as the resistance of the film to permeation by water vapor. An interesting relationship is observed when $(w/t)^{-1}$ is plotted against thickness of film; although no theoretical basis for such a plot can be proposed at the present time, we feel it is worth presenting. Figure 6 shows such plots for the room temperature permeation of simple films of CAP, CAB, and CAS. Figure 7 depicts the curves obtained for CAHP–PEG 4000 films. Whether these relationships are fortuitous or actual remains to be established with future work, but rather good linear plots are obtained. Of particular interest is that these plots extrapolate to the same point on the abscissa within experimental error. Thus, the virtual resistance of these films at zero thickness could be considered equivalent to the resistance of an additional 0.006 to 0.010 cm. for the propionate, butyrate, and stearate films and to an additional 0.025 to 0.028 cm. for the CAHP–PEG 4000 films.

The physical significance (if any) of the virtual resistance observed is obscure. One possibility might be proposed, however. Other workers have reported that when permeation occurs under a gradient where both sides of the film are exposed to water vapor, permeability constants obtained are greater than when water vapor pressure is zero on one side of the film (5, 6). It is known that cellulose fibers in relatively dry conditions shrink, and that this results in a lower permeability constant (8). Since the external environment of our system was an atmosphere of relative humidity equal to zero, the external surface of the film may be dehydrated and offer a significant resistance to the passage of water vapor. The authors intend to explore this observation in future work.

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Studies on Cell Growth and Cell Division III

Action of Azaserine on Cell Division

By KWAN-HUA LEE and YOKO YUZURIHA

The inhibitory action of azaserine on cell division of heat treated *Tetrahymena pyriformis* in a nutrient-free medium is not reversed by either glutamine or formylglycinamide riboside, but is completely reversed by phenylalanine. These findings support our previous report that azaserine exerts its selective inhibitory action on cell division by some means other than by inhibition of any one of those intermediate steps involved in purine synthesis. Diazo-oxo-norleucine (DON), an antibiotic with a close structural analogy to azaserine, has essentially the same biologic activity on cell growth and cell division. Diazouracil, a pyrimidine derivative, inhibits both cell growth and cell division.

In a previous report (1), it was shown that azaserine (*o*-diazoacetyl-*l*-serine) has a selective inhibitory action on cell division of heat treated *Tetrahymena pyriformis* in a nutrient-free medium after the necessary ribonucleic acid and deoxyribonucleic acid have been accumulated. However, the net result of several studies (2,3) was impressively integrated evidence that azaserine exerted its primary inhibition by blocking the *de novo* synthesis of purine. This was further supported by the findings that glutamine reversed azaserine inhibition in a competitive fashion (4) and that azaserine inhibition could be reversed by formylglycinamide riboside (FGAR) and some purines (5–9). In recent years, a large number of natural amino acids and some of their metabolic products have been reported as reversal agents to the inhibitory action of azaserine in various biologic systems (6, 7, 10–12). These findings, together with the result we reported previously (1), indicate that azaserine may block more than one site or it may act as an antagonist of a metabolite involved in a number of biochemical transformations. In the present study we chose to use glutamine, FGAR, and phenylalanine as typical representatives in the study of their effects on the inhibitory action of azaserine on cell division in heat treated *T. pyriformis* and expect to shed some light on the mechanism of action of azaserine. We also studied the action of DON (6-diazo-5-oxo-1-norleucine) and diazouracil (5-

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diazo-2,4-dihydroxypyrimidine) on cell growth and cell division.

METHODS

T. pyriformis GL was maintained in a proteose-peptone, liver extract fraction L and salt medium as reported before (1), except that ferrous ammonium sulfate was omitted since this salt caused the formation of a black precipitate after autoclaving. Stock cultures were transferred daily with an inoculating loop to 10 ml. of medium in test tubes (18 × 150 mm.) in a slanted position and maintained at 28°. For the experimental culture, 20 ml. of medium in a 300-ml. Erlenmeyer flask was inoculated with 0.03 ml. of a 2-day-old culture (containing about 10^4 cells) and maintained at 28°. At the end of 18 hours, the culture contained about 35,000 cells/ml. and was subjected for temperature treatment in the temperature controlled system described before (14). Each cycle consists of 30 minutes at 34.3°, followed by 30 minutes at 28.5°. At the end of the seventh cycle, the cells were separated in a hand centrifuge and washed three times with salt buffer solution (1). These latter manipulations took about 3 minutes in total. The washed cells were suspended in a suitable amount of salt buffer solution and initial samples for cell count were taken. One milliliter portions of the cell suspension were transferred to a series of 50-ml. round-bottomed centrifuge tubes containing appropriate amounts of testing agents with and without azaserine in salt buffer solution. The final volume of each tube was 2 ml. Salt buffer solution was used in the blank. These tubes were then clamped on a shaking device which has a horizontal movement of 4 cm. displacement at 50 strokes per minute in the temperature controlled water bath at 28.5° for 5.5 hours. At the end of incubation time, 0.6 ml. of formaldehyde reagent was added to each tube, and the cells were counted in a Sedgwick-Rafter counting chamber, using a Whipple ocular micrometer disk (15).

