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THE EFFECTS OF 5-FLUOROURACIL AND SOME OTHER DRUGS ON GROWTH, BRANCHING, AND PERITHECIAL FORMATION IN *SORDARIA FIMICOLA*

by

Howard F. Schoen

A dissertation
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in partial fulfillment for the requirements
for the degree of
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May 16, 1975
Date

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ABSTRACT

The initiation of perithecia was completely suppressed, and branching frequency reduced, without seriously lowering the linear growth rate, by growing the homothallic, ascomycetous fungus, *Sordaria fimicola*, on an agar medium (sucrose-nitrate with biotin and thiamine) containing one of the following additions: 5-fluorouracil (FU), 6-azauracil, 6-azaadenine, potassium cyanide, or 50 g/l casein hydrolysate. FU was active at the lowest concentration (1×10^{-6} M) of those drugs tested. Several other inhibitors of energy metabolism and of protein, RNA, and DNA synthesis were without such selective activity on perithecial initiation, although some of them did inhibit branching frequency selectively.

In spite of its relative lack of effect on linear growth rate, FU did inhibit dry weight accumulation in liquid culture. This is attributed to its effect on the branching frequency. The ability of several pyrimidines and pyrimidine

nucleosides to prevent this growth inhibitory effect was investigated. Uracil, deoxyuridine, and 5-bromouracil, at 5×10^{-4} M, completely or almost completely prevented inhibition by 5×10^{-6} M FU. Thymine and 5-bromodeoxyuridine, also at 5×10^{-4} M, restored growth to from 50 to 90 per cent of the control.

The accumulation of protein, RNA, and DNA with time was studied in cultures growing with or without FU. The amounts of all three parameters were depressed by FU; RNA was depressed slightly more than the others at 1×10^{-7} M through 5×10^{-7}M FU, but at higher concentrations all three, along with dry weight, were affected to approximately the same extent.

The correlations, between the known effects on cell metabolism of the various inhibitors used and the observed effects on perithecial production in S. fimicola, suggest that the inhibition of ribosome synthesis is most likely one cause of the inhibition of perithecial formation. However, other cellular loci for perithecial inhibition probably exist also.

To my friends, and especially to Rosanne,
this work is lovingly dedicated.

H.F.S.

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I.

INTRODUCTION

The advantages of using fungi for investigations on fundamental developmental problems have been well summarized by Raper (1971). In addition to the ease with which fungi can be cultured and manipulated in the laboratory and their rapid rate of growth and differentiation in many cases, they are, together with the slime molds, also the simplest eukaryotic organisms that possess distinct processes of differentiation. Their simplicity is perhaps best illustrated by the recent determinations that the genome size of fungi is only about five to ten times that of bacteria (Storck, 1972).

A. Initiation and Development of Fruiting Bodies

In spite of a considerable amount of research on the initiation of fruiting body formation in fungi, the biochemical bases for this process remain essentially unknown. Much of the work on this problem has been done on the Sordariaceae, a family of non-stromatic Pyrenomycetes of which Neurospora is the best known genus. The fruiting body in this family is a perithecium, a small, flask-shaped structure with a pore at the tip for discharging the ascospores. Hawker (1939) showed that the presence of thiamine in the medium was required for fruiting, but not for growth, in Sordaria fimicola

and Podosporo curvula. She also demonstrated that high levels of readily utilizable carbohydrate inhibited fruiting in S. fimicola (Hawker, 1957). The stimulative effect of certain slowly utilizable carbohydrates, primarily sucrose, was hypothesized to be due to the production of small amounts of phosphorylated intermediates in the medium (Hawker, 1957). Westergaard and Mitchell (1947) investigated the effect of media composition on fruiting in Neurospora crassa and demonstrated, among other things, the importance of the carbon to nitrogen ratio and the nature of the nitrogen source (NH_4NO_3, NO_3^- or organic) on whether or not fruiting will occur. Bretzloff (1954) showed that pH affects the initiation of perithecia in S. fimicola. Studies of this nature have, however, so far failed to provide a clue as to the internal controls of fruiting.

Westergaard and Hirsch (1954) found some mutants of Neurospora crassa lacking tyrosinase activity and failing to initiate perithecia formation. The existence of these mutants, and other evidence (Hirsch, 1954) suggested a relationship between melanin synthesis and perithecial initiation. The nature of this relationship is uncertain but other evidence (Horowitz et al., 1960) indicates that tyrosinase activity can be induced in these mutants without inducing perithecial production. Additional mutations completely preventing perithecial initiation in S. fimicola (El-Ani,

1964, El-Ani and Olive, 1975), in N. crassa (Ho, 1972) and in other species (e.g. Esser and Straub, 1958) have also been described. The presence of these and similar mutations opens new possibilities towards investigating and biochemical bases of the fruiting process, but to date very little has been achieved in this area. Given the existence of a developmental mutant, one usually has no clues as to where to search for the specific biochemical lesion caused by the mutant gene (Srb, 1972).

We therefore decided to reinvestigate the problem of fruiting body initiation in the homothallic species Sordaria fimicola (Rob) Ces. et de Not. Except for some experiments on the effects of media constituents, the bulk of our investigations concerned the effects of inhibitors on the growth and fruiting of the fungus. Since there has frequently been a failure on the part of workers in this area to distinguish between fruiting body initiation and fruiting body development and maturation, we have attempted throughout our study to clarify precisely what aspect of fruiting body production we were observing. The perithecium of S. fimicola originates as a single hyphal branch. The cytology of the early stages in the development of the perithecium has been described (Carr and Olive, 1958).

B. Use of Antimetabolites as a Probe in Morphogenesis

Purine and Pyrimidine analogs have been found to intervene at crucial developmental stages in both plants and animals. One of the most striking of these effects was the suppression by some of the RNA base analogs of the normal sheet-like growth of fern gametophytes while permitting their continued filamentous growth (Hotta and Osawa, 1958; Hotta et al., 1959; Raghavan, 1965a). None of the thymine analogs had such an effect. Actinomycin D was effective also, and so were two amino acid analogs, 5-methyltryptophane and ethionine, but not any of the common respiratory and glycolytic inhibitors (Hotta and Osawa, 1958; Hotta et al., 1959; Hotta, 1960; Raghavan, 1965b). The leaf morphology of a water fern could be affected by application of 5-fluorouracil and 2-thiouracil, in that normal lobation was suppressed and the characteristic sunken stomata of the land form were favored (White, 1966). Similarly, 2-thiouracil affected leaf morphogenesis in flowering plants (Heslop-Harrison, 1962) and 5-fluorouracil has been shown to inhibit flower induction (Cherry and van Hystee, 1965).

A great deal of work has been done on the affects of the base analogs and of RNA synthesis inhibitors on differentiation in animal systems (Brachet, 1964; Brockman and

Anderson, 1963; Hitchings and Elion, 1963). In general, the development of amphibian, tunicate and echinoderm embryos seems to be specifically interrupted just before gastrulation by these compounds.

Among the fungi, light-induced sporulation of Trichoderma viride was prevented by 5-fluorouracil and 8-azaguanine, with only slight reduction in its growth rate. Conidiophore development was specifically inhibited by 2-thioguanine, and spore formation by 6-ethylpurine in Aspergillus niger (Behal and Eakin, 1959). Transformation from yeast-like to mycelial growth was induced by 5-fluorodeoxyuridine in Ophiostoma multiannulatum (Hofsten, 1964), and by cycloheximide in Mucor rouxii (Haidle and Storck, 1966). Cycloheximide induced orange, gametangium-like structures on the sporophyte of Allomyces in place of the normal sporangia (Whiffen, 1951).

Summarizing, there appears to be considerable evidence that development can often be altered, without seriously impairing growth in general, in a wide variety of organisms, with a variety of metabolic inhibitors.

Although in many cases the precise mode of action of these drugs is unknown, in some cases the opposite is true, and in other cases some correlations have been obtained between morphological effects and alterations in metabolism.

We have attempted to correlate the observed effects on growth and perithecial formation of various inhibitors

with the known or suspected biochemical actions of these inhibitors. Three general problems must be kept in mind, however, An inhibitor which, under short term incubations, has a single site of action, may have numerous secondary effects under the long term incubations that we used (Brock, 1966). There may also be numerous effects resulting from an alteration by the inhibitor in the organism's physiological or developmental state (Wright, 1973). Finally, most of the work on the mechanisms of action of many of these drugs has been done on organisms other than fungi, and it may be expected that at least some of the drugs will have different effects in fungi. An additional complicating factor may be that few of these drugs have only one primary effect. For example, 5-fluorouracil (or its metabolites) not only inhibits RNA metabolism, it also inhibits DNA biosynthesis and bacterial cell-wall synthesis (Heidelberger, 1965). Nevertheless, we feel that use of inhibitors is a valuable probe in the elucidation of biochemical factors involved in development. For one thing, use of low concentrations of drug can frequently limit the latter's action to fewer primary sites, and for another, the action of specific normal metabolites in preventing the inhibitory action of analogs can often help pinpoint the nature of the actual biochemical lesions.

C. Outline of the Study

The initial part of our work involved testing a variety of drugs for their effects on growth and fruiting (Schoen and Lindenmayer, 1966; Lindenmayer and Schoen, 1967). We were interested mainly in finding drugs which specifically prevented fruiting without inhibiting vegetative growth. The most selective drug in this sense was the uracil analog 5-fluorouracil (FU), at least when the index of vegetative growth was the linear growth rate. (The lack of effect on vegetative growth was not seen with FU when growth was measured as dry weight accumulation in liquid culture, however [unpublished data].)

Because of the insensitivity of linear growth rate to concentrations of FU which completely prevented fruiting, more detailed studies on the effects of FU were done. These included the kinetics of perithecial inhibition and the kinetics of inhibition of dry weight, protein, RNA, and DNA accumulation in liquid culture. In an attempt to determine the site of action of FU, the ability of various natural and unnatural pyrimidines and derivatives to prevent the inhibitory effects of FU was studied in some detail. The latter experiments also allow one to make tentative conclusions about the ability of *S. fimicola* to carry out various interconversions among the pyrimidines and their derivatives.

II.

MATERIALS AND METHODS

A. Organism

A wild-type strain of Sordaria fimicola (Roberge) Cesati et de Notaris was obtained from the American Type Culture Collection (culture 14517) and was maintained on slants of basal medium solidified with 2% agar. Transfers were allowed to grow and develop at room temperature (20-28°) until mature perithecia were present, about 6 days, and then were stored at 4-6°.

B. Inocula and Incubation Conditions

For growth on agar, blocks (ca. 5 mm on each side) of an agar stock culture were used as inoculum. (For perithecial counts, where a more uniform inoculum was desired, a young culture growing in a Petri dish was used instead of a stock culture.)

For growth in liquid culture, an inoculum was prepared by transferring scrapings from a stock slant to a 250 ml Erlenmeyer flask containing liquid basal medium, allowing this to grow for 5-6 days, and then blending this in a Sorvall Omnimixer or Virtis homogenizer; 0.4 ml of the resulting suspension was then added to each experimental flask.

In both cases cultures were incubated at 24° ± 2° in constant fluorescent white light (Sylvania Powertube, Cool White, FA8T12-CW-VHO).

C. Media

A sucrose-nitrate medium, with thiamine and biotin added, has been reported to promote perithecial production in S. fimicola (Bretzloff, 1954; Hawker, 1939). The basal medium used was modified slightly from Bretzloff (1954) and contained: 20.0 g sucrose, 1.0 g KNO_3, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.12 g $CaCl_2 \cdot 2H_2O$, 0.1 mg $FeCl_3$, 0.4 mg $MnSO_4 \cdot 4H_2O$, 0.4 mg $CuSO_4 \cdot 5H_2O$, 0.9 mg $Zn(NO_3)_2 \cdot 6H_2O$, 0.5 mg thiamine · HCl, and 12.5 µg biotin in 1.00 liter glass-distilled water. For experiments on agar medium, 2% agar (Difco or BBL) was added to the basal medium. The pH of the basal medium is 4.6 before autoclaving. It was adjusted to 6.0 with KOH before adding agar for the experiments in section III.B. The pH otherwise was not adjusted except for experiments in which additions to the medium altered the pH, in which cases it was readjusted to 4.6.

The medium was autoclaved at 121° for 15 minutes. Additions to the basal medium were incorporated into the medium before autoclaving, except for some experiments with amino acid analogs in which the analogs were dissolved and autoclaved separately and then added, and except also for

actinomycin D and trifluorothymine which were dissolved and filter-sterilized separately and then added to the cooled, autoclaved medium.

D. Growth Measurements

1. Experiments on Agar

a. Growth - The growth parameter measured for cultures growing on agar was that of the rate of growth in one dimension. This was measured in two ways. One was in growth tubes (30 cm long, 1.2 cm diameter) (Ryan et al., 1943) in which the growth rate was determined by measuring the advance of the growth front from one day to the next, over a period of several days. As has been observed repeatedly for fungi growing under such conditions (Ryan et al., 1943; Gillie, 1968b; Trinci, 1969), we found that after a short lag the advance per day is constant, i.e. growth is a linear function of time.

The second way the growth on agar was measured was in petri plates, where the growth of individual surface hyphae was followed for 2 hours. Measurement was facilitated by drawing the hyphae to scale at 15 or 20 minute intervals (rotating among several plates set up on microscopes at the same time). The measurements of individual hyphae were not performed in controlled temperatures, and were done probably at a few degrees higher than 24° in the illuminated petri

plates on microscope stages.

When the rates of growth of leading hyphae in plates were compared with the rates of the entire front in the growth tubes, the former values were usually found to be about 20% higher. This is not unexpected, since the leading hyphae are the fastest growing elements of the mycelium, and they may occasionally be growing at angles to the axis of the growth tube, which would result in a slower advance for the front than that for its components. In addition, illumination of the petri plates during observation of the individual hyphae may have increased the growth rate by warming the plates (this effect was minimized by turning off the light between observations). Occasions when the observed tube growth rate was greater than the hyphal growth rate are assumed to result from experimental error.

The results in Tables I through IV are from single experiments; most of the experiments were repeated, with similar results.

b. Branching - The advancing front of a S. fimicola colony on an agar plate consists of rapidly growing leading hyphae oriented radially at about 1 mm intervals, with the rest of the area filled in by more flowly growing branch hyphae (Lindenmayer and Schoen, 1967). Examples of the growth of such branch systems under our control conditions are shown in Figures 1 and 9, where successive 15 or 20

Figures 1-13. Time lapse drawings of cultures growing on agar and examined under a dissecting microscope. All figures were drawn to the same scale. The bar at the bottom indicates 500 μ. From Lindenmayer and Schoen (1967).

Fig. 1. Growth in a control culture, of the same experiment as that of Figure 2; 15 min intervals.

Fig. 2. Growth in the presence of 1.0×10^{-6} M FU; 15 min intervals.

Fig. 3. Growth in the presence of 2.0×10^{-4} M 6-azauracil. Average values for fast growing portion; hyphal growth rate, 7.6 μ/min; branch density, 4.4 branches/mm; branching frequency, 2.8 branches/100 min. Average values for slow growing portion: 1.4 μ/min; 32 branches/mm; 5.5 branches/100 min.

Fig. 4. Growth in the presence of 3.0×10^{-6} M 8-azaguanine; 20 min intervals.

Fig. 5. Growth in the presence of 5.0×10^{-5} M potassium cyanide; 20 min intervals. Different experiment from that reported in Table III. Average values: hyphal growth rate, 9.4 μ/min; branch density, 7.0 branches/mm; branching frequency, 6.1 branches/100 min.

Fig. 6. Growth in the presence of 5.0×10^{-5} M sodium azide; 20 min intervals. Different experiment from that reported

in Table III. Average values: hyphal growth rate, 6.0 μ/min; branch density, 5.5 branches/mm; branching frequency, 2.7 branches/100 min.

Fig. 7. Growth in the presence of 1.0×10^{-5} M 8-azaadenine; 20 min intervals.

Fig. 8. Growth in the presence of 1.0×10^{-4} thiourea; 20 min intervals.

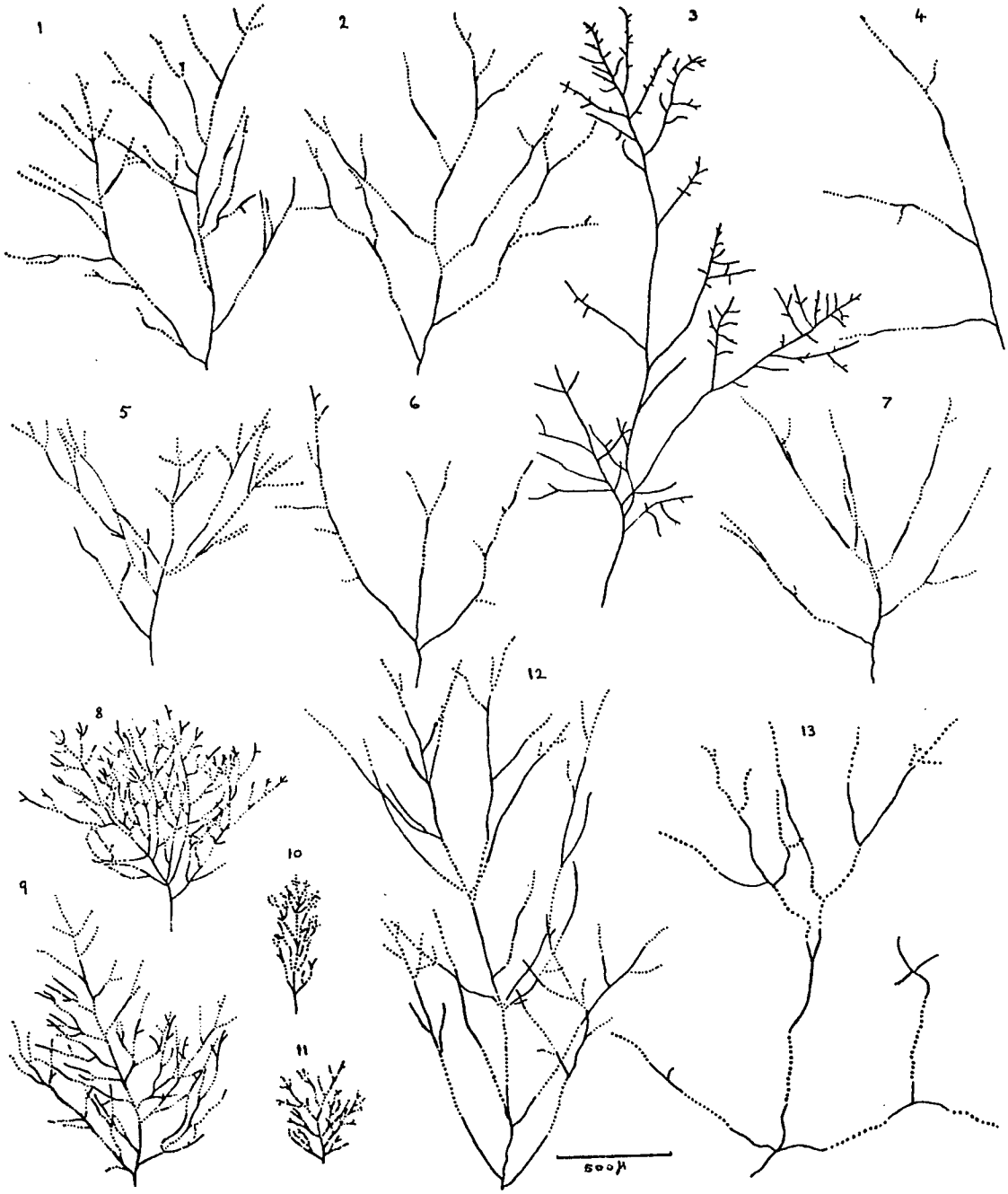
Fig. 9. Growth in a control culture of the same experiment as that of Figure 4; 20 min intervals.

Fig. 10. Growth in the presence of 5.0×10^{-3} M ammonium sulfate as sole source of nitrogen; 20 min intervals.

Fig. 11. Growth in the presence of 1.0×10^{-2} M glycine as sole source of nitrogen; 20 min intervals.

Fig. 12. Growth in the presence of 5.0×10^{-3} M asparagine as sole source of nitrogen; 20 min intervals.

Fig. 13. Growth in the presence of 50 g/l casein hydrolysate added to basal medium (including potassium nitrate); 20 min intervals.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500μ

minute increments of growth are indicated by alternating solid and dotted line segments. As a rule the branches themselves have no secondary branches within a region 200 μ from the leading hypha and extending back 1 to 2 mm from the tip of the leading hypha (e.g. Figure 9). All the hyphae lie on the surface at this point, subsurface hyphae being formed only several hours later. Leading hyphae are thicker than the branches, and besome septate sooner. Another distinction between leading hyphae and others is the rapid cytoplasmic streaming that can be seen proceeding always toward the tip only in leading hyphae. The branches may show no streaming, or the flow may be toward their base. The septa have central open pores at this stage (Buller, 1933, pp. 84-117), so they do not block cytoplasmic streaming.

The branch hyphae have a slower initial growth rate than that of the leading hyphae. Most of the branches eventually stop growing (usually before growing 1 mm) but some of them may accelerate their growth and become new leading hyphae. Since the relative rate of increase of the circumference of a colony is fairly small after the colony reaches a diameter of more than a few millimeters, the number of leading hyphae remains fairly constant over short time periods.

It should be noted that the type of branching we are referring to is subapical, or monopodial branching, where a branch occurs behind but fairly close to the apex, i.e.

usually within 100 μ of the hyphal tip. Dichotomous branching was seldom seen. Branching does occur on older portions of hyphae (Buller, 1933, pp. 4 ff.) but we did not study this type of branching.

A simple relationship holds between hyphal growth rate, branching frequency, and branch density (Lindenmayer and Schoen, 1967). If the growth of the hypha is constant, then

$$l = rt + l_o \qquad (1)$$

where l is the length of the hypha at time t, r is the linear growth rate, and l_o is the length of the hypha when t = 0. We define the branch density, d, as the number of branches, n, per unit length: d = n/l. Hence l = n/d and substitution in and rearrangement of equation (1) gives

$$n = rtd + l_o d \qquad (2)$$

We define the frequency of branching, f, as the differential $\underline{dn}/\underline{dt}$, hence, by differentiating equation (2),

$$\frac{dn}{dt} = f = rd \qquad (3)$$

assuming d is constant. [It may be argued that f may not properly be defined as a differential, but the relation f = rd can also be derived from equation (2) if f is defined as $\Delta n/\Delta t$.] For convenience in representing the data we have used μ/min as the units for r, number of branches per 100 minutes for f, and number of branches present on a hypha

per millimeter for d. Adjusting equation (3) for these units gives

$$f = \frac{rd}{10} \qquad (4)$$

or

$$d = \frac{f}{r} \times 10 \qquad (5)$$

If time-lapse drawings of branch systems are prepared as in Figure 1, all three of the above variables (f, r and d) can be determined independently, and they will then exhibit, within the limits of measurement, the relationship to each other that is expressed by equation (4). However, we feel that the data for r and f are more reliable than those for d, so in most cases in our tables the d values were obtained by taking the average of the directly observed branch density values and the ones calculated from equation (5); this has the effect of giving extra weight to the observations for r and f.

