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THE RESPONSE OF *CANDIDA UTILIS* AND *ESCHERICHIA COLI* TO 4-AMINOPYRAZOLO(3,4-*d*)PYRIMIDINE*

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Abstract—The inhibition of growth of *Candida utilis* by 4-aminopyrazolo(3,4-*d*)-pyrimidine (4-APP) has been observed in simple glucose-nitrate and glucose-ammonia media. Effective inhibition by the antimetabolite may be obtained at concentrations as low as $5{\cdot}4 \times 10^{-6}$ M. The inhibition can be overcome by adenine, adenosine, hypoxanthine, thiamine, or pyridoxine. The rates at which these metabolites act lead to the proposal that the primary site of action of the inhibitors is in purine metabolism. Spectrophotometric data indicate that no measureable alteration or oxidation of 4-APP by *C. utilis* or *Escherichia coli* occurs.

IT HAS BEEN OBSERVED by Skipper *et al.*[1] and by Hsu *et al.*[2] that 4-aminopyrazolo-(3,4-*d*)pyrimidine (4-APP) can serve as an effective inhibitor of certain types of mouse carcinoma. These findings prompted an investigation of the effect of 4-APP on several microorganisms. While growth of Neurospora mutants,[3] *Lactobacillus arabinosus*,[4] *Candida albicans*,[5] and *Escherichia coli*[6] is inhibited, the response to 4-APP in the presence of purines, their ribosides, and some vitamins has been varied.

This paper deals primarily with the results of studies on *Candida utilis*, the response of which 4-APP is different from those organisms studied by other groups. Since *C. utilis* requires only glucose and inorganic salts for growth, both glucose-nitrate and glucose-ammonia broths were used in order to determine the effect of 4-APP on the microorganism. The results of some metabolic studies on *E. coli* are also presented.

MATERIALS AND METHODS

Nutrient medium

C. utilis was grown on glucose-nitrate and glucose-ammonia media containing other salts;[7] *E. coli* was grown on a medium containing 1·2 g NH_4Cl, 10 g glucose 10·7 g K_2HPO_4, 5 g NaCl and 1·2 g KH_2PO_4 per liter.

Growth studies

The growth studies were performed in matched tubes 13 × 170 mm, containing 4·5 ml of nutrient medium and a sufficient volume of test solutions, or 0·9 per cent NaCl, or both, to bring the total volume to 5·5 ml.

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Growth of *C. utilis* was measured on a Bausch and Lomb colorimeter, model Spectronic 20, by recording the optical density at 420 mμ. Readings were taken regularly during the incubation time. The yeast cells were incubated at 27–28°C; *E. coli* was incubated at 34°C.

Spectrophotometric studies

Aliquots of 2 ml of medium were taken, and 0·1 ml of 2·1 N HCl was added to them. After centrifugation, the supernatant fluids were diluted 1 : 4 or 1 : 6·5 with 0·1 N HCl and measured for their absorption in the ultraviolet range with the Perkin–Elmer spectrocord.

Paper chromatographic studies

Aliquots of 0·05 ml of the cell suspension were applied to Whatman no. 1 sheets. Descending chromatograms were run in *n*-butanol : NH_4HCO_3 : H_2O.[8] The purines and their analogs were detected by their absorption in the ultraviolet range.

Test compounds

The 4-APP, adenine, guanine sulfate, xanthine, uric acid, hypoxanthine, uracil, cytosine, thymine, pyridoxine·HCl, riboflavin, folic acid, nicotinic acid, or DL-methionine was added to the nutrient media before autoclaving. Adenosine, adenosine-2′,3′-(mixed)phosphates, adenosine-5′-phosphate, adenosine diphosphate, adenosine triphosphate, glycinamide·HCl, and 4-amino-5-imidazolecarboxamide·HCl were filtered under sterile conditions and added subsequent to autoclaving.