The procedures used in the study of selective activity of DON and diazouracil were essentially the same as described in a previous report (1).

The formylglycinamide riboside (4-amino-5-imidazole carboxamide riboside) used is a product of the California Corp. for Biochemical Research; glutamine, phenylalanine, and diazouracil are prepared by the Nutritional Biochemicals Corp. Azaserine and DON were graciously supplied by Dr. E. L. Wittle, Parke Davis Research Laboratories, Detroit, Mich.

TABLE I.—EFFECT OF FGAR, GLUTAMINE, AND PHENYLALANINE ON THE INHIBITORY ACTION OF AZASERINE ON CELL DIVISION OF HEAT-TREATED *T. pyriformis*

Additions, mcg./ml.		Cell Count, 1,000/ml. Initial	Final
FGAR	Azaserine		
None	None		166.3
None	1.5	69.3	114.8
100	1.5		103.0
200	1.5		101.3
Glutamine	Azaserine		
None	None		245.5
None	1.5	80.1	171.7
200	1.5		167.0
200	None		243.0
Phenylalanine	Azaserine		
None	None		453.0
None	1.5		294.5
25	1.5		341.9
50	1.5	156.8	440.0
100	1.5		472.0
25	None		451.0
50	None		468.0
100	None		448.1

RESULTS

Effect of FGAR.—The reversal effect of FGAR to azaserine inhibition on purine synthesis has been studied in several laboratories (5, 7). It is well established that *T. pyriformis* requires pre-formed purines and pyrimidines (16); however, there is no information on the catabolism or the resynthesis of purine from its metabolic intermediates in these protozoa. For this reason, we studied the possible reversal effect of FGAR to azaserine inhibition. It was found that FGAR failed to reverse the cell division inhibited by azaserine. The results are shown in Table I. The results support the finding that *T. pyriformis* requires pre-formed purines and also indicate that purines in these protozoa are not degradated to simpler metabolites that can be used for synthesis of FGAR or purine. However, in pigeon liver preparations (3, 4) and in *E. coli* (17), it was shown that the primary site of inhibitory action of azaserine was at the step in which glutamine is used to amidinate formylglycinamide ribotide (FGARP) to formylglycinamidine ribotide (FGAMP). Since FGARP is not available, glutamine can be used for testing the above mentioned mechanism of action of azaserine.

Effect of Glutamine.—Azaserine blocks primarily the *de novo* synthesis of purine at the step in which glutamine is used to amidinate FGARP. At higher concentrations, azaserine also blocks the other two steps where glutamine is involved in purine synthesis (4, 5, 8). The ability to nullify azaserine inhibition and the close resemblance of the molecules of azaserine and glutamine have led several workers (3, 18) to believe that azaserine blocks the transfer of nitrogen from glutamine to a purine precursor. It has also been shown that azaserine inhibits the amination of xanthosine-5-phosphate to guanosine-5-phosphate in which glutamine is the donor of the amino group (19). It is possible that glutamine may reverse azaserine-inhibited nitrogen transfer reactions other than those reactions mentioned

TABLE II.—CELL DIVISION INHIBITORY ACTION OF AZASERINE IN THE PRESENCE OF CULTURE MEDIUM

Medium	Cell Count, 1,000/ml. Initial	Final	Final/ Initial
Salt buffer		175.7	3.1
Fresh medium[a]	56.7	306.3	5.5
Original medium with azaserine[b] (2 mcg./ml.)		213.7	3.8
Salt buffer		197.6	3.2
Fresh medium[c]	62.5	357.2	5.7
Fresh medium with azaserine (2 mcg./ml.)		234.1	3.5

[a] Heat treated cells with azaserine added at the beginning of heat treatment. [b] Medium from *fresh medium, footnote a*. [c] Heat treated cells with no azaserine added at the beginning of heat treatment.

above. Since the biologic system we used does not involve purine synthesis, it is a suitable system to test other possibilities. It was found that glutamine, even at higher concentrations failed to reverse azaserine inhibition on cell division. The results are shown in Table I.