In early experiments, on the other hand, only d was determined, by simply counting the number of branches on apical 1 mm segments of leading hyphae. By using the growth rate determined from growth tubes, an approximate value for f can be calculated from equation (4); f values obtained this way are enclosed in parentheses in the tables. The associated d values, therefore, represent direct measurements, not derived figures.

2. Measurement of Growth in Liquid Culture

Growth Conditions - Cultures were grown in 250 ml Erlenmeyer flasks containing 40 ml of liquid basal medium. The flasks were inoculated and incubated as described above without shaking. Under these conditions the mycelium grows submerged for several days. It ultimately reaches the surface of the medium, at which point perithecia typically begin to develop at the surface of the mycelium.

Dry Weight Determination -

Method 1 - This is the simpler method and was used when no further determinations of mycelia constituents were to be made. The mycelium was collected on a tared filter paper circle in a Buchner funnel, washed twice with water, dried overnight in an oven at 90°, and weighed.

Method 2 - This method was performed when subsequent determinations of protein, RNA, and DNA were to be made on the mycelium. It is not really a simply dry weight that is measured, but actually the weight of the mycelium after removal of most of the acid soluble and lipid soluble components. Hence where this method was used the dry weight is referred to as the residual dry weight. The details of the procedure are given in the next section.

E. Protein, RNA, and DNA Determination

1. Extraction

The method used is a modified Schmidt-Thannhauser procedure (Schneider, 1946, 1957; Munro and Fleck, 1966). Mycelium grown in liquid culture was harvested by filtration on a Buchner funnel, washed twice with water, transferred to a tared conical centrifuge tube and extracted with cold 0.2 M perchloric acid (PCA). The extraction was speeded by homogenizing the mycelium by hand with a glass rod. The mycelium was next centrifuged in the cold and washed twice with cold 0.2 M PCA. For the lipid extraction the mycelium was next treated for one hour each with cold 95% ethanol, saturated (at 4°) with sodium acetate (approximately 2%); ether : 95% ethanol (3:1) at room temperature; and ether at room temperature; all with occasional stirring. The residue was finally washed twice with ether and dried in a stream of nitrogen. The residues were stored for up to 1 month in a desiccator at room temperature. The centrifuge tubes were weighed; the weight of the dried extracted residue is referred to as the residual dry weight.

RNA was extracted by hydrolyzing the residues with 4.0 ml 0.3 M NaOH for one hour at 37°. The tubes were next chilled on ice and protein and DNA precipitated by adding 2.5 ml 1.2 M cold PCA and allowing the tubes to stand on ice for 10-20 min. The precipitate was centrifuged down and

washed twice with 5.0 ml 0.2 PCA. The supernatant and washings were combined, 5.0 ml 0.2 M PCA added, and the whole made up to 50 ml with water (=RNA extract). Longer hydrolysis times did not give a significantly higher yield.

DNA was extracted by adding 2.5 ml of 5% trichloroacetic acid (TCA) to the residue from the preceding step and hydrolyzing for 30 min in a boiling water bath. In early experiments the mixture at this stage was simply chilled in ice, centrifuged and the residue washed twice with 5% TCA. A significant amount of protein is extracted along with the DNA by this procedure (Hutchison and Munro, 1961) and while this protein does not appear to interfere with the subsequent DNA determination, its removal from the residue means, of course, that the subsequent determination of protein will be too low. (Attempts to account for this lost protein by measuring protein present in the hydrolysate were unsuccessful. The biuret reaction was not sensitive enough to be quantitative and the Lowry et al. (1951) method for protein could not be used because acid hydrolysates of DNA contain a substance, presumably adenine, which gives a positive reaction with this method.) The problem was reduced, but by no means eliminated, by adding 10% TCA instead of 5% TCA to the mixture after hydrolysis, and letting the resulting mixture stand overnight in the refrigerator. This results in slightly higher amounts of protein in the residue used for

subsequent protein determinations. The residue was then washed twice with 5% TCA and the extract and washings combined and made up to 10 ml with 5% TCA (=DNA extract).

The residue from the last wash was finally washed twice with acetone, dried in a stream of nitrogen, and saved for protein determination.

2. RNA Determination

Initially we attempted to measure RNA in the RNA extract by reacting with orcinol in the orcinol reaction of Mejbaum (Schneider, 1957). The RNA extracts contain, however, a substance or substances other than RNA hydrolysis products which give a brown color or form a precipitate in this test. Using acid instead of alkali to hydrolyze and extract the RNA also extracted such additional substances. Attempts to remove the contaminants by precipitation with ethanol or by heating with 1.3 N acid in a boiling water bath followed by heating with 0.1 N alkali in a boiling water bath prior to the determination were unsuccessful. RNA was therefore determined by measuring the O.D. of the RNA extract at 260 nm (Munro and Fleck, 1966). Since a small amount of protein is extracted along with the RNA under these conditions, this was corrected for by subtracting 0.001 $O.D._{260}$ unit per ml for each µg protein per ml present (Munro and Fleck, 1966), as determined on neutralized extract by the method of Lowry

et al. (1951), using bovine gamma globulin as a standard. This correction never amounted to more than 10% and was usually less than 5%. Examination of the absorption spectrum of the extract indicated that protein was the only UV-absorbing contaminant. This was further checked in preliminary experiments by specifically absorbing the nucleotides and oligo-nucleotides of the extract on Dowex 1 (De Deken-Grenson and De Deken, 1959). The $O.D._{260}$ of the resulting solution was equal to 0.001 times the number of µg/ml of protein as previously determined, confirming that protein was the only contaminant and that the correction factor being applied is a reasonable one.

3. DNA Determination

Neither the diphenylamine reaction according to the method of Dische (Schneider, 1957) nor of Burton (1956) was sensitive enough for the determination of DNA in the DNA extract. The Burton method worked well when used directly on the mycelial residue without prior hot TCA extraction, but this made subsequent protein determination on the same replicate unsatisfactory. The p-nitrophenylhydrazine method of Webb and Levy (1955) was ultimately chosen and, with minor modifications, proved acceptable, although the color obtained with the DNA extract of mycelium was slightly different from that obtained with commercial, purified DNA.

The more complex p-nitrophenylhydrazine method of Martin and Donohue (1972) was also tried but there was little improvement, so the method of Webb and Levy was chosen as the best compromise of accuracy and simplicity.

4. Protein Determination

The acetone-washed and dried residue from the hot acid extraction was treated with 1.0 ml 1.0 M NaOH for 1 hour at room temperature. Protein was then determined on the suspension by the biuret method (Layne, 1957).

F. Perithecial Counts

In Tables I - IV the degree of fruiting is described semi-quantitatively by a series of signs, "+++" indicating normal fruiting as seen in control cultures, "++" and "+" indicating a reduction in the number of perithecia or a notable reduction in perithecial size, and "-" indicating the complete absence of perithecia and microscopic perithecial initials. In most of these experiments the culture was grown in growth tubes, but in some cases data from cultures growing in petri dishes was also used.

In the experiments of Section III.B.1 quantitative data on perithecial formation were obtained. For these experiments cultures were grown on 40 or 50 ml of medium solidified with agar in 10-cm petri dishes. The plates were

inoculated in the center, sealed with parafilm, and incubated until perithecia developed. The perithecia in 1-cm squares were counted while the plate was observed under a dissecting microscope at 10X or 24X. An attempt was made to distinguish mature from immature or arrested perithecia on the basis of their appearance. This was only partly successful because there appear to be intermediate forms and it is difficult to decide how to classify some of these. An attempt was also made to count submerged perithecia. This was exceedingly difficult because of the opacity of the agar and the necessity to focus up and down through the agar to see submerged perithecia at all depths. The attempt was finally abandoned except that in some experiments, submerged perithecia which were just below the surface of the agar and therefore visible without refocusing were counted.

The counts at the lower magnification had the disadvantage of not including the very immature perithecia which may be important in the interpretation of the results, but they had the advantage of ease of counting, and hence more squares could be counted per plate, giving results less influenced by the non-randomness of the distribution of perithecia in the plate (the perithecia frequently occur in loose clusters or in radially directed tree patterns).

III

RESULTS

A. The Effects of a Variety of Inhibitors and Media Compositions on Growth, Perithecial Production, and Branching on Agar Media

1. Inhibitors

Our findings (Schoen and Lindenmayer, 1966; Lindenmayer and Schoen, 1967) with the four purine and pyrimidine analogs which proved to be the best selective inhibitors of perithecial formation are given in Table I. For each of these compounds there is a minimum concentration at and above which perithecial initiation was completely inhibited. This concentration is indicated by the vertical arrows in Figures 14 and 15. Figure 14 indicates that these minimal concentrations, except for 8-azaguanine were considerably below those concentrations causing a sharp decrease in the growth rate.

In Figure 14 the curve describing linear growth rate for 6-azauracil (azauracil) is somewhat different than those for FU and 8-azaguanine (azaguanine), in that before the growth rate drops steeply there is a prolonged, slower decline with increasing concentration. This may be related to the alternating rapid and slow hyphal growth rates produced

TABLE I

Effect of RNA-Base Analogues on Growth, Branching, and Perithecial Initiation*

Inhibitor and Concentration (M)	growth rate: tube (μ/min)	growth rate: hyphal (μ/min)	branching: density (branches per mm)	branching: frequency (branches per 100 min)	perithecial density	comments
azauracil						
0	9.9	14.7	7.0	10.6	+++	
1.0×10^{-5}	9.1	11.0	5.3	5.8	++	submersed perithecia,
1.0×10^{-4}	8.1	8.2	2.5	1.9	+	delayed development
2.6×10^{-4}	7.7**	...	5.0**	(3.8)**	-	sparse growth
1.0×10^{-3}	7.2	5.9	10.5	4.0	-	very sparse growth
4.4×10^{-3}	6.8**	...	3.6**	(2.4)**	-	very sparse growth
fluorouracil						
0	11.0	15.4	7.0	11.3	+++	
1.0×10^{-7}	13.9	15.6	7.5	10.0	++	submersed perithecia, no delay
1.0×10^{-6}	13.3	15.7	5.3	8.0	-	
1.0×10^{-5}	15.7	13.4	3.0	4.0	-	sparse growth
1.0×10^{-4}	7.0**	...	3.4**	(2.4)**	-	very sparse growth
azaguanine						
0	9.1	10.0	14.0	14.0	+++	
3.0×10^{-6}	9.1	8.9	1.3	1.0	++	submersed perithecia
1.0×10^{-5}	10.2	8.7	1.1	<1	+	
3.0×10^{-5}	3.5	4.0	1.5	<1	-	sparse growth
6.7×10^{-5}	3.2**	...	...	...	-	very sparse growth
azaadenine						
0	6.3	12.6	6.2	8.0	+++	
3.0×10^{-6}	...	10.7	5.6	6.0	++	submersed perith., delayed devel.
1.0×10^{-5}	6.5	10.0	2.6	2.5	-	sparse growth
3.0×10^{-5}	5.4	11.2	1.5	1.9	-	very sparse growth

*From Lindenmayer and Schoen (1967).

**Data obtained in a different experiment, experimental values expressed in proportion to ratio of control values

Fig. 14. Tube growth rate (———) and branching frequency (- - -), as percent of control vs. concentration of inhibitor. Data from Table I. Vertical arrows indicate lowest concentration tested at which no perithecia were formed. Values for branching in parentheses were measured differently from the others (see Materials and Methods). ■, 6-azauracil; ●, 5-fluouracil; ▲, 8-azaguanine; o, 8-azaadenine.

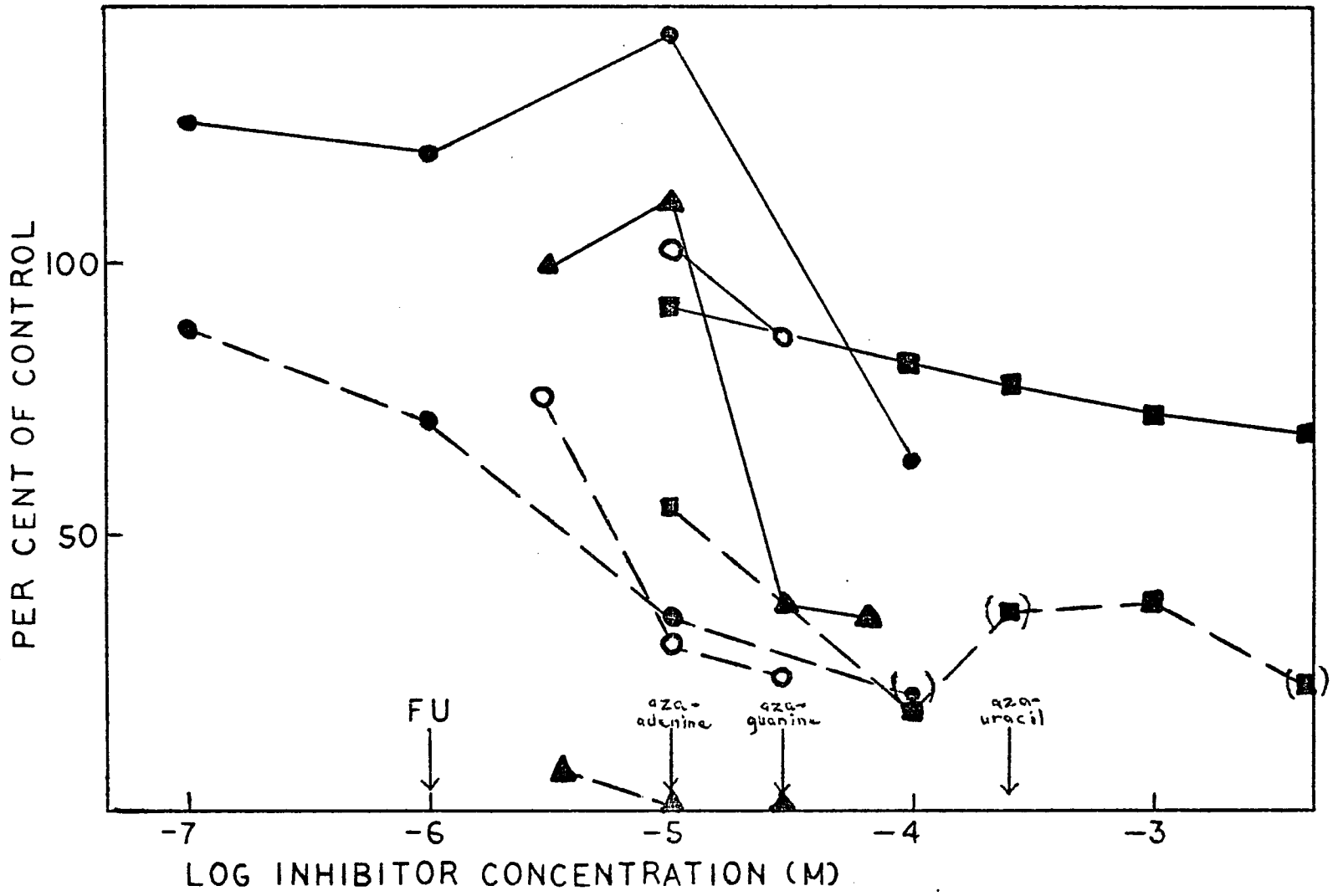
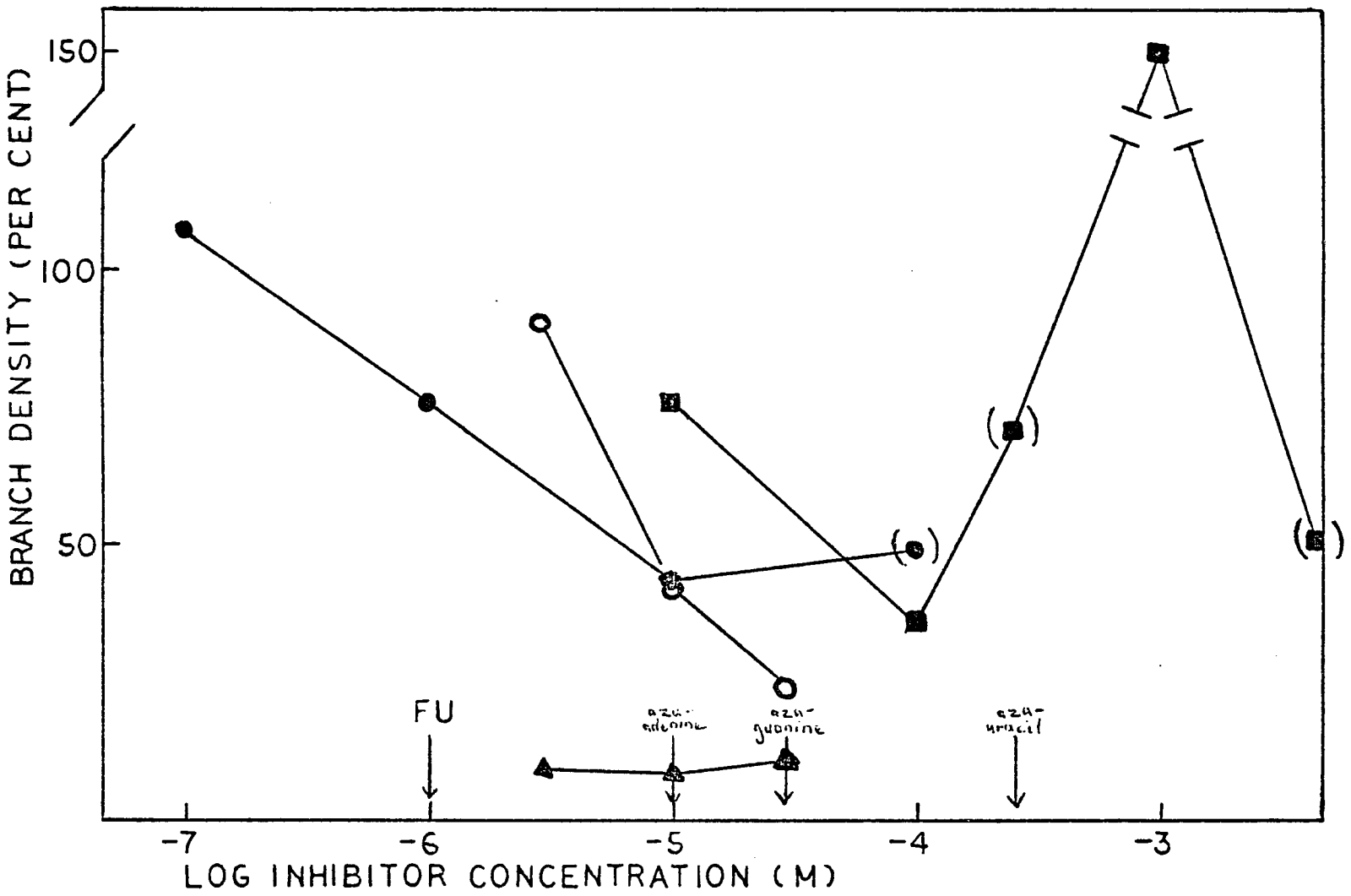

PER CENT OF CONTROL
100
50
FU
aza-adenine
aza-guanine
aza-uracil
-7
-6
-5
-4
-3
LOG INHIBITOR CONCENTRATION (M)

Fig. 15. Branch density vs. concentration of inhibitor. Data from Table I. Values in parentheses were measured differently from the others (see Materials and Methods). Vertical arrows and symbols for the different inhibitors as in Figure 14.


BRANCH DENSITY (PER CENT)
150
100
50
FU
azaadenine
azaguanine
azauracil
-7
-6
-5
-4
-3
LOG INHIBITOR CONCENTRATION (M)

by this compound (see below). The curve for azaguanine is quite similar to that for 5-fluorouracil (FU) but, significantly, the minimal dose for perithecial inhibition lies at the end of the growth rate drop, unlike the case with the other three analogs. The curve for 8-azaadenine (azaadenine) is presumably similar to that for azaguanine and FU, but there is insufficient data to establish this.

Branching frequency was inhibited at lower concentrations of all four analogs than was linear growth rate. For FU the minimal dose for total perithecial inhibition was lower than for 50% inhibition of branching frequency; for the other three analogs it was higher. The results for branch density (Figure 15) are similar, except that azauracil showed a pronounced increase in branch density above 1.0×10^{-4} M.

The minimal dose for total perithecial inhibition was lowest for FU (1.0×10^{-6} M). At this concentration, as indicated in Figure 14, the tube growth rate was higher than for the control, and the hyphal growth rate was about the same as for the control (Table I). The branch density and branching frequency values were about 70% of their control values. A time lapse drawing of a culture growing in 1.0×10^{-6} M FU is shown in Figure 2, which may be compared with that of a control culture in Figure 1.

The minimal concentration of azauracil at which complete perithecial inhibition was achieved, was 2.6×10^{-4} M.

A drawing of the hyphae grown at 2.0×10^{-4} M is shown in Figure 3. These cultures have a unique growth pattern, not seen with any of the other inhibitors in which hyphal growth was examined, in that the growth rates of individual hyphae seemed to change drastically from a high value near that of the control to a value 10 times less. The branching frequency was about the same in both the rapidly and slowly growing portions, resulting in alternately high and low branch densities (Figure 3). This unusual growth pattern bears some resemblance to the circadian "clock" mutants of N. crassa (Sussman et al., 1964) except that in our case the periods were of 2 to 3 hours duration and were not synchronous.

The last two inhibitors in Table I are azaguanine and azaadenine; these completely inhibited perithecial initiation at 3.0×10^{-5} M and 1.0×10^{-5} M respectively, at which points they also inhibited branching frequency and branch density considerably (Table I, Figures 4, 7, 14, and 15), especially azaguanine. Also, as noted above, azaguanine considerably inhibited growth at this concentration.

A common effect of all these inhibitors was the submersed production of perithecia at intermediate concentrations (Table I). Usually this took the form of well defined layers 2 to 3 mm below the agar surface. The necks of some of the perithecia reached the surface and discharged spores normally, but most of them did not come to the surface at all.