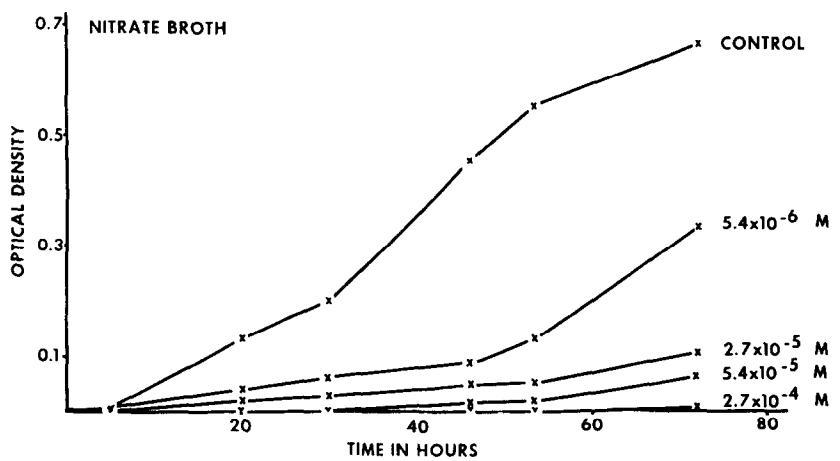

FIG. 1. The effect of various concentrations of 4-APP on the growth of *C. utilis*.

RESULTS

The effect of 4-APP on Candida utilis

Fig. 1 shows the effect of several concentrations of 4-APP on growth of *C. utilis* in both glucose-nitrate and glucose-ammonia broths. It was observed that $5{\cdot}4 \times 10^{-6}$ M 4-APP exerted a significant inhibition of growth during a 72-hr incubation time with both media.

The effect of various compounds on inhibition

Adenine, guanine sulfate, xanthine, uric acid, hypoxanthine, adenosine, adenosine-2′,3′-(mixed) phosphates, adenosine-5′-phosphate, ADP, ATP, glycinamide·HCl, 4-amino-5-imidazolecarboxamide·HCl, guanosine, inosine, thymine, cytosine, uracil, uridine, riboflavin, nicotinic acid, pyridoxine·HCl, pyridoxamine·HCl, thiamine·HCl,

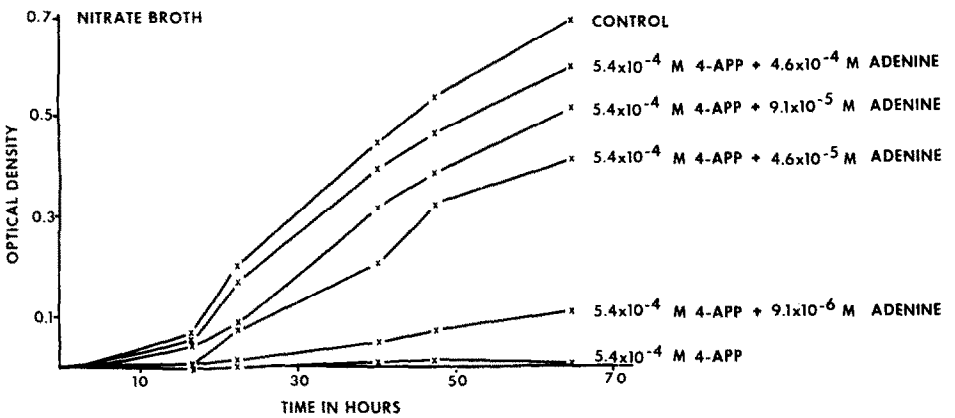

FIG. 2. The effect of various concentrations of adenine on overcoming the inhibition of growth of *C. utilis* in the presence of 4-APP.

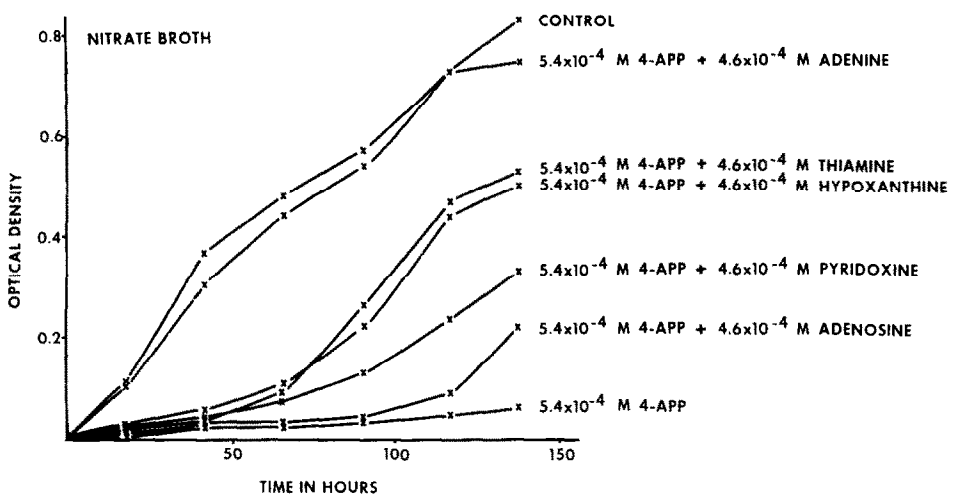

FIG. 3. Comparative effect of several metabolites capable of overcoming inhibition caused by 4-APP of growth of *C. utilis*.