Effect of Phenylalanine.—A large number of natural amino acids have been reported to be reversal agents to azaserine inhibition; phenylalanine was reported, in some biologic systems, to be a more potent reversal agent than glutamine (6, 7, 13). This fact suggests the possibility that phenylalanine may reverse azaserine inhibition on a site where glutamine is not involved. We found, indeed, that phenylalanine reversed completely the inhibitory action of azaserine on cell division in *T. pyriformis*. The results are shown in Table I.

Cell Division Inhibitory Action of Azaserine in the Presence of Culture Medium.—The reversal action of phenylalanine raised the question of whether the culture medium used has any reversal activity on the inhibitory effect of azaserine. To test this, the following experiment was performed. Azaserine was added to a culture of *T. pyriformis* at the beginning of temperature treatment (2.0 mcg./ml.), and the culture medium was separated at the end of the seventh temperature cycle; the heat treated organism was collected and washed with salt buffer. Aliquots of these heat treated cells, with known cell count, were then introduced to a series of centrifuge tubes and centrifuged. The supernatants were carefully removed. Measured portions of (*a*) original medium used in heat treatment, which contained 2 mcg./ml. of azaserine added at the beginning of heat treatment, (*b*) fresh culture medium, and (*c*) salt buffer were added to the series of tubes containing heat treated cells separately. These samples were then incubated at 28° for 5.5 hours. At the end of the incubation period, the cell counts of all samples were taken. A parallel control, without the addition of azaserine, was carried out at the same time. The culture medium of the control was also separated at the end of the seventh temperature cycle and the heat treated *T. pyriformis* was collected and washed. The cell division of these heat treated cells were studied essentially in the same way as described above in (*a*) fresh medium with the addition of azaserine, 2 mcg./ml. final concentration, (*b*) fresh medium, and (*c*) salt buffer. It was found that the cell counts of samples containing azaserine were practically the same and both were about 65% of that of the control. These results definitely indicated that the cell division inhibitory action of azaserine can be demonstrated in the presence of culture medium. It also suggested that azaserine is very stable during temperature treatment even in the presence of *T. pyriformis*. These experiments, with samples in duplicate, have been repeated three times, and the results obtained were practically the same. The results of a representative experiment are shown in Table II.

Selective Activity of DON.—DON is another antibiotic isolated from a streptomyces with a close structure analogy to azaserine. Both DON and azaserine are potent inhibitors of sarcoma 180, but with different inhibitory capacities. In the present study we intended to see if there was a difference in biologic action between these two antibiotics, and we found that DON acted essentially in the same way as azaserine. The results are shown in Fig. 1.

Effect of Diazouracil on Cell Growth and Cell Division.—Diazouracil is an analog of both uracil and thymine. It may also be considered as an alkylating agent. Diazouracil inhibited both cell growth and cell division. The results are shown in Fig. 2.

DISCUSSION

In an earlier report (1), we were able to demonstrate for the first time with experimental evidence the selective inhibitory action of azaserine on cell division in *T. pyriformis*. It should be mentioned again that the protozoa we used does not synthesize purine and is suitable to study the actions of azaserine and its inhibitory action on purine synthesis. It is a system in which cell growth is not involved, so any inhibitory action of drug on heat treated *T. pyriformis* in a nutrient-free medium is most probably on cell division only.

There is no doubt that azaserine, in several biologic systems, exerts its action on the *de novo* synthesis of purine at the steps whereby glutamine is involved (4, 5, 8). The competitive relationship of azaserine and glutamine in pigeon-liver enzyme system (4) and the reversal effects of glutamine and FGAR on azaserine inhibition in microorganisms (5–9) further support this proposed mechanism of action. The hypothesis of inhibition of purine synthesis by azaserine may explain its action on the overall

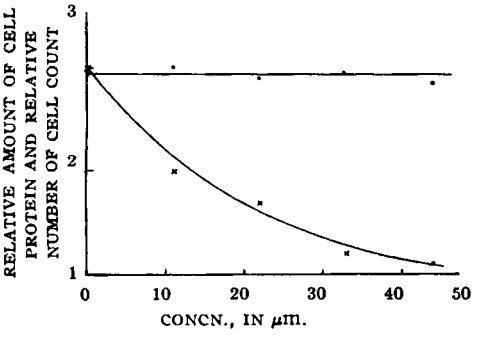

Fig. 1.—Effect of various concentrations (μM. per liter) of DON on cell protein and cell count. Key: ●, relative amount of cell protein at the end of heat treatment period in nutrient medium; X, relative cell count 5.5 hours after heat treatment in nutrient-free medium.