The development of asci and ascospores appeared normal, nevertheless. There was also a noticeable degeneration of the surface hyphae in these cultures after 1 or 2 days, in the course of which they became highly vacuolated or empty. The hyphae that grew under the surface did not appear to be affected in this way.

There was no appreciable delay in the initiation or formation of the perithecia in the presence of the lower concentrations of these compounds except as noted in Table I.

Summarizing, the two pyrimidine analogs were more selective in suppressing perithecial formation than the 2 purine analogs, and FU was particularly active in suppressing perithecial formation at very low concentrations with relatively minor effects on vegetative growth.

The next question is whether any of the analogs of thymine or thymidine also are active in this system. We tried four such analogs, 6-azathymine (azathymine), 5-bromouracil (bromouracil), trifluorothymine, and 5-bromodeoxyuridine (bromodeoxyuridine), and none had any activity. Concentrations up to 3×10^{-3} M of azathymine and bromouracil did not affect perithecial density or growth rate in agar culture, nor did a concentration of 3.3×10^{-5} M of the nucleoside analog (Lindenmayer and Schoen, 1967). 1.0×10^{-3} M trifluorothymine had no effect on growth or perithecial production in liquid culture.

There is a group of base analogs, the sulfo- and sulfhydro-derivatives, which also showed little or no activity. 6-Mercaptopurine seemed to stimulate perithecial formation and increase branching frequency at concentrations between 1 and 5 x 10^{-4} M. 2-Thiocytosine and 2-thiouracil were relatively ineffective at concentrations as high as 3.5 and 2 x 10^{-4} M, respectively. These inhibitors also caused the submersed formation of perithecia, as was the case with the analogs in Table I. It was also noted that in the presence of 2-thiouracil more than the usual number of dichotomous branches were formed (Lindenmayer and Schoen, 1967).

Another sulfo-derivative, 6-thioguanine, was interesting in that at intermediate concentrations (2 to 6 x 10^{-5} M) it inhibited perithecial initiation in the light but not in the dark. At high concentrations (1.8 x 10^{-3} M)perithecia formed in the light but in reduced numbers, branching frequency was unaffected, and the growth rate was stimulated by 80% (Lindenmayer and Schoen, 1967).

Another analog, N-6-benzyladenine, had little effect up to 1 x 10^{-4} M, but inhibited growth and perithecial formation at 1 x 10^{-3} M (Lindenmayer and Schoen, 1967).

We were further interested to find out whether inhibitors of nucleic acid synthesis other than the base analogs also affected perithecial development. Actinomycin D reduced the number of perithecia but at the same time reduced

the growth to 40% of the control at a concentration of 50 mg/l, so it could not be considered a selective inhibitor of perithecial formation. It also reduced branching frequency to 15% of the control. At 10 mg/l it had no effect on any of these parameters (Lindenmayer and Schoen, 1967). Nalidixic acid had no effect on linear growth rate or perithecial production at 10 mg/l. At 100 mg/l, linear growth was severely inhibited and there were no perithecia visible to the naked eye; vegetative growth was abnormal. Sarcomycin had no effect at concentrations up to 1 g/l. Hydroxyurea reduced the number of perithecia slightly but greatly reduced the growth rate at 1 to 3 x 10^{-4} M. Perithecial development was delayed and the perithecia were submerged when formed.

The inhibitors of de novo purine and thymidylic acid synthesis, amethopterin and sulfanilamide, were also tested. Amethopterin at 20 mg/l caused perithecia to be submersed. At 40 mg/l the linear growth rate was not reduced but growth was sparse; some perithecia were still produced at 500 mg/l. Sulfanilamide had no effect at 1.0 x 10^{-3} M. At 1.0 x 10^{-2} M linear growth was inhibited; perithecia were still produced but they were mostly small and submersed.

Azaserine, which inhibits de novo purine synthesis, gave complete growth inhibition at 1.0 mg/l but no perithecial inhibition at lower concentrations that permitted growth (Lindenmayer and Schoen, 1967).

We also tried some inhibitors of protein synthesis (Schoen and Lindenmayer, 1966; Lindenmayer and Schoen, 1967) (Table II). Puromycin did not inhibit perithecial formation or growth appreciably at up to 2×10^{-4} M (108 mg/l), but it did reduce the branching frequency to below 50%. Cycloheximide inhibited growth almost completely at 5×10^{-6} M (1.4 mg/l), and there were no perithecia in these cultures, but at lower concentrations which still greatly inhibited growth, there were many undeveloped and submerged perithecia in the culture. The branching frequency was also inhibited by cycloheximide, even at 5×10^{-8} M (0.014 mg/l).

A number of different amino acid analogs were tried, with the results for two of them shown in Table II. p-Fluorophenylalanine almost completely inhibited growth when added to the medium at 5.5×10^{-6} M, yet perithecia were still formed. Similarly, DL-ethionine suppressed both growth and perithecia formation at 1×10^{-4} M but at lower concentrations it did not inhibit the latter while still significantly inhibiting the growth rate and branching frequency. 5-Fluorotryptophane hardly affected fruiting at 2.25×10^{-6} M, while growth was reduced to 60% of the control. 4-Azaleucine did not diminish perithecial density even at 100 mg/l, where growth was inhibited by 50%.

D-Leucine had the interesting effect of allowing the development of perithecial initials, or what perhaps would

TABLE II

Effect of Protein Synthesis Inhibitors on Growth, Branching, and Perithecial Initiation*

Inhibitor and Concentration (M)	growth rate: tube (μ/min)	growth rate: hyphal (μ/min)	branching: density (branches per mm)	branching: frequency (branches per 100 min)	perithecial density	Comments
cycloheximide						
0	6.3	12.6	6.2	8.0	+++	
5.0×10^{-8}	4.5	6.9	8.3	5.0	+++	somewhat submersed perithecia
2.0×10^{-7}	2.8	5.7	8.5	3.5	+++	many submersed immature perithecia only
5.0×10^{-7}	1.9**	2.5**	10.9**	3.0**	++	only microscopic initials, dense growth
5.0×10^{-6}	...	...	...	...	-	very slow, dense growth
puromycin						
0	8.5	13.8	6.1	10.0	+++	
2.0×10^{-6}	6.5	11.8	6.5	5.9	+++	
2.0×10^{-5}	10.4	...	...	...	+++	some delay in spore discharge
2.0×10^{-4}	9.3	8.8	5.1	4.6	++	spores present but not discharged; no delay in perithecial formation
p-fluorophenylalanine						
0	11.5	12.0	7.9	9.6	+++	
1.0×10^{-8}	9.2	8.3	5.7	4.4	++	
1.0×10^{-7}	10.3	11.2	7.8	8.5	++	
1.0×10^{-6}	9.2	9.3	8.6	7.7	+	perithecia not submersed, well developed
5.5×10^{-6}	...	...	...	...	+	very slow, dense growth
DL-ethionine						
0	...	9.7	11.5	10.8	+++	
1.0×10^{-6}	9.8	10.7	5.6	5.9	+++	
1.0×10^{-5}	...	5.6	6.0	3.0	++	normal perithecial developm.
1.0×10^{-4}	...	...	...	...	-	very slow growth

*From Lindenmayer and Schoen (1967)

**Data obtained in a different experiment, experimental values expressed in proportion to ration of control values.

better be described as naked ascogonia, but, at 1.0×10^{-2} M, preventing the latter's further development. At this concentration linear growth was inhibited and surface hyphae were nearly absent. At 5.0×10^{-3} M growth was more normal and some of the ascogonia developed further, but almost none developed to the point where they were visible to the naked eye. At 2.0×10^{-3} M there were numerous visible perithecia but most were submersed. L-Leucine had no such effects.

Thus, none of these presumed inhibitors of protein synthesis showed any specificity towards inhibiting perithecial initiation.

We lastly tried several inhibitors of energy metabolism (Lindenmayer and Schoen, 1967) (Table III). Cyanide was selective, completely inhibiting perithecial production at 5.0×10^{-4} M, while only moderately reducing growth rate and branching frequency. 2,4-Dinitrophenol at 5.0×10^{-4} M and thiourea at 1.0×10^{-2} M also completely prevented perithecial production, but with more pronounced effects on growth and branching. (It should be noted that thiourea is not only a reported inhibitor, it is also utilizable as a sulfur source by many fungi [Foster, 1949, p. 532].) At 1.0×10^{-4} M thiourea, growth rate was affected much more than was branching frequency; the resulting high branch density can be seen in Figure 8. Azide was fairly effective as an inhibitor of fruiting, and inhibited growth moderately and branching

TABLE III

Effect of Metabolic Inhibitors on Growth, Branching, and Perithecial Initiation*

Inhibitor and Concentration (M)	growth rate		branching		perithecial density	Comments
	tube (μ/min)	hyphal (μ/min)	density (branches per mm)	frequency (branches per 100 min)		
sodium azide						
0	8.8	14.1	6.9	10.0	+++	
1.0×10^{-6}	7.0**	...	4.3**	(3.0)**	++	
5.0×10^{-6}	10.2	10.0	3.5	4.1	+	all perithecia submersed, some delay in development
5.0×10^{-5}	5.6	5.4	5.1	2.5	+	long delay in dev.; sparse, curly growth
potassium cyanide						
0	12.7	11.7	5.2	5.3	+++	
5.0×10^{-6}	10.5	...	5.6**	...	++	somewhat submersed perithecia,no dela
5.0×10^{-5}	8.8	9.2	4.8	4.3	+	same as above
5.0×10^{-4}	9.0	8.0	6.7	4.2	-	sparse growth
sodium fluoride						
0	10	...	8.0	(8)	+++	
1.0×10^{-6}	9	...	5.1	(5)	+++	
1.0×10^{-5}	9	...	6.0	(5)	+++	
1.0×10^{-4}	8	...	5.3	(4)	+++	abnormal growth
2,4-dinitrophenol						
0	8.8	14.1	6.9	10.0	+++	
5.0×10^{-5}	6.3	6.5	6.0	3.5	+	
5.0×10^{-4}	3.5	3.9	15.7	5.8	-	
thiourea						
0	...	9.7	11.5	10.8	+++	
1.0×10^{-4}	...	4.8	16.8	8.0	++	some delay in perithecial devl.
1.0×10^{-3}	...	3.6	17.5	5.7	+	abnorm.growth, long delay in perithecial development
1.0×10^{-2}	...	2.4	9.1	1.5	-	

*From Lindenmayer and Schoen (1967)

**Data obtained in a different experiment, experimental values expressed in proportion to ratio of control values.

frequency by 75% at 5.0×10^{-5} M. Fluoride did not inhibit fruiting at the concentrations tested, but did inhibit growth and branching somewhat. Figures 5 and 6 show the branching patterns in the presence of cyanide and azide, respectively.

In summary, of the inhibitors shown in Table III, only cyanide was a selective inhibitor of perithecial production. Along with azide, it also induced the submersed formation of perithecia, which is characteristic also of the selective base analogs.

2. Media

A series of experiments was performed using different kinds of nitrogen sources (Lindenmayer and Schoen, 1967). It is known that in fungi fruiting can be influenced by the nitrogen as well as the carbon source (Westergaard and Mitchell, 1947; Hawker, 1966). The sucrose-nitrate medium employed in our other experiments was selected precisely because it is favorable to perithecial development. It was of interest, however, to find out how other nitrogen sources would change the perithecial density, growth, and branching rates under our experimental conditions. The results are given in Table IV. The nitrogen compounds were added in amounts to give 1×10^{-2} M nitrogen in each case, except for casein hydrolysate, which was used either at 5 or 50 g/l,

TABLE IV

Effect of Different Nitrogen Sources on Growth, Branching, and Perithecial Initiation*

Nitrogen Source**	growth rate: tube (μ/min)	growth rate: hyphal (μ/min)	branching: density (branches per mm)	branching: frequency (branches per 100 min)	perithecial density	comments
1.0 g/l KNO_3 (basal medium)	...	9.4	14.3	13.7	+++	
5.0 g/l casein hydrolysate	22.1	...	....	...	++	few mature perithecia, some delay; mycelium pinkish
5.0 g/l casein hydrolysate +1.0 g/l KNO_3	21.7	...	...	...	++	no mature perithecia, some delay; mycelium pinkish
50 g/l casein hydrolysate +1.0 g/l KNO_3	...	19.9	3.0	5.0	-	heavy pink crust, segmented thick hyphae
0.66 g/l asparagine	...	21.4	6.7	12.5	++	immature perithecia appear after long delay and do not dev.further
0.75 g/l glycine	...	3.9	21	7.8	+	microscopic immature perithecia only, fluffy growth
0.30 g/l urea	17.1	17.3	5.7	9.4	++	few mature perithecia; mycelium pinkish
0.66 g/l $(NH_4)_2SO_4$	4.1	4.3	25	12.0	++	growth somewhat fluffy

*From Lindenmayer and Schoen (1967)

**Equivalent to 10^{-2} M nitrogen in each case, except for casein hydrolysate

which corresponds roughly to 5×10^{-2} M or 5×10^{-1} M nitrogen, respectively. The linear growth rates were approximately doubled at both concentrations of casein hydrolysate and also by asparagine and urea, as compared to the nitrate medium. On the other hand, glycine and ammonium sulfate reduced the growth rate to about 40% of that in nitrate medium. The branching patterns for some of these media are shown in Figures 10 through 13. Among the various media, only casein hydrolysate had a marked effect on the branching frequency, 50 g/l (with 1.0 g/l KNO_3) reducing the frequency to 36% of the control. 50 g/l casein hydrolysate was also the only medium in which no perithecia were formed. Glycine, as the sole nitrogen source, also inhibited perithecial initiation to a considerable extent, and lowered the branching frequency to 57% of that in nitrate. Forty g/l KNO_3 (not shown) partially inhibited perithecial development. A fair number of submerged, immature perithecia were present, and a few regions had very immature surface perithecia.

B. More Detailed Studies on the Effects of 5-Fluorouracil

1. Effects on Perithecial Density

Because FU inhibited perithecial production at the lowest concentration and appeared to be the most selective of all the inhibitors tested, its effects were investigated

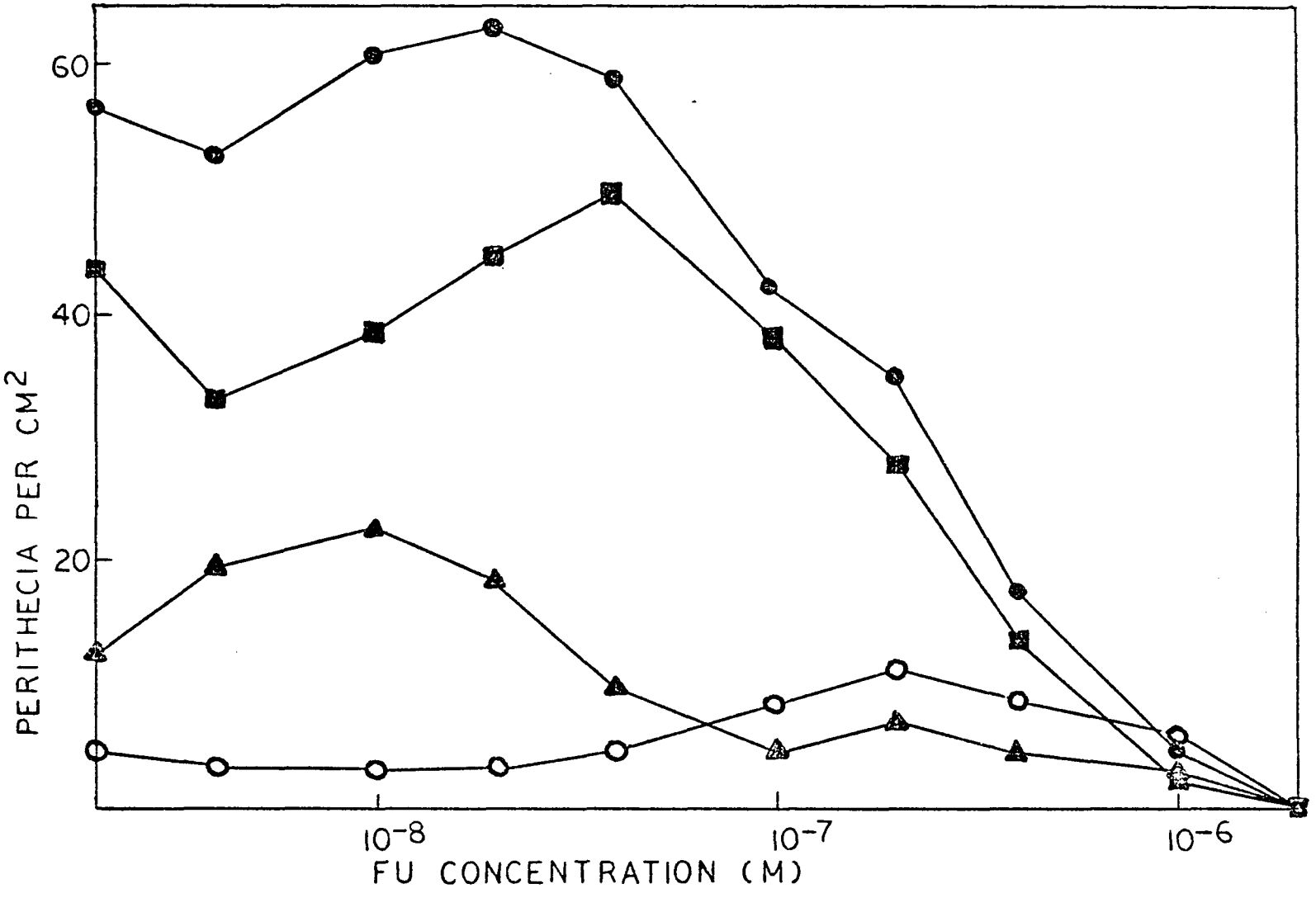
more fully. Counts of perithecial density at different FU concentrations typically yielded data like those shown in Figure 16, although the actual values differed in different experiments. Surprisingly, low doses of FU stimulated perithecial production. This effect was seen at the lowest dose tested (2×10^{-9} M), and rose to a peak between 10^{-8} and 10^{-7} M. From 10^{-7} M to 10^{-6} M there was a rapid reduction in perithecial density.

It is interesting that the peak stimulation for mature perithecia occurs at a different dose than that for immature perithecia. It is important to note, however, that not only is the density of the individual classes of perithecia stimulated, but the density of total surface perithecia is also stimulated; thus the increase in the density of one class is not occurring at the expense of another. Formation of subsurface perithecia is stimulated also, but in this case the stimulation does not peak until 2×10^{-7} M FU, at which point the density of surface perithecia is much below its respective peak.

The inhibition of immature perithecia is almost complete at 1.0×10^{-7} M, but the number does not decrease further with increasing concentration until 1.0×10^{-6} M FU, above which concentration it falls to zero.

If FU acts by binding to some receptor site, then the dose response data are expected to follow a certain pattern

Fig. 16. Perithecial counts per square cm vs. FU concentration. The points represent the average of four 1-cm^2 fields per plate, examined at 24X. One plate was counted for each concentration except 2 x 10^{-9} M, for which 2 plates were counted. The values for the control (four 1-cm^2 fields per plate; 2 plates) were: surface total, 41.3; surface mature, 26.8; surface immature, 14.5; subsurface, 2.3. (For what is meant by these terms, see Materials and Methods.)
●, surface total; ■, surface mature; ▲, surface immature, o, subsurface.


60
40
20
PERITHECIA PER CM2
10-8
10-7
10-6
FU CONCENTRATION (M)

when plotted as response vs. log dose (Goldstein et al., 1968). The stimulation of perithecial production at low FU levels indicates, however, that the relationship is complex and will not yield to any simple analysis. The slopes of the linear portions of the curves for surface total and surface mature perithecia in Figure 16 were calculated for between the values at 1×10^{-7} and 4×10^{-7} M FU. In separate experiments, however, entirely different values for these slopes were obtained; thus little significance can be attached to these values.

Further insight into drug action can sometimes be achieved by a double reciprocal plot. The data from one experiment plotted by this procedure are shown in Figure 17. From this figure and from other sets of data plotted this way it is apparent that a linear relationship is obtained above that concentration of FU giving 50% inhibition (approximately 10^{-7} M for the data in Figure 17). The distinct tailing at high levels of FU again indicates that the relationship beween FU level and perithecial inhibition is complex. (No particular significance should be attached to the slope or intercepts of this graph, as quite different values were obtained for these parameters in different experiments.)

2. Effects on Growth in Liquid Culture

a. The normal pattern and its correlation with

Developmental Events. The pattern of growth in standing liquid culture of S. fimicola is shown in Figure 18. This figure is a composite of data from several experiments. The results for one experiment which differed from the other results is shown separately. The growth in this case differed from that in the other experiments apparently by having a delayed start of about 2 days. Values for cultures younger than 2 days were not obtained because of the difficulty in handling such small amounts of growth. Between days 2 and 3.5 to 4, growth is rapid and approximately exponential, with a specific growth rate of approximately 1.2 day^{-1} and a doubling time of approximately 0.58 day. After this phase, growth rate suddenly slows down. Subsequent growth has a complex form. Typically there is a small spurt of growth between days 7 and 10. This is seen better in the graphs of the individual experiments (Figure 19), in which the residual dry weights are plotted on linear scales. Maximum growth is reached between 8 and 14 days, depending on the experiment. After this the residual dry weight remains fairly constant, rising slowly in some experiments and falling slowly in others (see also Figure 19).

Growth is initially submerged, remaining so until after the period of rapid growth has ended, although sometimes there are small pieces of mycelium floating while the bulk of the culture is at the bottom of the flask. At 6 to 7 days of

Fig. 17. Double reciprocal plot of perithecial count data. Data shown are for surface total only, and are from a different experiment than the one from which the data of Figure 16 were taken. The points represent the average of four 1-cm^2 fields per plate, examined at 24X. The denominator in the ordinate is expressed as 100 minus perithecial density (as percent of control) so that the slope of the resulting curve is positive, and the ordinate for 100% inhibition is 1.

