA was stable for at least 40 hr when plasmodia were incubated in EtBr. When EtBr was added to suspension cultures of microplasmodia, total protein continued to increase ninefold before leveling off. The increase occurred with time in a linear

rather than an exponential fashion and was prevented by the addition of 2,4-dinitrophenol or sodium azide. It is suggested that mitochondria of EtBr-treated plasmodia carry out oxidative phosphorylation but do not increase in number.

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