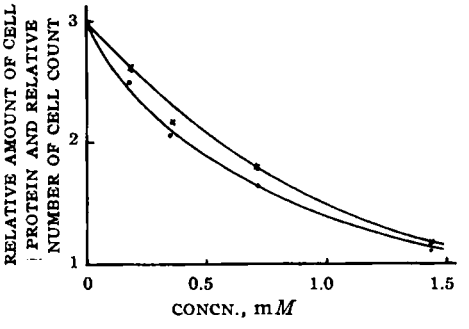

Fig. 2.—Effect of various concentrations (m*M* per liter) of diazouracil on cell protein and cell count. Key: ●, relative amount of cell protein at the end of heat treatment period in nutrient medium; X, relative cell count 5.5 hours after heat treatment in nutrient-free medium.

growth of microorganisms tested in mass cultures, but it does not explain the inhibitory action of azaserine on cell division of heat treated *T. pyriformis*, since these protozoa require pre-formed purines. Formylglycinamide riboside does not reverse azaserine inhibition on cell division in *T. pyriformis* which suggests that purines, if ever metabolized, do not catabolize beyond FGAR stage or the protozoa do not resynthesize purine from their metabolic products.

Gots and Gollub (6) failed to reverse azaserine inhibition by using naturally occurring purines in a purine requiring mutant strain of *Escherichia coli* and concluded that inhibition of purine biosynthesis is not sufficient in itself to account for the inhibition of growth.

It has been shown that azaserine inhibits the amination of xanthosine-5-phosphate to guanosine-5-phosphate by glutamine (19). This finding suggests that azaserine may inhibit other nitrogen transfer reactions in which glutamine is involved. In some cases, the glutamine-sparing mechanism has been applied in explaining the reversal effects of amino acids and other related compounds. It is not surprising that glutamine does not reverse the inhibitory action of azaserine on cell division in *T. pyriformis*, since glutamine has also been found without reversal effect in several other biologic systems (20–22). In some cases, an actual increase in glutamine pool by the addition of azaserine has been reported (7, 23).

Inhibition of transamination reaction in *Scenedesmus sp.* (23) has been suggested, but it was not reproduced in the cell-free preparations of *Azotobacter agilis* (24). Pyridoxal phosphate and several of its analogs do not affect azaserine inhibition as one would expect if inhibition of transamination reaction is involved (13).

The reversal effect of phenylalanine is of interest. From structural considerations, it would be difficult to assign to azaserine the role of a competitive inhibitor of phenylalanine.

It is possible that inhibition is brought about not by azaserine, but by a metabolic product of serine. This idea finds support in the observations that during inhibition of yeast systems, azaserine disappeared, and serine, a possible product of azaserine catabolism, appeared (12). Leucine, which prevented azaserine inhibition, also prevented the catabolism of azaserine. Azaserine was also found to be deaminated in mouse liver preparation (10) and in bacteria (13). Whether phenylalanine has anything to do in azaserine metabolism remains to be proved.

The possibility of a strictly chemical effect on the stability of azaserine was ruled out by Tomisek, *et al.* (7).

A hypothesis of lessened cell permeability toward azaserine in the presence of a reversal agent was shown to be inapplicable in *E. coli*, although an active transport of azaserine mechanism was observed for ascitic cells (25), neoplastic plasma cells, and lymphocytes (26). Whether phenylalanine has an effect on the permeability of *T. pyriformis* to azaserine remains to be studied.

The actual explanation of the reversal effect of phenylalanine is not available. It appears to act by preventing the inhibitory action of azaserine on cell division rather than by compensating for the inhibition. It is justified to suggest that a demonstration of the reversal effect of phenylalanine to azaserine inhibition in biologic system probably is related to cell division mechanism.

That azaserine does inhibit cell division in culture medium at 28° strongly supports the validity of the findings we reported earlier (1). The present results indicate that the failure of azaserine to inhibit cell growth in culture medium at the concentration it inhibits cell division in nutrient-free buffer is not due to any reversal agent present in the culture medium. It should be mentioned that when the selective activity of any drug on cell growth and cell division is to be tested with the system we described (1), one must first test the possible interference of the drug and the components in the culture medium.

The inhibitory actions of DON and diazouracil further demonstrate the usefulness of the biologic system we described (1) in the study of drug actions.

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