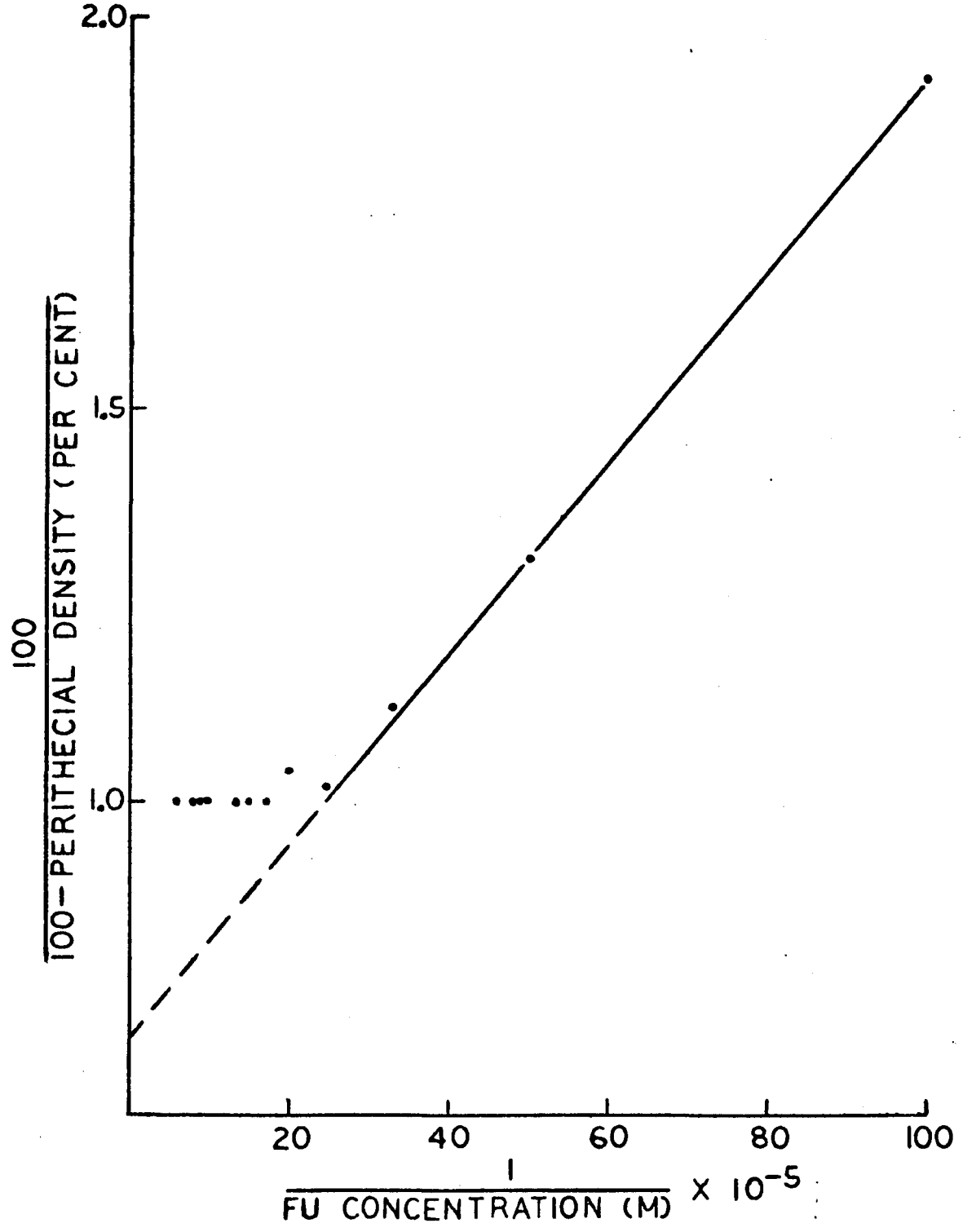
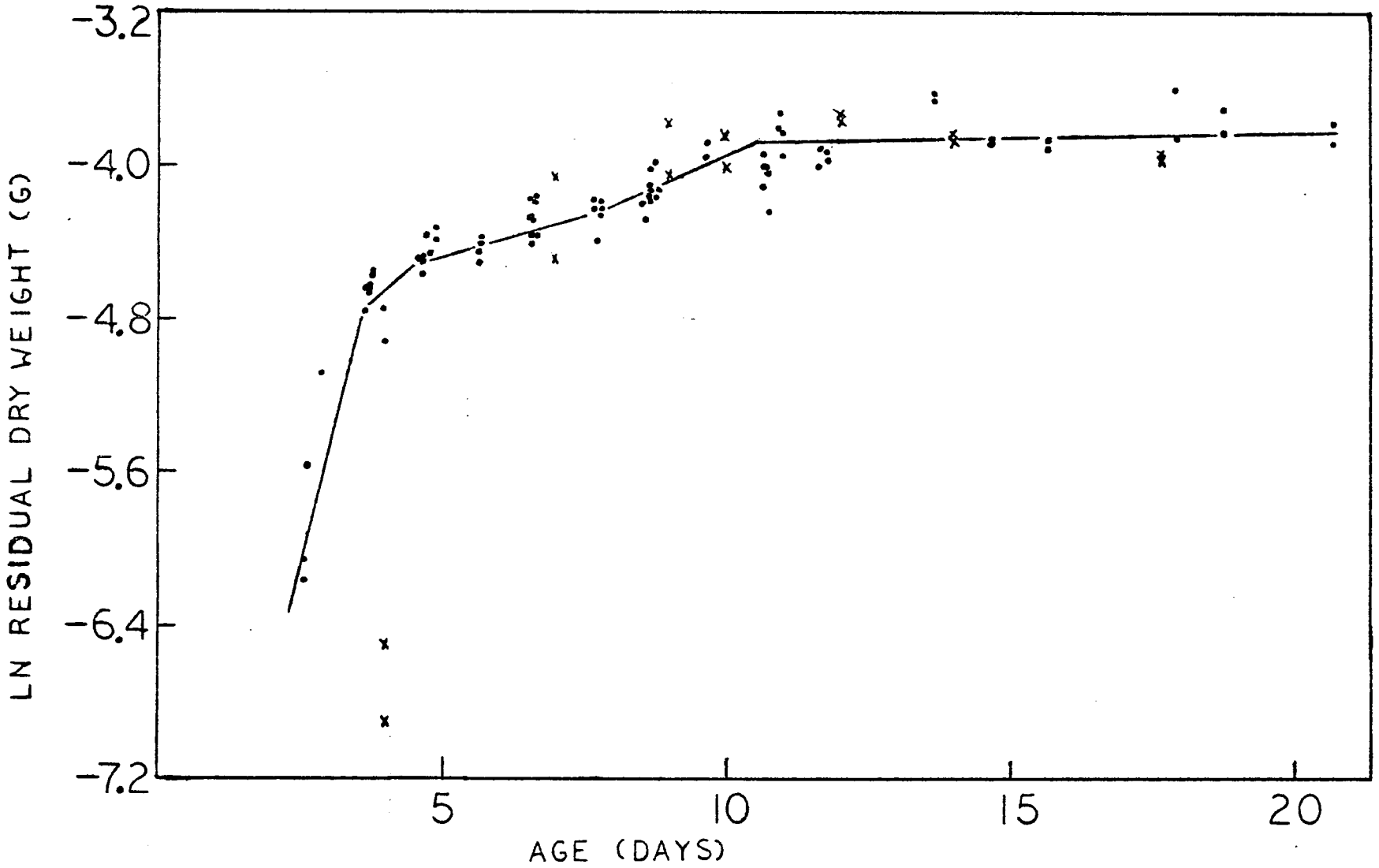

2.0
1.5
1.0
100
100−PERITHECIAL DENSITY (PER CENT)
20
40
60
80
100
1
FU CONCENTRATION (M)
X 10⁻⁵

Fig. 18. Natural logarithm of residual dry weight vs. age of culture at harvest. Results are from several different experiments; each point represents a single harvest. The drawn curve was fitted to the data visually. The results of one experiment (x) are noticeably different from the others (●).

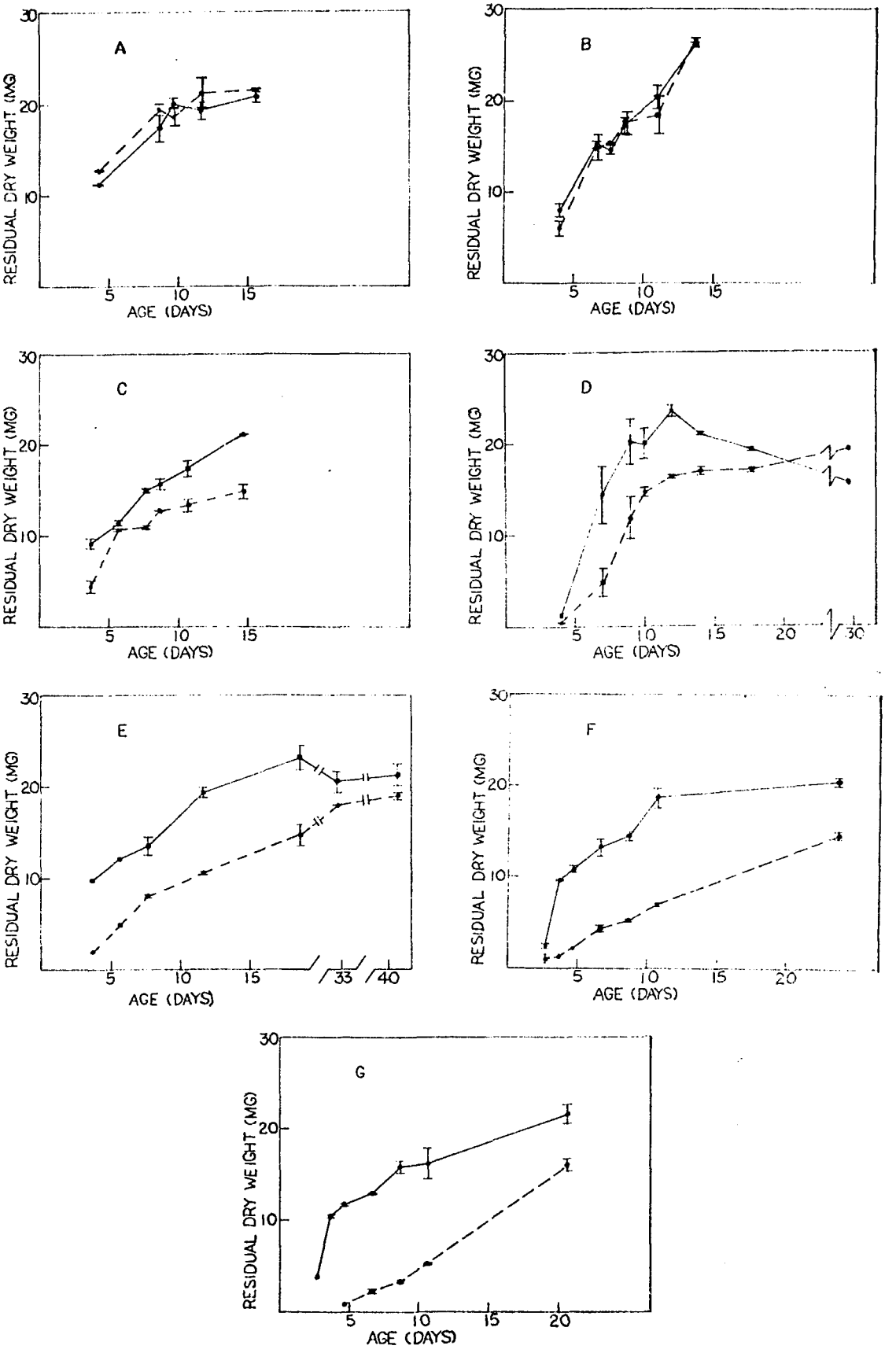

-3.2
-4.0
-4.8
-5.6
-6.4
-7.2
LN RESIDUAL DRY WEIGHT (G)
5
10
15
20
AGE (DAYS)

growth, the mycelium fills the medium and the top of the mycelium can be seen growing just above the air-liquid interface. Perithecia become visible to the naked eye at this time. The perithecia are mature by day 10 to 14. The maturation of the perithecia seems to be correlated with the end of the late spurt of growth. In one experiment the mycelium apparently never broke the air-liquid interface, but perithecia developed at the normal time anyway.

b. The Pattern in FU-Inhibited Cultures. A series of experiments on the effects of FU on liquid culture growth of S. fimicola was done. Results for several experiments using several different concentrations of FU are presented in Figure 19. (The values for the controls in these experiments are included in the data of Figure 18.) Harvests during the exponential period of growth were made in only a few cases. In general the effect of FU on growth is most pronounced in the younger cultures; in older cultures the dry weight reached in the FU cultures is reduced only slightly or not at all. The data are not sufficient to decide whether the inhibition is due to a reduction in the growth rate, an increase in the duration of a lag period, or both.

At the lower FU levels growth is not much different from control. Initiation of perithecia was not apparently delayed, although perithecial maturation was slightly retarded at 5.0×10^{-7} M. On the other hand, it was slightly advanced

Fig. 19. Effect of FU on residual dry weight accumulation in liquid culture. In most cases the points are the averages of two replicates, with the actual values of the replicates represented by horizontal bars and connected by vertical lines. Where a particular harvest did not have a replicate, only a point is plotted. _____, control; - - -, grown in the presence of FU. a. 1.0×10^{-7} M FU; b. 2.5×10^{-7} M FU; c. 5.0×10^{-7} M FU; d. 1.0×10^{-6} M FU; e. 2.5×10^{-6} M FU; f. 5.0×10^{-6} M FU; g. 1.0×10^{-5} M FU.


A
B
C
D
E
F
G
RESIDUAL DRY WEIGHT (MG)
AGE (DAYS)
30
20
10
5
10
15
20
30
33
40

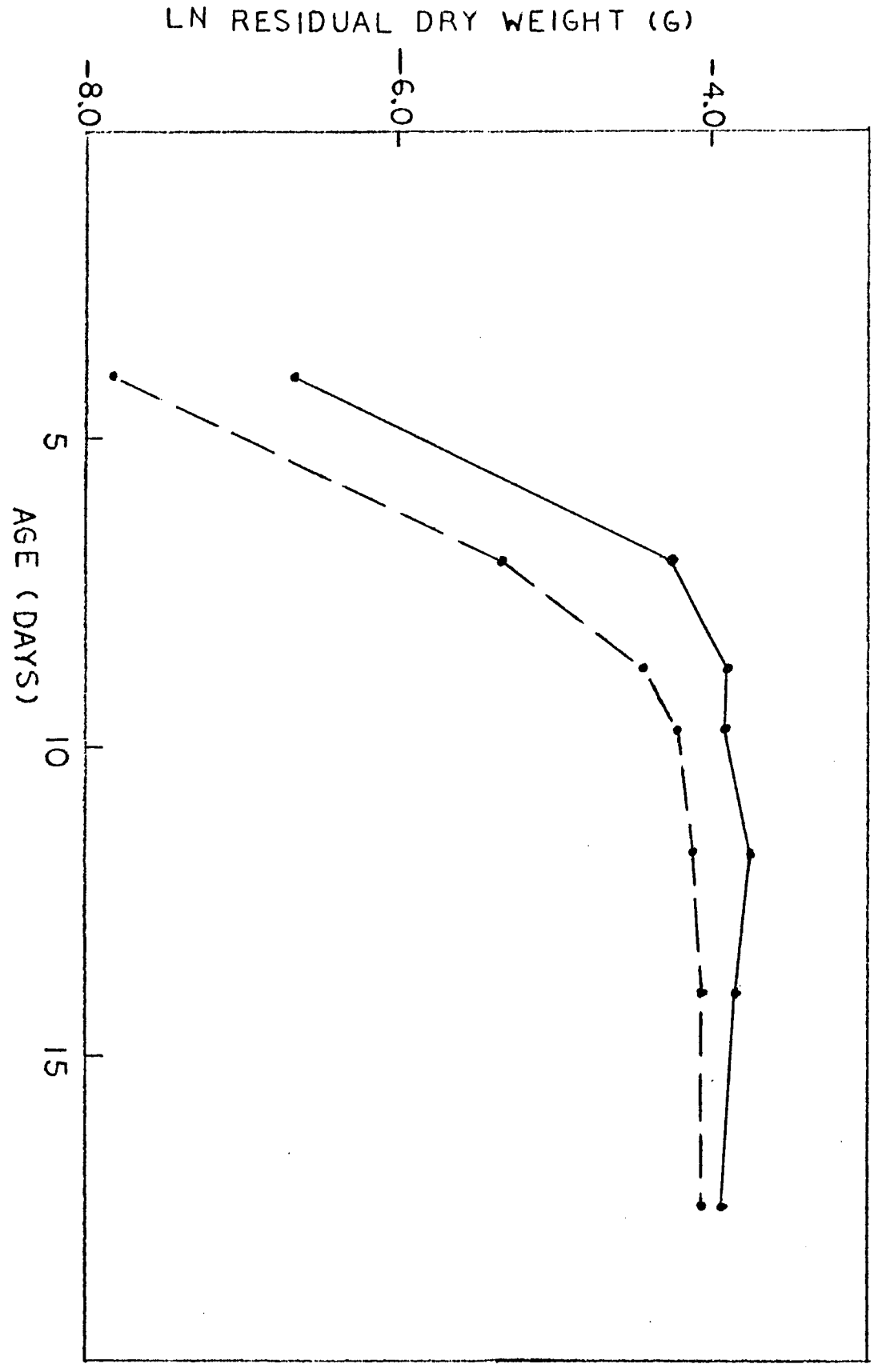
at 1.0×10^{-7} M. At 1.0×10^{-6} M FU and above, a major difference from the controls, aside from an overall reduction in growth, is the absence of a sudden slowing of the relative growth rate; instead there is a gradual reduction (e.g. Figure 20). (The relative growth rate is the absolute growth rate at any particular time divided by the amount of growth present at that time. Where growth is exponential, the relative growth rate is often called the specific growth rate. The relative growth rate is equivalent to the slope of the growth curve when growth is plotted on a logarithmic scale.)

At 1.0×10^{-6} M FU, the mycelium reached the surface of the medium by day 9, somewhat later than in the control flasks. This is apparently after the relative growth rate has begun its decline. In some flasks perithecia developed in patches, rather than over the entire surface as in the control flasks. A similar pattern was seen in 2.5×10^{-6} M FU, except that there were even fewer perithecia present. At 5.0×10^{-6} M perithecial formation was almost completely inhibited. At 1.0×10^{-5} M, only patches of mycelium broke the air-liquid interface, and only after 20 days.

For comparison, the results of one experiment with 1.0×10^{-5} M 6-azauracil are shown in Figure 21. See also Section III.B.3.c.

c. Dose-Response Relationships. The most fundamental measurement of growth for microorganisms, including fungi, is

Fig. 20. 1.0×10^{-6} M FU. Natural logarithm of residual dry weight vs. age of culture at harvest. Each point represents the average of two replicates. Same experiment as in Figure 19d. _____, control; ---, grown in the presence of FU.


LN RESIDUAL DRY WEIGHT (G)
-8.0
-6.0
-4.0
AGE (DAYS)
5
10
15

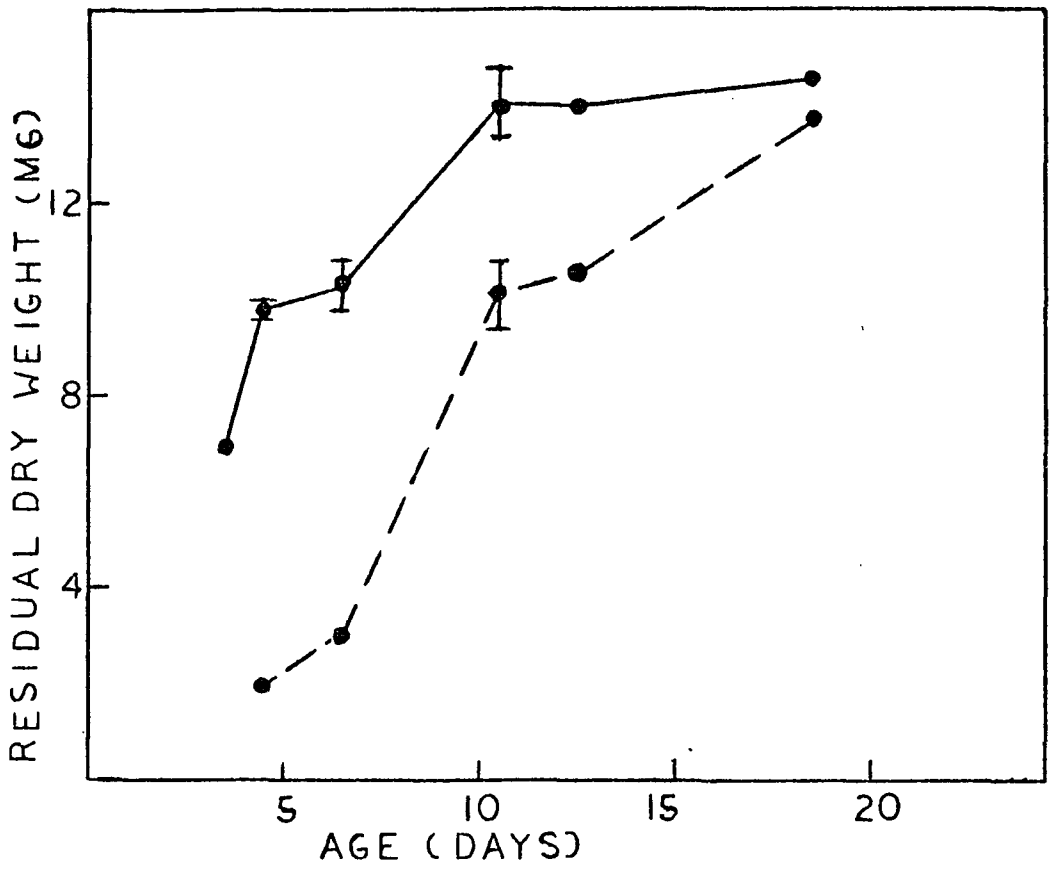


Fig. 21. 1.0×10^{-5} M azauracil. Residual dry weight vs. age of culture at harvest. Where the points are an average of 2 replicates, the actual values of the replicates are represented by horizontal bars connected by vertical lines. Where a particular harvest did not have a replicate, only a point is plotted. For the points at 4.5 and 6.5 days, the mycelium from 2 flasks was combined and the resultant residual dry weights halved. ___, control; ---, grown in the presence of azauracil.

probably the specific growth rate or, where the relative growth rate is changing during the course of the incubation, the maximum relative growth rate (for discussion see Trinci, 1969, 1971). Our data indicate that, for control cultures, the maximum relative growth rate is attained only prior to 4 days of incubation, after which the relative growth rate rather abruptly slows (Figure 18). The amount of growth that is present at this time under our conditions is only about 10 mg residual dry weight and it is difficult to get accurate determinations in sufficient number to determine the growth rate in separate experiments. This problem becomes more severe for cultures grown in the presence of FU, for which, where the data are available, the relative growth rate is seen to decline at much lower growth accumulations. For example, at 1.0×10^{-6} M and 2.5×10^{-6} M FU, the decline is first definitely observable at about 5 mg of residual dry weight, and at 1.0×10^{-5} M FU at 2 mg. And we do not even know whether higher relative growth rates would be observable if we had harvested younger cultures. In sum, we feel that, although theoretically preferable, the use of specific growth rate or maximum relative growth rate for comparing the inhibitory effects of different concentrations of FU would be impractical. We have, therefore, selected the maximum percent inhibition of growth accumulation observed from the various harvest times as our indication of the inhibitory

effect of FU. This maximum difference was generally obtained at or near 4 days of incubation. Since results with very young cultures tended to be erratic, results from cultures younger than 4 days were excluded for this purpose. A graph of the response, as determined in this manner, versus FU concentration is shown in Figure 29 (Section III.B.3.d).

For comparison, a single experiment was done to determine the effect of FU on dry weight (as opposed to residual dry weight) accumulation. For this experiment there was only a single harvest, after 5 days of growth, and the inhibitory effect was determined as the dry weight of each concentration as a per cent of the control. The results are shown in Figure 22.

3. Effects on Protein, RNA, and DNA Accumulation

a. The normal pattern and its correlation with developmental events. The results for several experiments are shown in Figure 23. (Also shown in these figures are the data for residual dry weight.)

Protein - During the phase of exponential increase in residual dry weight there appears to be also a very rapid increase in protein content. The protein level continues to increase rapidly after this phase, however, although at a slower rate. Between days 7 and 10, when a small spurt of growth is usually observed, there is usually a more pronounced

Fig. 22. Dry weight after 5 days of growth, as percent of the control value, vs. concentration of FU. In most cases the points are the averages of 2 replicates, with the actual values of the replicates represented by horizontal bars and connected by vertical lines. Where a particular concentration did not have a replicate, only a point is plotted.

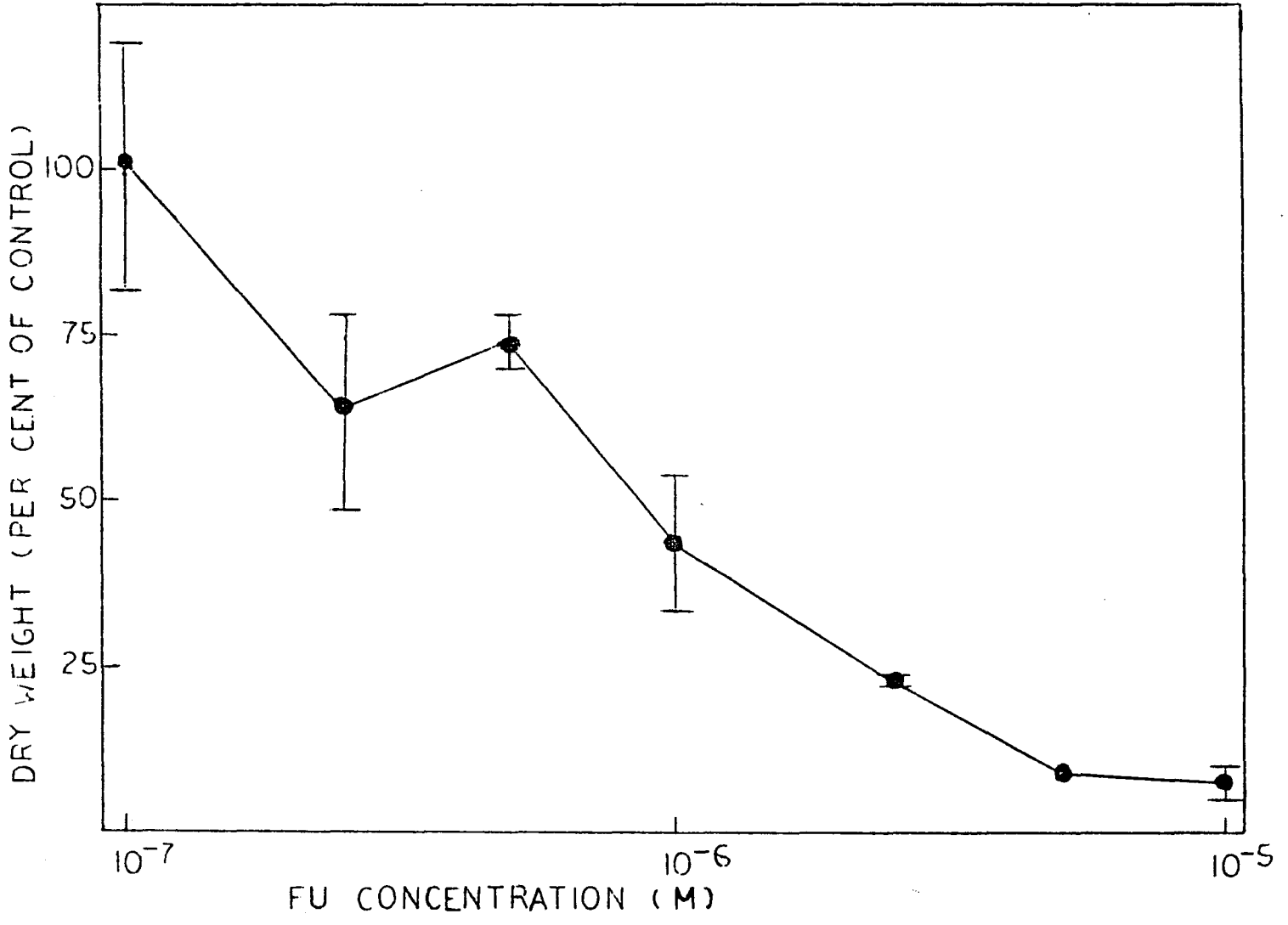
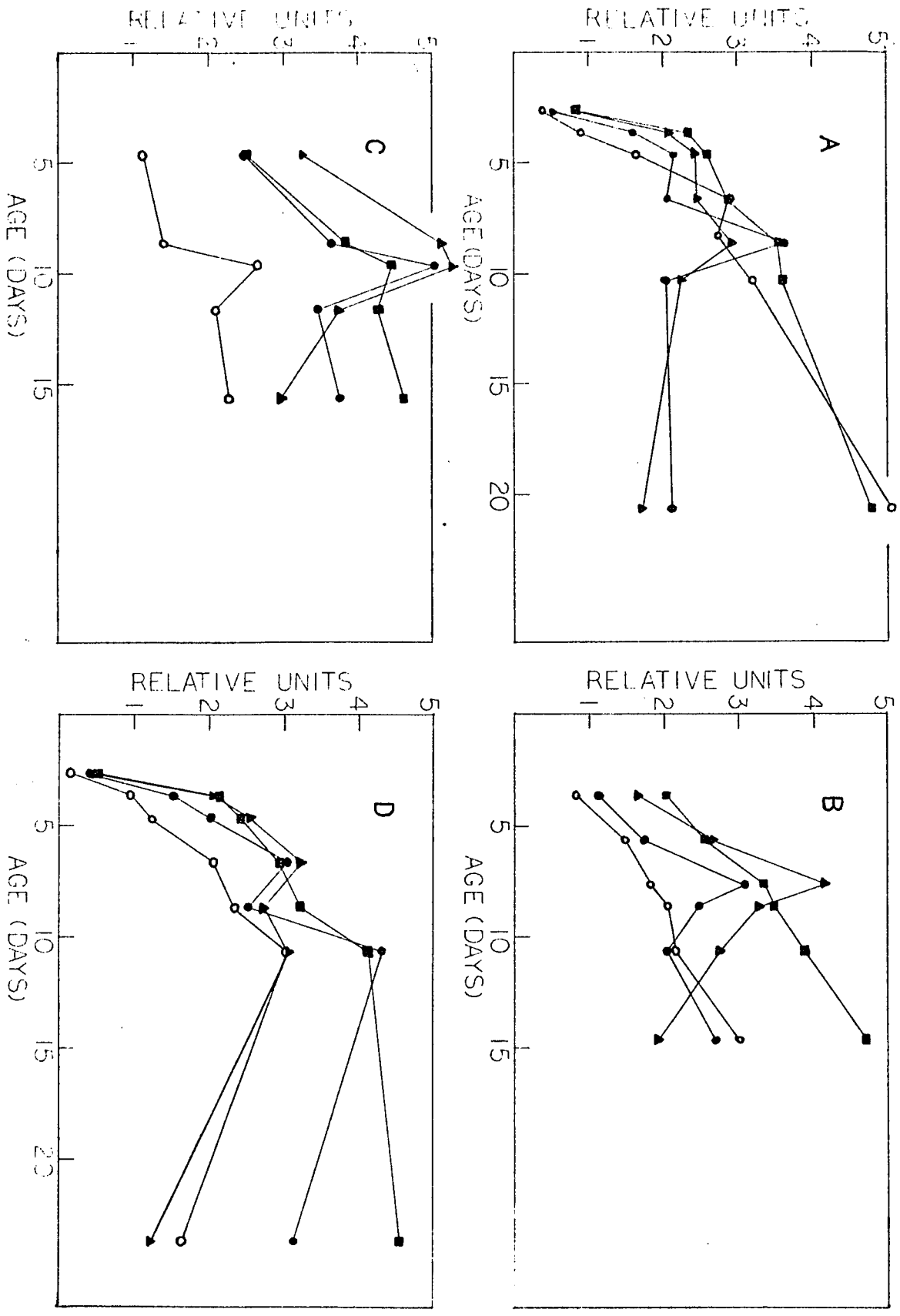

DRY WEIGHT (PER CENT OF CONTROL)
100
75
50
25
10^{-7}
10^{-6}
10^{-5}
FU CONCENTRATION (M)

Fig. 23. Amounts of residual dry weight, protein, RNA, and DNA, vs. age of culture at harvest. In most cases points are the average of 2 replicates. Each graph represents the results of a different experiment. ■, residual dry weight; ●, protein; ▲, RNA; o, DNA. Each unit on ordinate represents 4.5 mg residual dry weight per flask, 0.055 $O.D._{540}$ (biuret test), 0.08 $O.D._{260}$ (corrected), o. 0.04 $O.D._{560}$ (p-nitrophenylhydrazine test). (The results for the p-nitrophenylhydrazine test are not comparable between different graphs since different quantities of extract were used in the determinations in the different experiments.)


RELATIVE UNITS
1
2
3
4
5
A
B
C
D
AGE (DAYS)
5
10
15
20

increase in the protein level (Figure 23a). This is correlated with the development of the perithecia. Following this is a marked fall in the level (usually by day 12). Depending on the choice of harvest times, a subsequent rise is frequently seen around day 15 (Figure 23b), after the perithecia are already mature. It is clear from Figure 23 that the results vary considerably from one experiment to the next, making a precise description of the system difficult or impossible. If the protein levels in the culture do indeed vary as rapidly as the peaks and valleys of these figures suggest, then it is likely that in many experiments at least some peaks could be missed, depending on the actual choice of harvest times.

RNA - The pattern for RNA accumulation is similar to that for protein except the peak in RNA level frequently occurs prior to the peak in protein level, and a second peak occurring at day 15 was never seen (Figure 23). After day 12, and usually beginning before day 10, RNA almost always falls. This may be due to the sequestering of large amounts of RNA in the ascospores and the possible failure of our procedure to extract RNA from mature ascospores.

DNA - The results for DNA were even more variable than for the other parameters investigated, and there does not seem to be a typical pattern (Figure 23).

b. The Pattern in FU-inhibited Cultures. When graphs showing all 3 parameters are examined, the pattern appears similar to the controls at FU concentrations of 1×10^{-6} M and below (Figure 24a). At higher FU concentrations the early accumulation of protein, RNA, and DNA is reduced relative to the final levels achieved (Figure 24 b-d). DNA shows this early reduction markedly at an FU concentration as low as 2.5×10^{-6} M (Figure 24b), while protein still shows an earlier peak, although small, at 1.0×10^{-5} M FU (Figure 24d).

Although the overall pattern does not appear to be affected at concentrations of FU of 1.0×10^{-6} M and below, the quantity of each measured constituent, relative to the control values, is indeed reduced, and this reduction is first noticeable at 5.0×10^{-7} M FU (Figures 25a, 26a, and 27a). At lower concentrations there does not seem to be an effect, with the results sometimes showing a slight inhibition and sometimes a slight stimulation. The inhibition becomes more pronounced with increased FU levels (Figures 25-27). Even at the highest levels tested (1.0×10^{-5} M) however, there is still a "recovery" in older cultures, as shown clearly in Figures 25e, 26e, and 27e. This recovery for protein is less than for RNA and DNA, even at lower concentrations (compare Figure 25d and e with Figures 26d and e and 27d and e).

c. The Pattern in 6-Azauracil-inhibited Cultures. For

Fig. 24. Amounts of residual dry weight, protein, RNA, and DNA, in cultures grown in the presence of FU, vs. age of culture at harvest. In most cases points are the average of 2 replicates. a. 1.0×10^{-6}M; b. 2.5×10^{-6} M FU; c. 5.0×10^{-6} M FU; d. 1.0×10^{-5} M FU. Symbols as in Figure 23. Each unit on ordinate represents 6.1 mg residual dry weight per flask, 0.04 $O.D._{540}$ (biuret test), 0.07 $O.D._{260}$ (corrected), or 0.04 $O.D._{560}$ (p-nitrophenylhydrazine test). (The results for the p-nitrophenylhydrazine test are not comparable between different graphs since different quantities of extract were used in the determinations in the different experiments.)

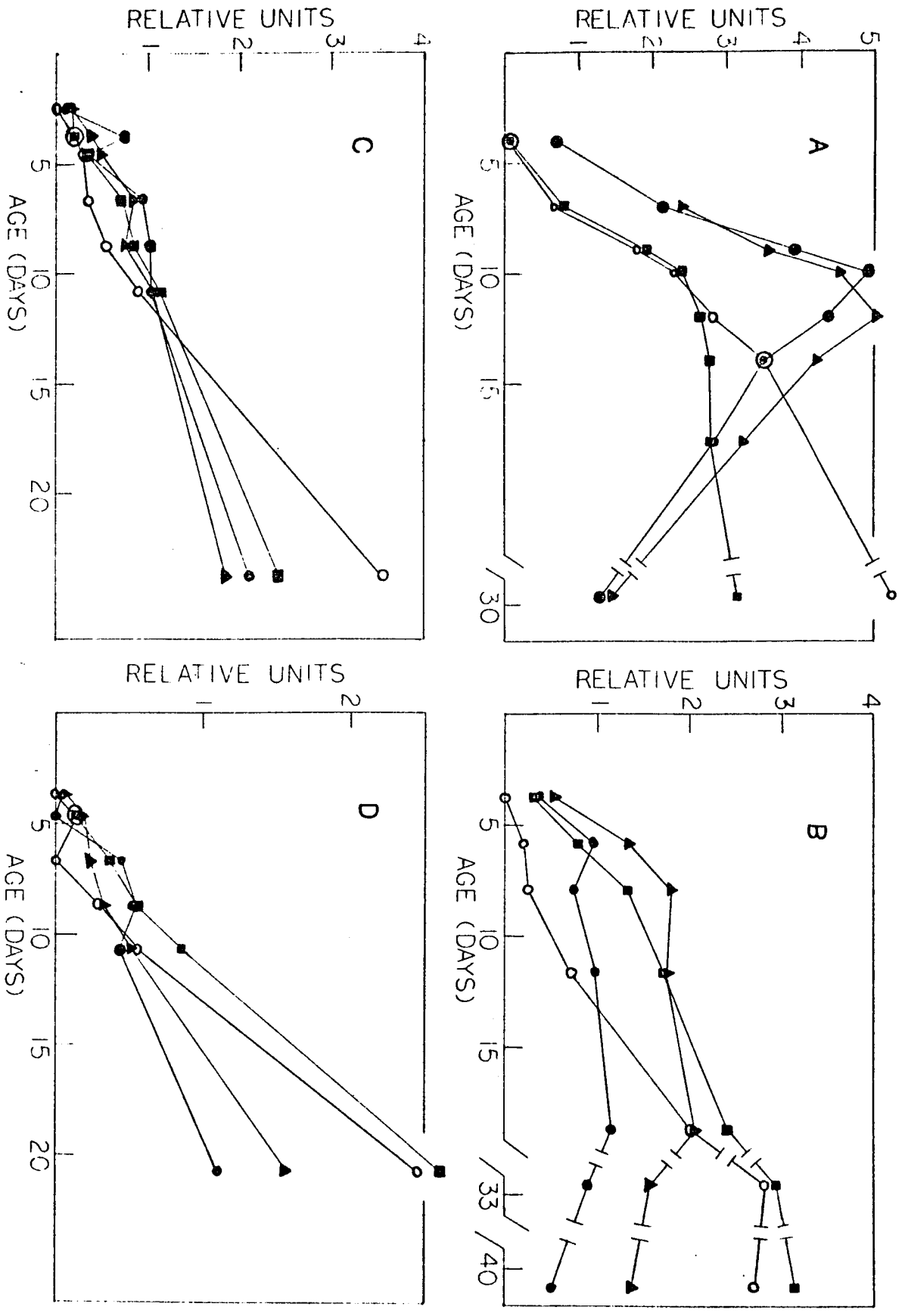

RELATIVE UNITS
AGE (DAYS)
A
B
C
D

Fig. 25. Effect of FU or azauracil on protein (biuret test) accumulation in liquid culture. In most cases the points are the average of two replicates, with the actual values of the replicates represented by horizontal bars and connected by vertical lines. Where a particular determination was done on only one sample, only a point is plotted. ____, control; ---, grown in the presence of inhibitor. a. 5.0×10^{-7} M FU; b. 1.0×10^{-6} M FU; c. 2.5×10^{-6} M FU; d. 5.0×10^{-6} M FU; e. 1.0×10^{-5} M FU; f. 1.0×10^{-5} M azauracil. (Control values in f may be depressed because the residues were subjected to an additional hot TCA extraction prior to the protein determination.)

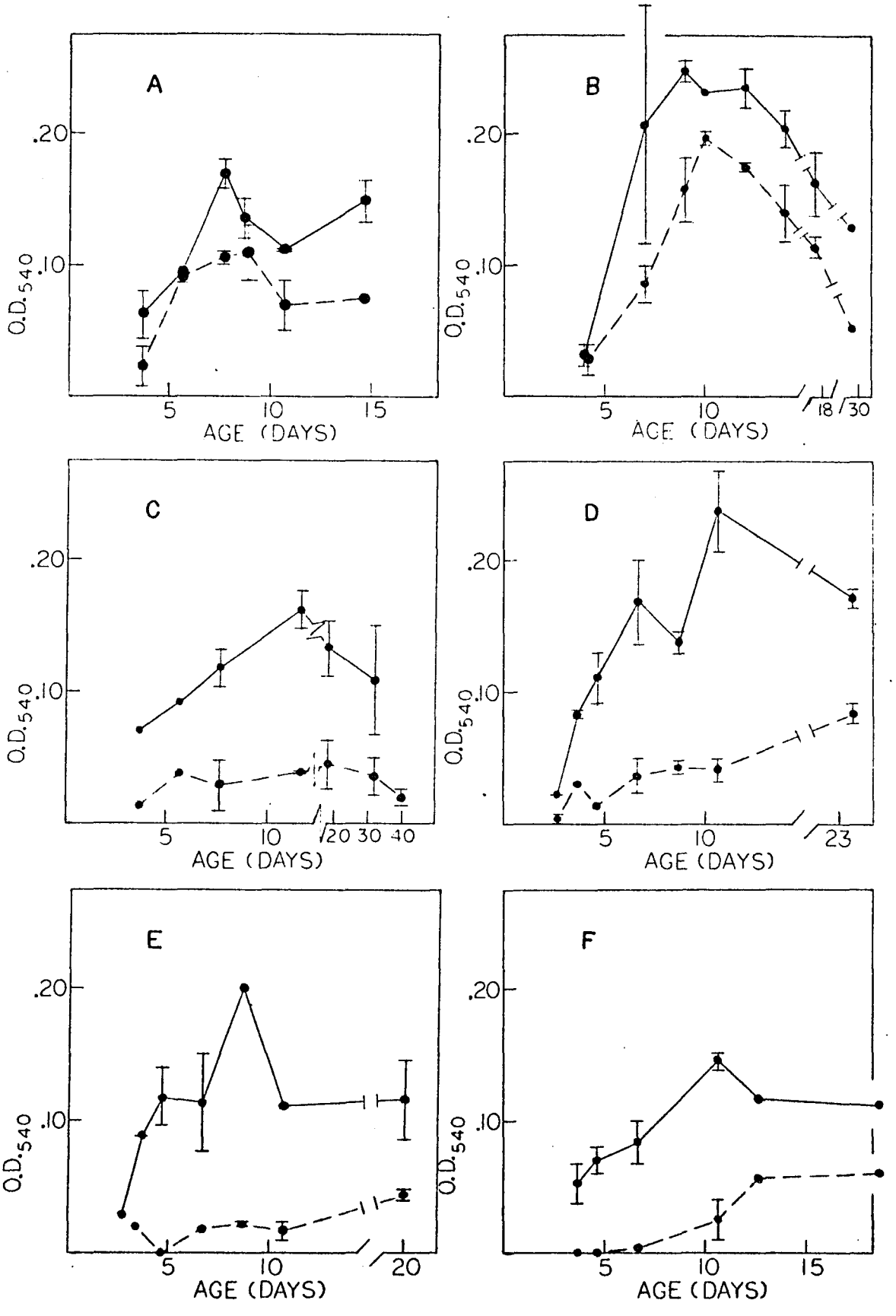

A
B
C
D
E
F
.20
.10
O.D.540
5
10
15
18
30
20
23
40
AGE (DAYS)

Fig. 26. Effect of FU or azauracil on RNA ($O.D._{260}$, corrected for protein) accumulation in liquid culture. In most cases the points are the average of two replicates, with the actual values of the replicates represented by horizontal bars and connectec by vertical lines. Symbols and concentrations as in Figure 25.

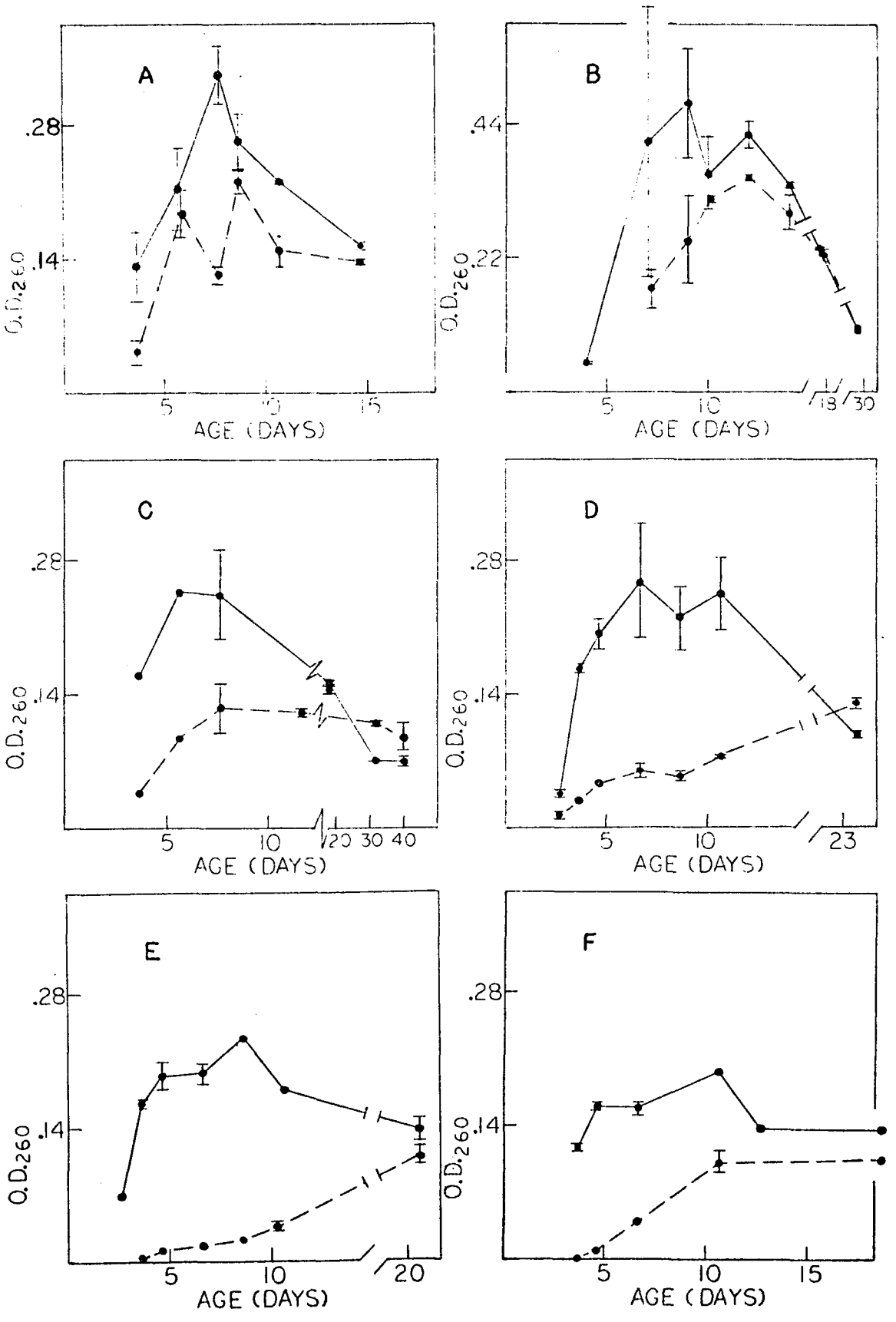
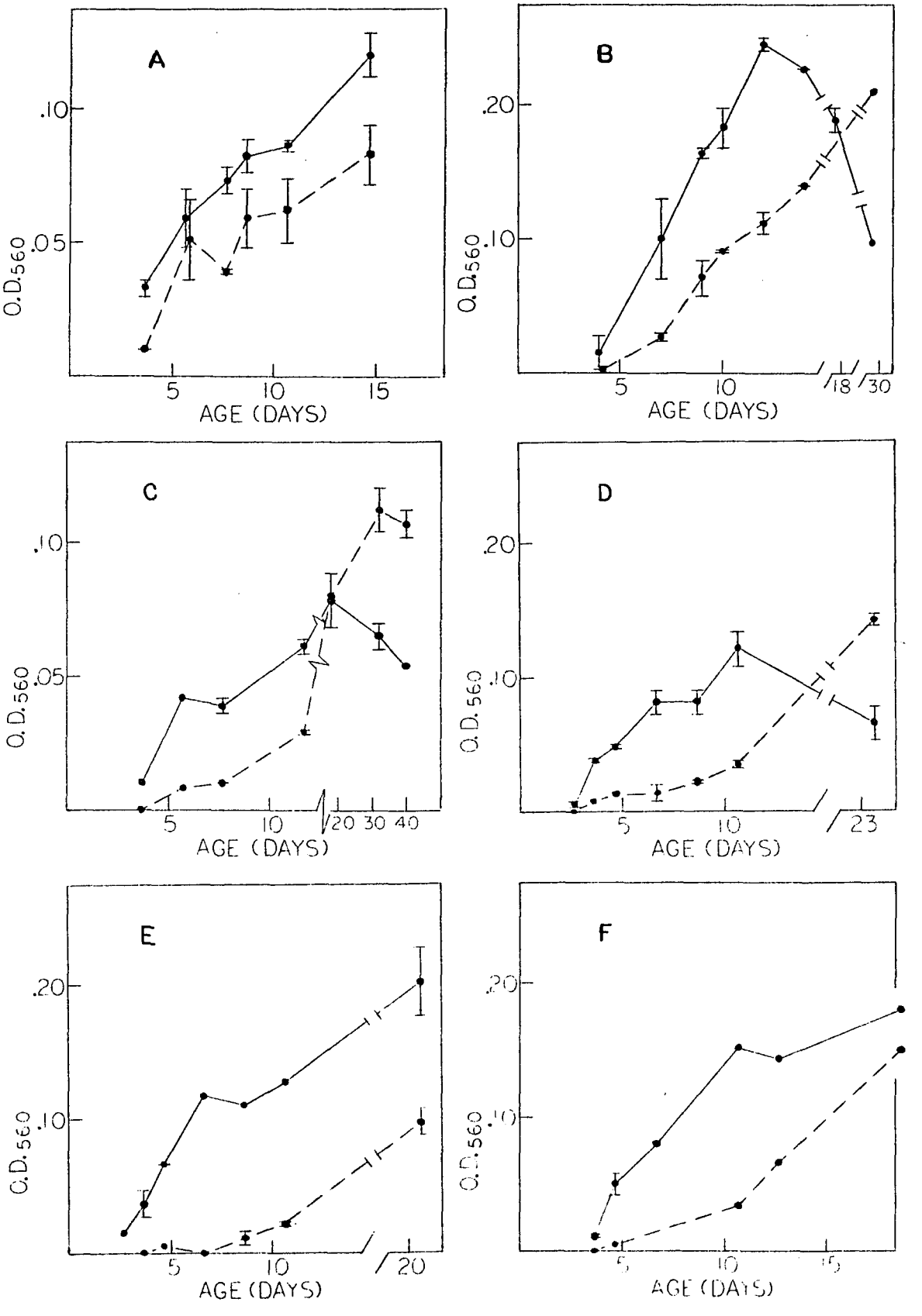

A
.28
.14
O.D.260
5
10
15
AGE (DAYS)
B
.44
.22
O.D.260
5
10
18
30
AGE (DAYS)
C
.28
.14
O.D.260
5
10
20
30
40
AGE (DAYS)
D
.28
.14
O.D.260
5
10
23
AGE (DAYS)
E
.28
.14
O.D.260
5
10
20
AGE (DAYS)
F
.28
.14
O.D.260
5
10
15
AGE (DAYS)

Fig. 27. Effect of FU or azauracil on DNA (p-nitrophenyl-hydrazine test) accumulation in liquid culture. In most cases the points are the average of two replicates, with the actual values of the replicates represented by horizontal bars and connected by vertical lines. Symbols and concentrations as in Figure 25. (The results are not comparable between different graphs since different quantities of extract were used in the determinations in the different experiments.)


A
B
C
D
E
F
O.D.560
AGE (DAYS)
.10
.05
.20
5
10
15
18
30
20
23
40

comparison, one experiment was done with 1.0×10^{-5} M azauracil. The results are shown in Figures 25f, 26f, 27f, and 28, and appear to be approximately similar to the results for 2.5×10^{-6} M FU. Perithecial formation was greatly delayed, but perithecia did eventually develop, although not as many as in the control.

d. Dose-Response Relationships. The problems in determining the maximum relative rates of increase in the levels of protein, RNA, and DNA are even greater than those for residual dry weight, which were discussed in Section 2.c. Consequently, the method used for describing the degree of inhibition by FU is the same here as that used for the residual dry weight, except that, in addition, results for cultures harvested after 11 days of growth were also eliminated in determining the maximum percent inhibition. A graph of the responses as determined in this manner is shown in Figure 29. There is no striking difference among the three parameters measured, or for that matter between those three and residual dry weight. RNA accumulation is more sensitive to low concentrations of FU than are the others; the ED_{50} for RNA is about 3.5×10^{-7} M, compared with 6 to 8 $\times 10^{-7}$ M for protein and DNA. At 1.0×10^{-6} M FU and above, however, all four curves are fairly similar.

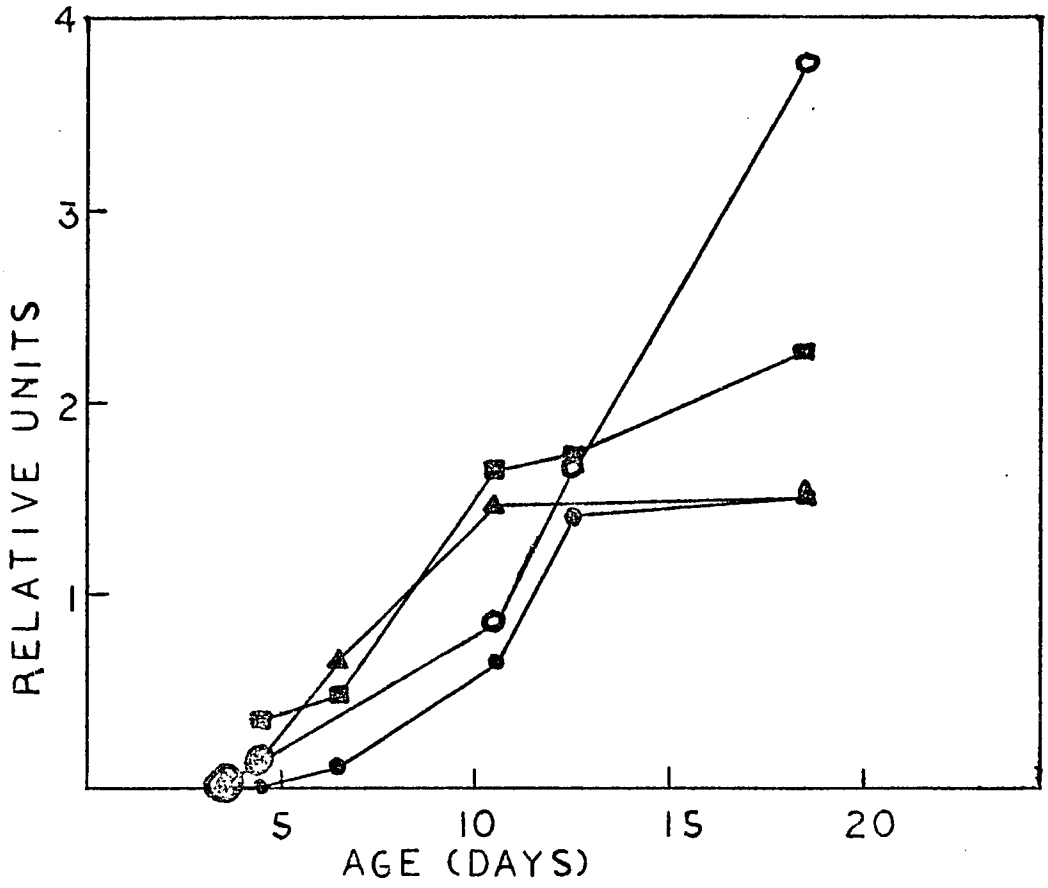
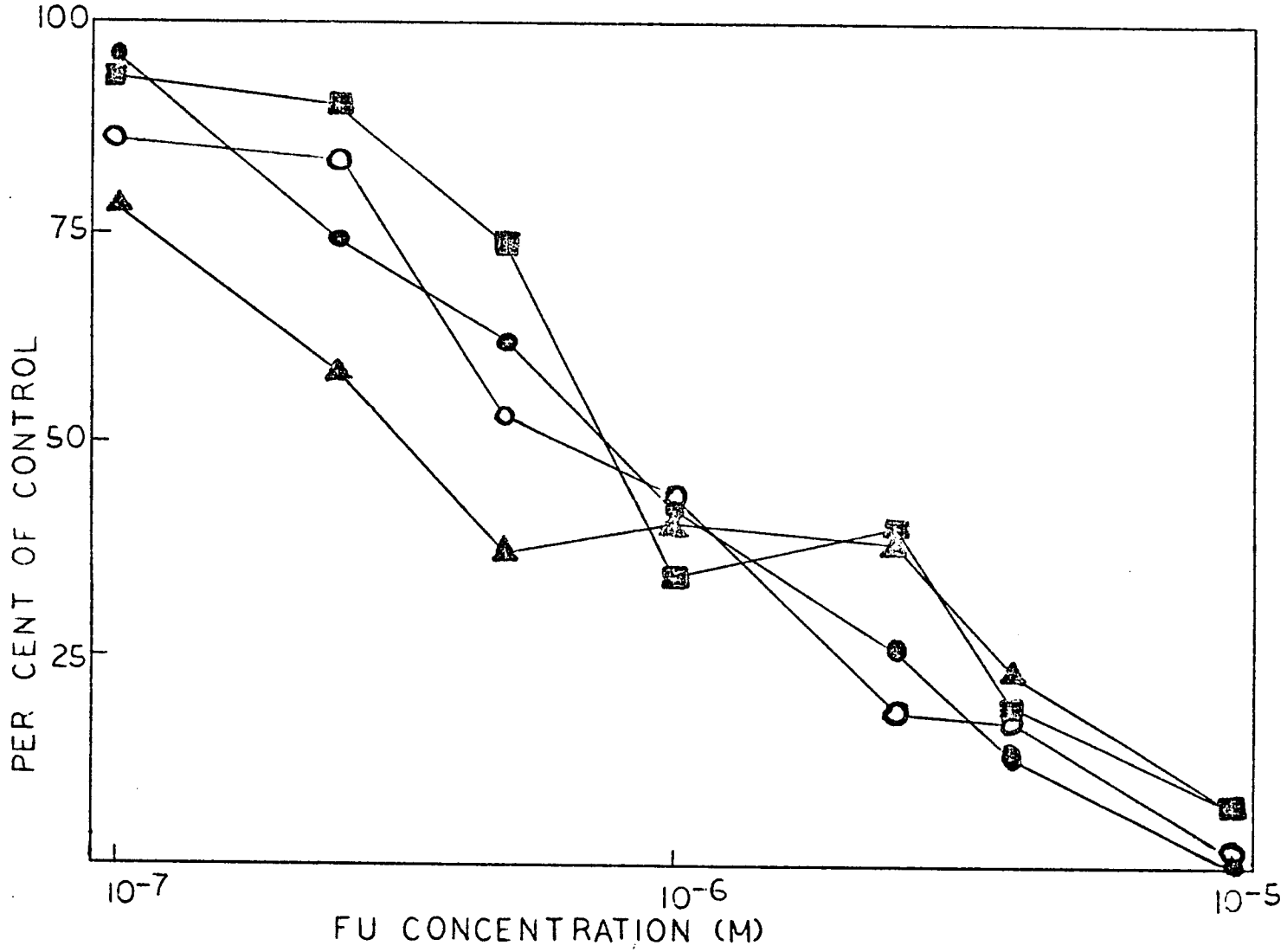


Fig. 28. Amounts of residual dry weight, protein, RNA, and DNA in cultures grown in the presence of 1.0×10^{-5} M azauracil, vs. age of culture at harvest. Points are either the average of 2 replicates, or, where a particular determination was done on only one sample, the value of that determination. Symbols as in Figure 23. Relative units as in Figure 24.

Fig. 29. Residual dry weight, protein, RNA and DNA, as per cent of control, vs. concentration of FU. Points represent average of 2 determinations on the day (between days 4 and 11) when the maximum average inhibition for each parameter was observed. One experiment was done for each concentration of FU. ■ , residual dry weight, ●, protein, ▲, RNA; o, DNA.


100
75
50
25
PER CENT OF CONTROL
10-7
10-6
10-5
FU CONCENTRATION (M)

4. Reversal of 5-Fluorouracil Inhibition by Pyrimidines and Pyrimidine Nucleosides

Another attempt to pinpoint the locus of FU inhibition was by determining which pyrimidines and pyrimidine derivatives would prevent the inhibitory effects of FU, and whether the inhibitory effects of FU on liquid-culture growth could be separated from those on perithecial formation by selectively preventing one inhibition or another. Thus, for example, whether the effect of FU was on RNA synthesis or DNA synthesis might be distinguishable by the differential ability of uracil or thymine and their derivatives to mitigate the inhibitory effects. Complicating factors, of course, are whether or not or to what degree the compounds are taken up and anabolized; these complications are considered in the Discussion.

In preliminary experiments on agar, the effect of normal bases on preventing the inhibitory action of the four base analogs in Table I on perithecial production was investigated with the following results (Lindenmayer and Schoen, 1967): (1) The inhibition of perithecial formation by 2.0×10^{-6} M FU could be prevented by double the molarity of uracil, but not by cytosine, thymine, adenine or guanine. (2) The effect of 2.0×10^{-4} M azauracil on perithecial initiation could similarly be counteracted by double the morality of uracil but not by any of the other bases (except that thymine and cytosine stimulated vegetative growth somewhat). (3) The

inhibition by 2.0×10^{-5} M azaadenine was prevented by double the molarity of adenine or guanine, while that by 2.0×10^{-5} M azaguanine was prevented by double the molarity of guanine but not by adenine; neither was prevented by cytosine, thymine, or uracil. Thus, the effects of all four active base analogs on perithecial formation could be prevented by the corresponding normal bases, and, except for azaadenine, by them only.

The results of experiments on cultures growing in liquid medium gave a more complex picture. Table V gives the results of a series of experiments on the prevention of FU inhibition. Uracil and deoxyuridine were the most effective compounds, completely preventing the growth (dry weight accumulation) inhibition by 5.0×10^{-6} M FU at 5.0×10^{-4} M. Uridine was less effective. Thymine was also quite effective, but, at 5.0×10^{-6} M FU, the effect reached a plateau at 5.0×10^{-4} M at less than complete restoration of growth. At 1.0×10^{-5} M FU, thymine was more effective at 1.0×10^{-3} M than at 5.0×10^{-4} M, but the response was still less than complete. The thymine nucleosides were much less effective, the ribonucleoside being apparently without any effect.

Some analogs of thymine were also tried as preventives. 5-Bromouracil was even more effective than thymine, and 5-bromodeoxyuridine was quite effective even though thymidine was not.

TABLE V

Prevention of FU Inhibition by Pyrimidines and Pyrimidine Nucleosides

FU concn (M)	base series and concn (M)		growth (%)* free base		ribonucleoside		deoxyribonucleoside	
5.0×10^{-6}	uracil	1.0×10^{-6}	15.3	(1.8)	...		...	
		5.0×10^{-6}	24.7	(1.0)	12.5		19	
		5.0×10^{-5}	76.5		21		47	
		5.0×10^{-4}	113	(33.2)	28.8	(8.0)	90.6	(28.9)
		1.0×10^{-3}	87		51.3	(32.9)	108.5	
	thymine	5.0×10^{-6}	0		...		...	
		5.0×10^{-5}	26.3	(0.4)	...		...	
		5.0×10^{-4}	65.1	(14.4)	...		...	
		1.0×10^{-3}	68.0	(5.3)	5.5		19	(9.9)
	bromouracil	5.0×10^{-6}	19.5		...		5	
		5.0×10^{-5}	38.5		...		9.5	
		5.0×10^{-4}	95		...		51	
		1.0×10^{-3}	87	(16.3)	...		82.5	
	azathymine	1.0×10^{-3}	14.3	(0.4)	...		...	
	trifluorothymine	7.5×10^{-4}	28		...		...	
		1.0×10^{-3}	32.8	(3.2)	...		...	
	cytosine	5.0×10^{-4}	25.5		26.5		22	
		1.0×10^{-3}	37.5		...		...	
	5-methylcytosine	5.0×10^{-4}	28		...		...	
1.0×10^{-5}	thymine	5.0×10^{-5}	12		...		...	
		1.0×10^{-4}	17	(1.4)	...		...	
		5.0×10^{-4}	37		...		...	
		1.0×10^{-3}	58.5	(16.3)	...		...	

*Dry weight after 5 days, as percent of control without FU, with standard error of the mean in parentheses where experiment was repeated. Two replicates per experiment. For FU alone growth was, for 5.0×10^{-6} M, 10.6 (6.7) %; for 1.0×10^{-5} M it was 6.5 (3.1) %.

Finally, cytosine, its nucleosides, and 5-methylcytosine showed a slight preventive effect.

Dry weights were determined also in older cultures (15 to 25 days old). The overall pattern of results was similar to those seen in the 5-day cultures. The percent inhibition by FU alone was less, as might be expected from the data presented in Section B.2.b. Where the growth as a percent of control for a preventive was high (as, for example, with 5.0×10^{-4} M thymine), there was in general no further increase, relative to the control, in older cultures. Where the degree of reversal was smaller (as with 5.0×10^{-6} M uracil), there was an increase in the percent dry weight in older cultures. However, since the culture with FU alone also increased in percent dry weight, there was no increase in the relative degree of prevention (in fact, there was usually a decrease).

There was, however, one exception to this pattern. The growth with deoxyuridine, as percent of control, typically fell in older cultures (for 5.0×10^{-4} M deoxyuridine, the average in cultures aged 15 to 25 days was 72%, and for 1.0×10^{-3} M it was 76%).

In general, the degree of perithecial development was correlated with the degree of restoration of growth. Even in cases where growth restoration was complete, however, there were usually noticeable differences in overall appearance

between the control and the cultures with additions. The only preventive restoring the appearance of the culture to one identical to the control was uracil at 5.0×10^{-4} M and above. 1.0×10^{-3} M uridine gave almost normal perithecial development but the overall appearance of the culture was still noticeably different from the control. In one experiment, 5.0×10^{-4} M bromouracil and 1.0×10^{-3} M bromodeoxyuridine each restored the appearance to one very similar to that of the control, but, in another experiment, 5.0×10^{-4} M bromouracil and 5.0×10^{-4} M bromodeoxyuridine each failed to restore perithecial production altogether. Thymine and uridine together also gave an appearance like the control's (see below).

Two additional control experiments were performed. In one, the ability of purines and a purine ribonucleoside (inosine) at 100 times the molarity to prevent inhibition by 5×10^{-6} M FU in liquid culture was tested. They were totally without effect. In the other, 5.0×10^{-4} M of several pyrimidine compounds were tested in the absence of FU for their effect on growth. Uracil, uridine, deoxyuridine, and bromouracil were slightly stimulatory (ca. 20% more growth after 5 days than with no additions) and thymine and bromodeoxyuridine were without effect). At 18 days no effects were seen on growth, but bromouracil and bromodeoxyuridine inhibited perithecial development somewhat. (Cytosine compounds were

not tested in this manner.)

In order to account for the effects of uptake and usage peculiarities in S. fimicola of these compounds, the ability of the above compounds to prevent the inhibitory effects of two inhibitors of pyrimidine anabolism was tested. The inhibitors were azauracil, which inhibits the de novo synthesis of pyrimidines (Škoda, 1963) and hydroxyurea, which inhibits the synthesis of deoxynucleotides (O'Donovan and Neuhard, 1970). The ability of a compound to prevent the inhibition would indicate that that compound was taken up and anabolized to the extent that it could replace the needed components that were not being made de novo because of the inhibitor. (Here again, there is also the possibility that it interferes with the transport of the inhibitor itself and exerts its preventive effect that way.) The procedure is not foolproof, as intermediate steps involved in the inter-conversions are not known for S. fimicola.

The results of the experiment with hydroxyurea are shown in Table VI. Although no combinations tested gave complete reversal, it is apparent that when the purine deoxyribonucleosides are present, deoxycytidine is the necessary and sufficient pyrimidine deoxyribonucleoside for maximum reversal. This experiment unfortunately fails to suggest possible metabolic fates for thymidine and deoxyuridine in S. fimicola.

TABLE VI

Prevention of Hydroxyurea Inhibition

by Deoxyribonucleosides*

Preventives**	Growth (%)+
none	31
AdR + GdR	34
" + UdR	35.5
" + CdR	51
" + TdR	35
" + UdR + TdR	35
" + CdR + TdR	40.5
" + CdR + UdR	53
" + CdR + TdR + UdR	48

*Hydroxyurea at 1.0×10^{-3} M. Abbreviations:
AdR - deoxyadenosine; GdR - deoxyguanosine;
CdR - deoxycytidine; UdR - deoxyuridine;
TdR - thymidine

**Preventives at 5.0×10^{-4} M each

+Dry weight after 16 days, as percent of control without hydroxyurea. Average of 2 replicates.

The results of the experiment with azauracil are given in Table VII. Surprisingly, all the pyrimidines tested gave some stimulation of growth compared to 6-azauracil alone. Uracil and uridine completely prevented inhibition; deoxyuridine was less effective. Thymine gave relatively less growth here than in the FU experiments; bromouracil, as in the FU experiments, was more effective than thymine, but bromodeoxyuridine was much less effective than in the FU experiments.

One additional experiment was performed to try to determine whether the effects of thymine and deoxyuridine are separable from the effects of uridine. That is, are thymine and deoxyuridine stimulating growth by a different mechanism (presumably by providing an increased endogenous supply of thymidylic acid) than is uridine (presumably by increasing the endogenous supply of uridylic acid)? The experiment consisted of testing the effects of combinations of thymine, uridine, and deoxyuridine. The results are shown in Table VIII. The effects of thymine plus deoxyuridine were intermediate between those of thymine alone and deoxyuridine alone. Thymine plus uridine and uridine plus deoxyuridine had a greater effect than either alone. Of the combinations only thymine plus uridine was like the control in perithecial development and overall appearance. Although these results seem to indicate that thymine and uridine do act in different

TABLE VII

Prevention of Azauracil Inhibition by Pyrimidines and Pyrimidine Nucleosides*

Preventive**	Growth (%)+
none	1
uracil	117
uridine	112.5
deoxyuridine	78.5
thymine	21
thymidine	14
bromouracil	47.5
bromodeoxyuridine	27.5
cytosine	29.5
cytidine	76.5
deoxycytidine	28
5-methylcytosine	47

*Azauracil at 2.0×10^{-4} M.

**Preventives at 5.0×10^{-4} M each.

+Dry weight after 5 days, as percent of control without azauracil. Average of 2 replicates.

TABLE VIII

Prevention of FU Inhibition by Thymine, Uridine, and Deoxyuridine, Separately and in Combinations*

Preventive Concentration (M)		growth (%)** 5 days	15 days
none		14	27
thymine	5.0×10^{-4}	57.5	67
deoxyuridine	5.0×10^{-4}	100.5	56.5
uridine	5.0×10^{-4}	23	81
thymine + deoxyuridine	2.5×10^{-4} 2.5×10^{-4}	86	70
thymine + uridine	2.5×10^{-4} 2.5×10^{-4}	73.5	94
deoxyuridine + uridine	2.5×10^{-4} 2.5×10^{-4}	128	100.5

*FU at 5.0×10^{-6} M.

**Dry weight as percent of control without FU. Average of two replicates.

ways in preventing inhibition by FU, the differences are not necessarily the same as those presumed above (see Discussion).

IV.

DISCUSSION

A. Relation Between the Types of Inhibitors Used and the Nature of the Inhibition Observed on Agar

1. Effect of Inhibitors on Perithecial Formation and Linear Growth Rate

Perithecial initiation was inhibited in S. fimicola, without seriously inhibiting linear growth rate, by the following: (1) azauracil and azaadenine at 1×10^{-5} to 1×10^{-4} M and by FU at 1×10^{-6} M, (2) cyanide at 5.0×10^{-4} M, and (3) large amounts (50 g/l) of casein hydrolysate. Inhibition of perithecial initiation was associated with moderately severe inhibition of linear growth rate with: (1) azaguanine at 3.0×10^{-5} M and (2) azide at 5.0×10^{-5} M, dinitrophenol at 5.0×10^{-4} M, and thiourea at 1.0×10^{-2} M. None of the other inhibitors tested showed such selectivity. Actinomycin D and cycloheximide reduced the number of perithecia but also severely reduced the linear growth rate. Nalidixic acid completely inhibited perithecial formation, but at the same time severely inhibited growth. With several of the inhibitors, growth was severely inhibited but perithecia were still produced.

One might assume that perithecia are initiated only

when a generally high level of metabolism is maintained, and that any inhibitor that puts a strain on the synthetic processes of the organism will also suppress perithecial formation. This is essentially one of the assumptions that Hawker (1957, p. 72) makes to account for the various nutritional effects on reproduction in fungi. Our results are more likely to be explained by assuming that there is a particular aspect of metabolism that is required for perithecial initiation and not required for growth, since many of the inhibitors which we studied greatly reduced vegetative growth yet permitted the production of perithecia. The rapid falling off to zero of the dose-response curves with FU (Figures 16 and 17) suggests that there is a threshold of some metabolic activity or product below which no perithecial production occurs. Thus one must try to find some biochemical effect common to the selective inhibitors and either absent or overshadowed by other effects of the nonselective inhibitors. It may of course also be the case that there are several loci of inhibition which will show the same selective effect on perithecial formation. Gillie (1968b) showed that starvation for a required amino acid in N. crassa caused a reduction in total dry weight accumulation without reducing linear growth rate. There may be numerous agents that can have deleterious effects on fungi, including inhibiting perithecial formation, yet which do

not inhibit linear growth rate. These various agents will not necessarily act by the same mechanism. In the discussion that follows, it should be borne in mind that the actual mode of action in S. fimicola of the inhibitors used is unknown, and inferences must be made from work in other organisms.

Of the RNA-base analogs that we used, FU has been studied the most extensively. FU is incorporated in place of uracil, as 5-fluorouridylic acid residues, into all fractions of RNA in a wide variety of organisms (Heidelberger, 1965; Mandel, 1969) including fungi (de Kloet and Strijkert, 1966; Gressel and Galin, 1967). Its most profound effect on RNA metabolism is to inhibit ribosomal RNA synthesis and ribosome accumulation (de Kloet and Strijkert, 1966; Mandel, 1969) or to result in abnormal or unstable ribosomes (Hills and Horowitz, 1966; Saunders et al., 1968; Hahn and Mandel, 1971; Mayo and de Kloet, 1972) apparently due to the inability of FU-containing ribosomal RNA precursors to undergo normal maturation processes (Willén, 1970; Mayo and de Kloet, 1972; Wilkinson and Pitot, 1973).

Although FU is incorporated into messenger RNA, it apparently does not significantly alter the functioning of the latter. There have been occasional reports that FU-containing messenger RNA has altered coding properties. Bussard et al. (1960) reported that the incorporation of FU into

messenger RNA in Escherichia coli leads to the synthesis of β-galactosidase with reduced specific enzyme activity. Such reports are few, and all attempts to demonstrate the insertion, caused by FU-containing messenger RNA, of an incorrect amino acid residue into a polypeptide chain have been unsuccessful (Rosen et al., 1969). Heidelberger (1965) cited a study by Rothman which purported to show such an alteration in alkaline phosphatase in E. coli, but the details of this experiment were apparently never published. On the other hand, studies with synthetic messenger RNA indicate that FU always codes like uracil (Heidelberger, 1965) and positive evidence for the normal functioning of FU-containing natural messenger RNA has been reported (Rosen, 1965; Horowitz and Kohlmeier, 1967; Ossowski and Reich, 1972). Good evidence for one type of miscoding caused by the incorporation of FU into messenger RNA is available, however, namely for the misreading of certain nonsense codons as sense (Champe and Benzer, 1962; Garren and Siddiqi, 1962; Rosen et al., 1969) as evidenced by phenotypic reversion of nonsense mutants by FU. (Attempts to get suppression of missense mutations by FU were unsuccessful [Rosen et al., 1969].) While such an action could conceivably cause mistranslation of polycistronic messenger RNA containing FU, the degree of phenotypic reversion observed was very small (apparently never more than 0.5%) and consequently such translation errors would likewise be expected to have only a minor effect.

The picture for FU effects on transfer RNA is less clear. Alterations in the levels of modified bases in FU-containing transfer RNA have been reported (Lowrie and Berquist, 1968; Kaiser, 1972). Changes in some of the biochemical and biophysical properties of such altered transfer RNA (e.g., thermal denaturation, amino acid acceptor activity, stimulation of in vitro protein synthesis) also have been reported (Lowrie and Berquist, 1968; Giege et al., 1969; Kaiser, 1969). The changes in properties do not appear to be severe enough to cause major alterations in protein synthesis in vivo; however, until more is known concerning transfer RNA functions one cannot conclude that the incorporation of FU into transfer RNA is without major consequences to the organism and could not be involved with the selective inhibitory effects observed in S. fimicola.

Another well established effect of FU, or of its metabolic product 5-fluorodeoxyuridylic acid, is the inhibition of de novo synthesis of thymidylic acid and consequent inhibition of DNA synthesis (Heidelberger, 1965). While we cannot rule out this effect as the mechanism for the selective action of FU, the failure of other presumed inhibitors of DNA synthesis, for example hydroxyurea, to have a similar selective effect, argues against it. It may, however, play a role in the inhibition of linear growth rate observed at the higher FU concentrations.

Other effects of FU that have been observed in other organisms and that could play a role in the inhibition observed in S. fimicola are (1) inhibition of sugar nucleotide metabolism, as demonstrated by the inhibition of bacterial cell wall synthesis (Heidelberger, 1965; Stickgold and Neuhaus, 1967) and (2) catabolism to fluoroacetic acid with consequent inhibition of the tricarboxylic acid cycle (Koenig and Patel, 1970). The second effect would account for the similarity in action in our systm between FU and cyanide, but would not explain the action of the other selective base analogs.

The other three RNA-base analogs of Table I have been studied much less extensively than FU but there is some information as to the modes of action of two of them. Azauracil, after anabolism to 6-azauridylic acid, inhibits orotidylic acid decarboxylase, an enzyme of de novo pyrimidine synthesis (Škoda, 1963). This in turn inhibits nucleic acid synthesis. Whether it has other effects is less clear although it apparently can, as does FU, interfere with sugar nucleotide metabolism (as indicated in Discussion section by Rogers and Perkins, 1960). Azauracil, as 6-azauridine 5'-diphosphate, has also been reported to inhibit amino acid acceptor activity and terminal CCA exchange in transfer RNA in vitro (Škoda, 1963). Whether the concentration of 6-azauridine 5'-diphosphate is ever high enough

in vivo to have this effect is not clear.

It would seem, because of a general inhibition of nucleic acid synthesis, that azauracil would be unlikely to inhibit perithecial production selectivity. It may be that general inhibition of RNA synthesis can be responsible for such selectivity, or it may be that one fraction of the organism's RNA, for example ribosomal RNA, is more sensitive to lowered endogenous nucleotide levels than the other fractions. The inhibition of pyrimidine synthesis would also be expected to inhibit DNA synthesis. Again, it may be that DNA synthesis is less sensitive to lowered rates of nucleotide synthesis than is RNA synthesis.

Azaguanine acts primarily by being incorporated into RNA. It inhibits synthesis of ribosomal RNA by interfering with the normal processing of the ribosomal precursors (Attardi and Amaldi, 1970). It also reportedly affects the normal functioning of messenger RNA and thereby inhibits protein synthesis directly (Roy-Burman, 1970); this may account for its relative lack of specificity compared with FU and azauracil. Very little work has been done with azaadenine, and virtually nothing is known about its mechanism of action.

Like FU, actinomycin D has also been reported to selectively inhibit ribosomal RNA synthesis (but the data on this are mostly from mammalian cells, e.g. Perry and Kelley

[1970]). This selectivity, however, is only at low doses. At higher doses other fractions of RNA are inhibited as well (Perry and Kelley, 1970). The doses required for inhibition in our system were much higher than those necessary for inhibiting RNA synthesis in mammalian cells; in the latter, RNA synthesis is completely inhibited by 1 mg/l actinomycin D (Reich et al., 1961). Assuming that in our system FU is acting primarily by its effect on ribosomal RNA synthesis it is then not certain why we did not see a selective effect with actinomycin D at lower doses. However, actinomycin D seems to act differently in fungi than in mammalian cells, at least with regard to sensitivity. In N. crassa, Horowitz et al. (1970) found only 37% inhibition of nucleic acid (presumed to be RNA) synthesis under short term (one hour) incubations with 20 mg/l actinomycin D. Under the same conditions protein synthesis was inhibited 21%. The low degree of inhibition in these experiments may be due to low permeability of N. crassa mycelia to the drug (Urey and Horowitz, 1967). Under long term conditions growth was inhibited 50% by only 1.3 mg/l actinomycin D. Thus the precise effects of actinomycin D in fungi remain to be clarified.

One would not expect that the actions of the various inhibitors on RNA synthesis per se would have deleterious effects on the organism, but rather that such effects would

arise from the resulting indirect inhibition of protein synthesis. If this were the case, however, it would be expected that inhibitors of protein synthesis should have the same effects on growth, branching, and perithecial formation as the inhibitors of RNA metabolism. We do not understand why there are differences in these parameters, especially linear growth rate, among cultures grown in the presence of the different classes of inhibitors.

Amethopterin, sulfanilamide, and azaserine are expected to block de novo purine (and, for amethopterin and sulfanilamide, thymidylic acid) synthesis and, hence, RNA and DNA synthesis. We attribute the lack of selectivity of these drugs to their known "side effects," that is, they are known to inhibit several reactions in addition to those involved in purine and thymidylic acid synthesis, primarily reactions concerned with amino acid synthesis (White et al., 1964). The lack of selectivity could also be due to the induced deficiency of purine nucleotide coenzymes.

Cyanide, azide, and thiourea, three other selective inhibitors of perithecial initiation, are inhibitors of metallo-enzymes, including the enzymes of mitochondrial electron transport and of nitrate assimilation (Cochrane, 1958, p. 245; Hewitt and Nicholas, 1963; Stark and Dawson, 1963). Dinitrophenol, another selective drug, inhibits energy metabolism by uncoupling mitochondrial electron

transport from ATP formation (White et al., 1964). It appears also to inhibit transport properties of membranes independently of its effect on aerobic energy metabolism; for example, it (as well as azide) inhibited ammonia and glucose assimilation in anaerobically grown yeast (Kovác and Greksák, 1966). The reason for the similarity in effect between the selective base analogs and these inhibitors is not clear. The base analogs could, by virtue of possible inhibition of nucleotide coenzyme metabolism, interfere with energy metabolism, but this is just conjecture. We have already mentioned the possibility that FU interferes with the tricarboxylic acid cycle, and this could, of course, then inhibit energy metabolism. Obviously, a block in energy metabolism may affect many cell functions including RNA and protein synthesis.

We do not understand the significance of the submersed production of perithecia.

2. Effect of Inhibitors on Branching

Our data indicate that branching frequency is a more stable parameter to follow than is branch density (Lindenmayer and Schoen, 1967). For instance, while the branch density varies 3- to 5-fold between urea- and asparagine-grown cultures on the one hand, and ammonium sulphate-grown ones on the other (Table IV), the branching frequencies of

these cultures are all between 9.4 and 12.5 branches per 100 minutes, a variation of only about 10 to 30% from the control. Similarly, in the presence of various inhibitors the branch density may fluctuate quite erratically with increasing concentration of the inhibitor (or with time as in Figure 3), while the branching frequency usually shows a steady decrease, or remains fairly constant, but it almost never increases over the control (Lindenmayer and Schoen, 1967). These observations suggest that branching frequency is under an independent physiological control, while branch density is a dependent variable determined jointly by the local branching frequency and hyphal growth rate. In other words, a leading hyphae seems to be determined to branch periodically, irrespective of what the growth rate happens to be; this results in fewer branches per unit length when the growth rate is high (Figures 9 and 12), or in more branches per unit length when the growth rate is low (Figures 8 and 10)(Lindenmayer and Schoen, 1967). This effect is especially striking in the case of azauracil (Figure 3).

All the compounds that inhibited perithecial formation, except cyanide, also reduced the branching frequency, and all the compounds which did not reduce the branching frequency did not inhibit the formation of perithecia (viz. azauracil, azaadenine, azaguanine, FU, azide, dinitrophenol,

thiourea, and 50 g/l casein hydrolysate in the former case, and 2-thiouracil, 6-thioguanine, N-6-benzyladenine, 6-mercaptopurine, and p-fluorophenylalanine in the latter case). The experiment with cyanide had an unusually low branching frequency for the control (Table III), which may account for the exception. Not every compound that was a good inhibitor of branching frequency was also a good inhibitor of perithecial formation, however, since, for instance, actinomycin D, puromycin, and cycloheximide lowered the branching frequency, yet exerted only a weak inhibition of perithecial initiation. In other words, all good perithecial inhibitors were found to be good branching inhibitors, but not all branching inhibitors were good perithecial inhibitors (Lindenmayer and Schoen, 1967). It seems, therefore, that some physiological factor(s), which is (are) necessary for the production of perithecia, is (are) also needed for the maintenance of a normal rate of branching, but that some additional factor(s) which are necessary for branching are not necessary for perithecial formation (Lindenmayer and Schoen, 1967).

B. Dose-Response Relationships

1. The Effect of 5-Fluorouracil on Perithecial Density

Figure 30 compares the effect of FU on perithecial density with that on hyphal growth rate, branch density,

and residual dry weight accumulation in liquid culture. It is seen that the perithecial density is more sensitive to FU than is liquid culture growth at the concentrations shown, It will be recalled that at concentrations of FU below 1×10^{-7} M, the perithecial density was frequently found to be stimulated; the reason for this is not understood (see next paragraph). The other parameters were not studied at these lower concentrations.

A subjective observation was that the perithecia in the presence of FU were less randomly distributed than in the controls. They also tended to appear first near the inoculum, and later at successively greater distances from the inoculum, whereas in control plates the perithecia usually arose either over the whole plate simultaneously, or else near the edge of the plate first, and later nearer the inoculum. Also, in the controls, there was frequently a zone around the inoculum that had a greatly reduced perithecial density. While the fields used for counting were chosen in such a way as to minimize the effects of such differences in perithecial distribution, there is a possibility that the "stimulation" at the lower FU concentrations (below 1×10^{-7} M) was an artifact resulting from these differences. Computation of the coefficient of variation (the standard deviation as a percentage of the mean) for each plate did not, however, reveal any pattern of

differences between control and FU. In most plates, for controls as well as FU, the variance exceeded the mean, indicating that perithecia were not distributed randomly, but rather were clumped (see Section II.F).

2. The Effect of 5-Fluorouracil on Growth in Liquid Culture

While we were initially surprised that FU caused considerable inhibition of liquid culture growth even though the linear growth on agar was only slightly inhibited, upon further consideration we realized the importance of the branch density to the overall mass increase of a culture. Since the growth of a fungal mycelium, especially a young culture, is almost entirely restricted to the hyphal tips (Robertson, 1965), it is clear that the number of hyphal tips can play a major role in determining the growth of the colony as a whole. Furthermore, most of the hyphal tips produced in a young mycelium result from the subapical branching that we described earlier.

Whether or not filamentous fungi have an exponential phase of growth, comparable to that seen in unicellular organisms, is still uncertain, but evidence is accumulating that young cultures of at least some filamentous fungi can have an exponential growth phase under certain growth conditions (Borrow et al., 1964; Gillie, 1968; Trinci, 1969).

A model can be constructed which describes this situation and which takes into account the unique form of growth seen in filamentous fungi, namely the limitation of growth to the hyphal tips. The model is modified from Buchanan (1953). We assume that all hyphal tips grow at the same rate (or that there is an average growth rate for all the tips), that this rate remains the same throughout the experiment, and that measured growth is due entirely to growth by the hyphal tips. We also assume that the proportion, p, of the mycelium that is growing (i.e., that is represented by tips) is the same throughout the experiment. If y is the total amount of growth at any time, t, then py is the total quantity of hyphal tip at that time, and the growth rate, $\underline{dy}/\underline{dt}$, is proportional to py, viz.:

$$\frac{dy}{dt} = k(py)$$

where k is the proportionality constant and is presumably related to the hyphal tip growth rate. Integration gives

$$y = y_o e^{kpt}$$

where y_o is the amount of growth when t = 0.

It is clear that an inhibitor which does not reduce k will still reduce the growth rate if it lowers the proportion of tips in the mycelium. It is also clear that a reduction in the branch density will also result in a reduction in p, provided that there is no simultaneous increase

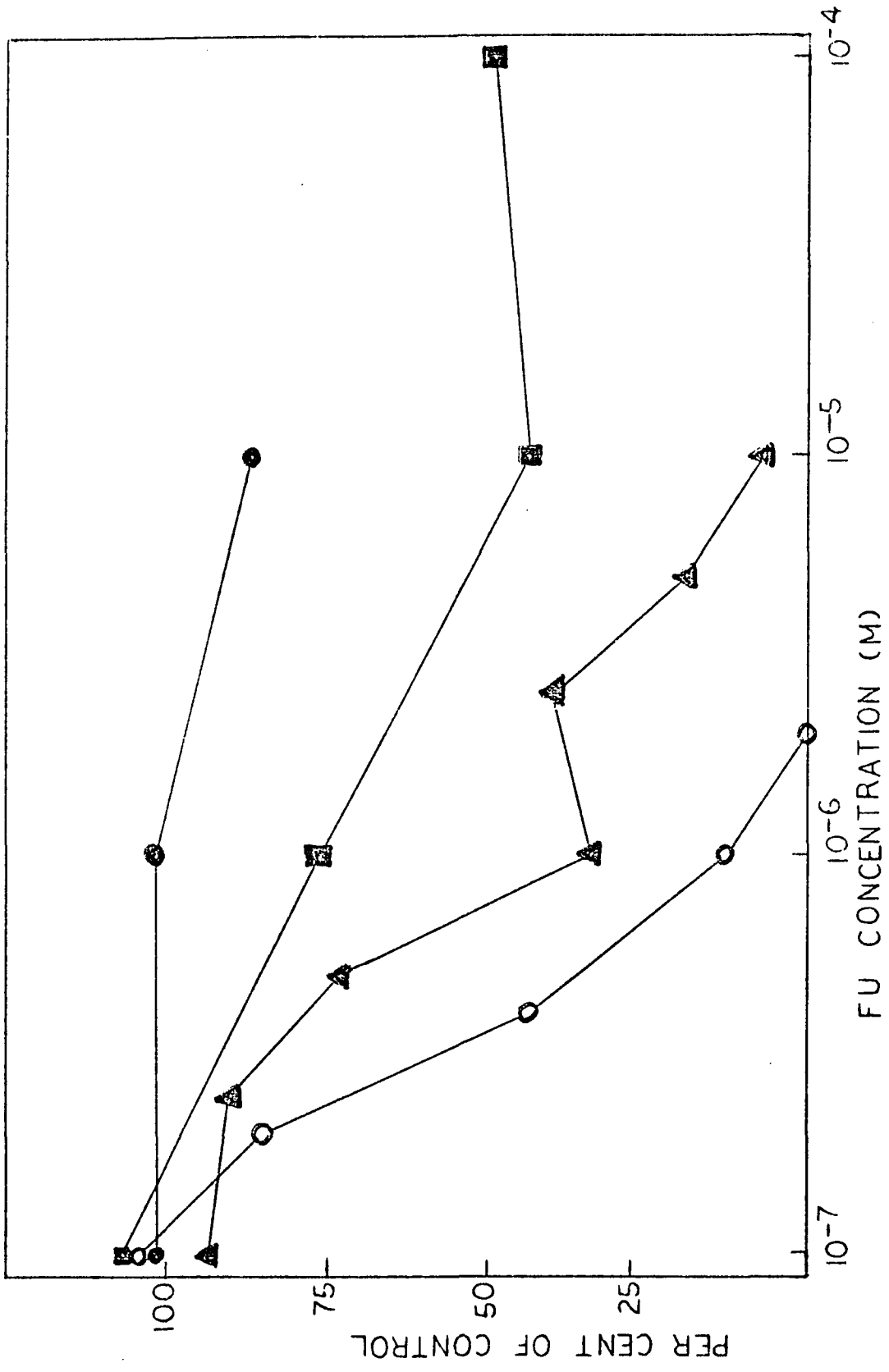
in the number of branches which continue to grow once formed (Trinci, 1969).

Trinci (1969) has attempted to relate branch density which specific growth rate, but to date there is, unfortunately, no treatment which precisely relates the various parameters: linear growth, branch density, hyphal density, specific growth rate, etc.

A graph comparing the effect of FU on branch density, hyphal growth rate, and liquid culture growth (residual dry weight) is shown in Figure 30. It is seen that, indeed, there does seem to be a relationship between branch density and liquid culture growth, although the curve for growth falls more sharply, especially after 5×10^{-7} M FU, than the curve for branch density. At 1×10^{-5} M FU the hyphal growth rate is also reduced somewhat, but this does not have any obvious effect on liquid culture growth.

The fact that the curve for liquid culture growth declines more sharply than that for branch density does not necessarily mean that the model described is incorrect or that p is not directly proportional to the branch density which was observed on agar, since in general the data for liquid culture growth were obtained after the period of exponential growth was over and thus are, strictly speaking, outside the scope of the model. Thus, even if the model is correct, and even if p is directly proportional to branch

Fig. 30. Hyphal growth rate and branch density (from Table I), residual dry weight (from Figure 29), and perithecial density (surface total, from Figure 16), as a percent of control, vs. FU concentration. ●, hyphal growth rate; ■, branch density; ▲, residual dry weight; o, perithecial density.


PER CENT OF CONTROL
100
75
50
25
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
FU CONCENTRATION (M)

density, we do not expect the curves for per cent inhibition of liquid culture growth and per cent inhibition of branch density to coincide. Without a knowledge of the specific growth rates (see Section III.B.3.d) it is not possible to test the model further.

3. The Effect of 5-Fluorouracil on the Accumulation of Protein, RNA, and DNA

Figure 29 indicates that the accumulation of protein, RNA, and DNA, as well as residual dry weight, is inhibited by FU. One would like to know which of these parameters are inhibited directly by FU and which are inhibited indirectly. In the long term incubations that we employed, secondary effects are to be expected, and such effects may even have unexpected tertiary effects on the synthesis of those components which are also inhibited directly. For example, a direct inhibition of ribosomal RNA synthesis may result in the failure to accumulate ribosomes, which may in turn result in a reduced level of protein synthesis, which may in turn have inhibitory effects on RNA synthesis in general, and so forth. (Aspects of this general problem are discussed by Brock [1966]).

As discussed previously, in other organisms, FU has been shown to inhibit directly the synthesis of RNA and of DNA, but not of protein. In longer term experiments

protein is also inhibited indirectly due to failure to synthesize ribosomes. Thus, in our experiments, which did involve long incubations, one might expect FU to inhibit the accumulation of all three macromolecular components to a similar extent. This indeed does appear to be the case at FU concentrations between 1×10^{-6} and 1×10^{-5} M. However, between 1×10^{-7} and 5×10^{-6} M, RNA accumulation appears to be more severely inhibited than does protein or DNA accumulation.

The shapes of the dose response curve in Figure 29 are atypical. In the typical log dose-response curve, one commonly sees a sigmoid curve, with shallow regions at the end and a steep decline in between (Goldstein et al., 1968, Chapter 1). In Figure 29 the protein curve shows instead a fairly linear decline over the entire range of FU concentrations tested. The DNA curve is approximately horizontal between the two lowest concentrations and then it too shows a fairly linear decline over the remaining concentrations. The RNA curve (and the residual dry weight curve) both seem to be composed of two separate sigmoid curves connected by a plateau between 5×10^{-7} and 2.5×10^{-6} M FU for RNA and between 1×10^{-6} and 2.5×10^{-6} M FU for residual dry weight. In view of the various secondary and tertiary alterations in metabolism that may be caused by long incubation with FU, the finding of such "atypical" curves is not

surprising.

It should be noted that measurement of the levels of macromolecular components tells us little about the actual rates of synthesis of these components. Although during exponential growth in microorganisms there is, in general, quantitatively little breakdown of macromolecular components (e.g. Venkov et al., 1972), and consequently measures of increases in the levels of macromolecular components give a good idea of the rates of synthesis, this situation does not commonly obtain during growth under more restrictive conditions. Thus, in starving filamentous fungi, yeast, green algae, and cellular slime molds, protein and RNA synthesis may continue in the face of declining levels of total protein and RNA (Turian, 1966; Sussman and Sussman, 1969; Jones, 1970; Mandelstam, 1971; Venkov at al., 1972). The situation with regard to DNA has not been well studied in filamentous fungi, but one may suppose that there will be DNA degradation in older, senescing portions of the mycelium, while DNA synthesis continues in younger, growing portions or in differentiating and growing fruiting bodies.

C. Prevention of 5-Fluorouracil Inhibition with Pyrimidines and Pyrimidine Derivatives

1. On the Mode of Action of 5-Fluorouracil

There are several mechanisms by which a compound

can prevent an inhibitor from acting. The following ones have been reported for the prevention of FU inhibition. (1) Inhibition of uptake. Kempner (1961) showed that uracil inhibits the uptake of FU by yeast cells. No other possible antagonists of FU were investigated by Kempner (1961), but Polak and Grenson (1973) showed that the uptake of 5-fluorocytosine by yeast can be inhibited by cytosine (and by adenine and hypoxanthine), but not by uracil or uridine. (2) Inhibition of anabolism to the active derivative. Although the precise locus of action was not determined, clear evidence was presented by Kempner (1961) that the conversion of FU to a FU-nucleotide is inhibited by exogenously supplied uracil. Polak and Scholer (1973) were unable to find any affect on anabolism of fluorocytosine by exogenously supplied uridine. (3) Inhibition of incorporation into RNA. Transfer of radioactive label from the FU-nucleotide to RNA was also suppressed by exogenously supplied uracil in Kempner's (1961) study, although the degree of suppression here was smaller than the suppression of uptake and of anabolism to the nucleotide. In Polak and Scholer's (1973) study, exogenously supplied uridine severely suppressed the incorporation of labelled fluorocytosine into RNA, even though there was no effect on uptake or conversion to nucleotide. Since FU is incorporated into RNA only as 5-fluorouridylic acid residues (Heidelberger, 1965; Polak and Scholer, 1973),

presumably only those compounds which are ultimately converted to uridine triphosphate or an analog thereof can prevent FU inhibition by this method. (4) Bypass of the block in thymidylic acid synthesis. Numerous examples exist in the literature for the prevention of FU inhibition by supplying the cell with an alternate source of thymidylic acid, usually thymine or thymidine (Heidelberger, 1965; Reich and Mandel, 1966; Ullman et al., 1972, Hahn and Mandel, 1974).

Of the above four methods, the first two potentially and the third exclusively, concern the effect of FU on RNA synthesis. On the basis of our data it is not possible to distinguish which of these three mechanisms is involved for any particular preventive.

The greater preventive effects of uracil and uridine, compared with cytosine and cytidine, could be due to the action of the former two compounds by all three mechanisms, while the action of the latter two is restricted to methods (2) and/or (3), presumably after being deaminated to uracil or a uracil derivative.

The situation with regard to deoxyuridine and deoxycytidine is less clear. They may act by somehow reversing the block in thymidylic acid synthesis, possibly by raising the endogenous levels of deoxyuridylic acid. However, the block in thymidylic acid synthesis would by itself by expected

to cause the endogenous level of deoxyuridylic acid to rise, and it is doubtful that any exogenously added compound would raise this level much further. We are not aware of any evidence that FU inhibition is ever overcome in this manner in vivo. We feel, therefore, that deoxyuridine and deoxycytidine act by being ultimately converted to uracil or uridylic acid, rather than deoxyuridylic acid, and act through method (2) or (3).

Thymine would be expected to act by method (4). Similarly, bromouracil, which in some organisms is incorporated into DNA in place of thymine without severe effects on DNA function (Brockman and Anderson, 1963) might also, along with bromodeoxyuridine, be expected to act this way. In N. crassa, however, there are no known enzymes for thymine anabolism (Fink and Fink, 1962; O'Donovan and Neuhard, 1970). Thymine and thymidine are, however, converted in vivo to uracil (Fink and Fink, 1962; O'Donovan and Neuhard, 1970; Liu et al., 1973). If this is the case in S. fimicola, then thymine could not act by method (4), and would presumably act by method (2) or (3). Bromouracil and bromodeoxyuridine could presumably also act this way if they were debrominated (Brockman and Anderson, 1963). But if thymine, bromouracil, and bromodeoxyuridine can be converted to uracil, then one would expect them to prevent inhibition caused by azauracil also. As shown in Table VII, however, these compounds are

much less effective in preventing azauracil inhibition than FU inhibition (Table V). This suggests that at least part of the preventive effects of these compounds with FU is due to the bypassing of the block in thymidylic acid synthesis [method (4)]. (If the enzymes for thymine anabolism are indeed present, it is surprising that the analogs trifluorothymine and azathymine do not inhibit growth or perithecial production [Section III.A.1]. On the other hand, if azathymine were converted to azauracil, it should also have an inhibitory effect. Azathymine and trifluorothymine did have a slight preventive effect with FU [Table V].) Additional possibilities for the preventive effect of thymine, bromouracil, and bromodeoxyuridine are antagonism of the uptake or anabolism of FU (and doing so more effectively than that of azaurazil) and inhibition of catabolism of FU to fluoroacetic acid-inhibition by thymine of the early steps of FU catabolism has been reported (Brockman and Anderson, 1963).

In the experiment shown in Table VIII, where pairs of preventives were used, it was noted that some pairs gave better prevention than the individual preventives, viz., at 5 days, thymine plus uridine, and, at 15 days, thymine plus uridine and deoxyuridine plus uridine. This too suggests that the inhibition of both RNA and DNA synthesis is involved in the inhibition of growth by FU. It may, however,

merely indicate that the conversion of the members of each pair to uridine triphosphate occurs via different pathways, and that the ultimate endogenous level of uridine triphosphate achieved is greater when both pathways are utilized. (We have already mentioned that deoxyuridine and uridine likely act by the same method.)

If FU inhibition does indeed involve inhibition of both RNA synthesis and DNA synthesis, then this of course would mean that at least a portion of the inhibition observed at 5.0×10^{-6} M FU is caused by this block in thymidylic acid synthesis and its attendant inhibition of DNA synthesis. Whether this also would play a role at lower FU concentrations, where branch density and perithecia formation are still inhibited, is not known. (DNA accumulation is inhibited at the lower concentrations but this could be due to secondary effects.)

2. Inferences about Pyrimidine Interconversions in S. fimicola

In view of the paucity of data on pyrimidine interconversions in fungi, it seems worthwhile to attempt a synthesis of what the data from Section III.B.4 may tell us about such reactions in S. fimicola, in spite of the fact that conclusions from this type of data can be only tentative without supporting enzyme and genetic data and

experiments with labeled precursors.

Starting with the experiment on preventives of azauracil-caused growth inhibition (Table VII), it will be recalled that all the preventives tried were successful to come degree in preventing growth inhibition. Since azauracil is believed to act by inhibiting de novo pyrimidine synthesis, this implies that either all these compounds are capable of ultimately being converted to a uracil ribonucleotide, at least to some degree, or that they interfere with the uptake or anabolism of azauracil itself. On the basis of our data there is no way of clearly distinguishing between these two possibilities.

Since azauracil inhibits by blocking the de novo synthesis of pyrimidines, those compounds which are converted to a uracil compound past the block (e.g. uridylic acid) to only a very slight degree may nevertheless have a large effect on allowing growth to proceed. On the other hand, FU is converted to 5-fluorouridine triphosphate and is subsequently incorporated into RNA. Uridine triphosphate formed from potential preventives will thus have to compete with the 5-fluorouridine triphosphate for incorporation and we therefore can expect that high levels will be required for the competition to be successful. In addition, uridine ribonucleotides presumably continue to be made de novo in the presence of FU (although possibly at a reduced rate, since

anabolites of FU exert feedback inhibition on de novo synthesis of uridylic acid [Heidelberger, 1965]) and hence additions to this pool resulting from transformation of exogenous pyrimidine may not have a great effect. Thus, we assume that prevention of FU inhibition is indicative of a major conversion of the preventive to a uridine ribonucleotide. Complications in this interpretation are the possible relief of the thymidylic acid block by certain preventives and the inhibition of the uptake and anabolism of FU by the preventive. It should also be borne in mind that differences in the effectiveness of the preventives may be due to differences in the rate of uptake of the preventives themselves.

Keeping in mind the reservations discussed above, we draw the following conclusions regarding pyrimidine interconversions in S. fimicola:

1) Uracil is converted readily to uridylic acid. This is based not only on the ability of uracil to prevent FU inhibition, but also on the ability of FU itself to inhibit growth, since in general it has been observed that the same enzymes involved in the anabolism of uracil also work on FU (O'Donovan and Neuhard, 1970).
2) Uridine is converted to uridylic acid, but less readily than is uracil, since uridine was not as good a preventive for FU as was uracil. Whether

the conversion is direct by the action of a kinase or indirect via uracil is not distinguishable by our data.

3) Deoxyuridine is converted to a uridine ribonucleotide, since it was a very good preventive for FU. What the pathway is, is not indicated by the data.

4) Thymine is converted to thymidylic acid and, presumably via uracil, very slightly to uridylic acid. The reasoning for this conclusion was discussed in Section 1. Bromouracil follows a pathway similar to that for thymine (i.e. is converted to 5-bromodeoxyuridylic acid and uridylic acid), except that it is converted to uridylic acid somewhat more readily than is thymine, since it prevented azauracil inhibition more readily than did thymine. Alternatively, the difference in effect between bromouracil and thymine could be due to a greater ability of bromouracil to compete with FU and azauracil in uptake or anabolism.

5) Thymine ribonucleoside appears to be inert, since it had no effect on FU inhibition. It was not tested, however, with azauracil. In N. crassa, endogenously generated thymine riboside is converted to thymine (O'Donovan and Neuhard, 1970;

Liu et al., 1973).

6) Thymidine is converted to a small extent to thymine. This is concluded because of the finding with both FU and azauracil that thymidine was less effective than thymine. Similar considerations apply to the results with bromodeoxyuridine. The possibility exists that thymidine and bromodeoxyuridine are also phosphorylated to thymidylic acid and 5-bromo-deoxyuridylic acid, respectively.
7) Cytosine is converted to a uracil ribonucleotide to a slight degree. What the pathway is, including where the deamination might occur, is not indicated by the data. 5-Methylcytosine is presumably also converted utlimately to a uracil ribonucleotide, following a pathway that combines features of the cytosine and thymine pathways, although it is not understood why it was more effective against azauracil than was cytosine. (Both compounds had the same effect against FU.)
8) Cytidine presumably is converted to a uracil ribonucleotide since it was a good preventive for azauracil, although it was only a slight preventive for FU. Again, the pathway is not known, although, since cytidine was more effective against azauracil than was cytosine, the pathway presumably does not

involve cytosine.

9) Deoxycytidine is converted to both a cytosine deoxyribonucleotide and a uracil deoxyribonucleotide, since it was the sole required pyrimidine deoxyribonucleoside for preventing hydroxyurea inhibition (Table VIII). The details of the pathway are not clear. Since deoxyuridine was not a preventive for hydroxyurea, the conversion of at least part of the deoxycytidine occurs via a pathway that does not involve deoxyuridine. Deoxycytidine may also be converted to a uracil ribonucleotide, since it was a slight preventive for azauracil.

The hypothesized interconversions are summarized in Figure 31. The complete lack of other information concerning pyrimidine metabolism in S. fimicola, plus the additional possibilities of differential effects of uptake of the various compounds and the multiplicity of possibilities for the preventive effects make this scheme very uncertain.

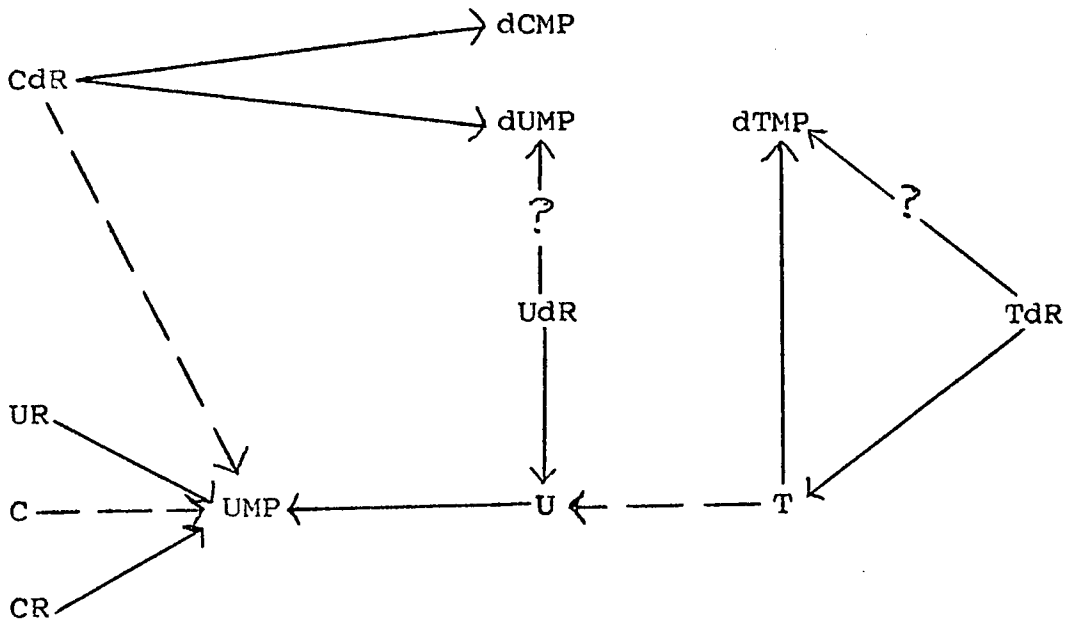


Fig. 31. Proposed scheme of pyrimidine interconversions in S. fimicola, as determined by studies of prevention of FU, azauracil, and hydroxyurea inhibition by various pyrimidines and pyrimidine derivatives. Dashed lines indicate that the proposed reaction occurs only slightly. Arrows connect only those intermediates that are implicated directly by the data (see text); they do not necessarily show the pathways involved. Abbreviations: U, uracil; UR, uridine; UdR, deoxyuridine; UMP, uracil ribonucleotide, presumably uridylic acid when derived from U or UR; dUMP, uracil deoxyribonucleotide; T, thymine; TdR, thymidine, dTMP, thymidylic acid; C, cytosine; CR, cytidine; CdR, deoxycytidine; dCMP, cytosine deoxyribonucleotide.

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