and DL-methionine at a concentration of $4{\cdot}6 \times 10^{-4}$ M were tested individually for their capacity to overcome inhibition caused by $5{\cdot}4 \times 10^{-4}$ M 4-APP on both media. *C. utilis* responded to the presence of adenine, adenosine, hypoxanthine, thiamine. HCl, pyridoxine·HCl, and pyridoxal·HCl. There was an immediate response to $4{\cdot}6 \times 10^{-4}$ M adenine on both glucose-nitrate and glucose-ammonia media containing

$5{\cdot}4 \times 10^{-4}$ M 4-APP (Fig. 2). Significant growth was observed with $4{\cdot}6 \times 10^{-5}$ M adenine in the presence of the same amount of inhibitor.

Adenosine overcame inhibition after an 80- to 90-hr lag in nitrate medium but had much less noticeable effect in ammonia medium* (Fig. 3). Hypoxanthine overcame inhibition in both media after a 70-hr lag phase. During a 70-hr incubation time, the initiation of visible growth in the presence of thiamine·HCl and pyridoxine·HCl (Fig. 3) was apparent on both broths. While pyridoxal also served to overcome inhibition, its effect was not nearly so noticeable as that of pyridoxine. The other vitamins, purines, and pyrimidines did not reverse the inhibition.

Spectrophotometric observations of broths

The data in Fig. 4 show the effect of incubation of *C. utilis* with adenine, 4-APP, and a mixture of the two compounds. There is a shift in the adenine curve corresponding to the formation of hypoxanthine by enzymic hydrolysis. There is no detectable shift in the 4-APP curve. The mixture shows a shift which has been attributed to the formation of hypoxanthine from adenine (Table 1).

TABLE 1. INCUBATION OF *Candida utilis* AND *Escherichia coli* WITH ADENINE, 4-APP, AND A MIXTURE THEREOF

Addition to medium	Rf in *n*-butanol : $(NH_4)HCO_3$: H_2O	
	C. utilis broths*	*E. coli* broths
4-APP before inc.	0·43	0·42
4-APP after inc.	0·43	0·42
Adenine before inc.	0·28	0·29
Adenine after inc.	0·13	0·16
Adenine + 4-APP before inc.	0·42; 0·28	0·42; 0·31
Adenine + 4-APP after inc.	0·42; 0·27, 0·12	0·42; 0·15
Controls		
Hypoxanthine	0·12–0·14	0·15
4-Hydroxypyrazolo-(3,4-*d*)pyrimidine		0·32
4,6-dihydroxypyrazolo-(3,4-*d*)pyrimidine		0·09
4-Amino-6-hydroxy pyrazolo(3,4-*d*)-pyrimidine†		0·09

* The solutions from which the data in Fig. 4 were drawn were lost. The data presented in this table are typical of several experiments in nitrate and ammonia media.

† Blue fluorescence.

Figure 5 shows the data obtained with 24-hr *E. coli* cultures in a similar experiment. These results are analogous to those obtained with *C. utilis*. The results obtained by paper chromatographic treatment of supernatant broths are given in Table 1.

There is no observable shift in the absorption curves when comparisons are made before and after incubation with broths containing 4-APP and pyridoxine, thiamine,

* Whereas the results reported for adenosine in Fig. 3 showed no effect over 100 hr, numerous experiments showed effects varying from small to none.

hypoxanthine, or adenosine. Confirmation of these results was obtained by paper chromatography.

DISCUSSION

By comparison with the microorganisms studied by others,[3, 4] *C. utilis* appears to have the greatest sensitivity to 4-APP. Although *L. arabinosus* is inhibited by 4×10^{-3} M 4-APP, and Neurospora mutants are inhibited by 4×10^{-4} M 4-APP, *C. utilis* is noticeably affected by 5×10^{-6} M 4-APP. Such sensitivity presents advantages in a study of the sites at which 4-APP will act on the subcellular level. The response to the addition of adenine in the presence of the inhibitor was immediate, whereas

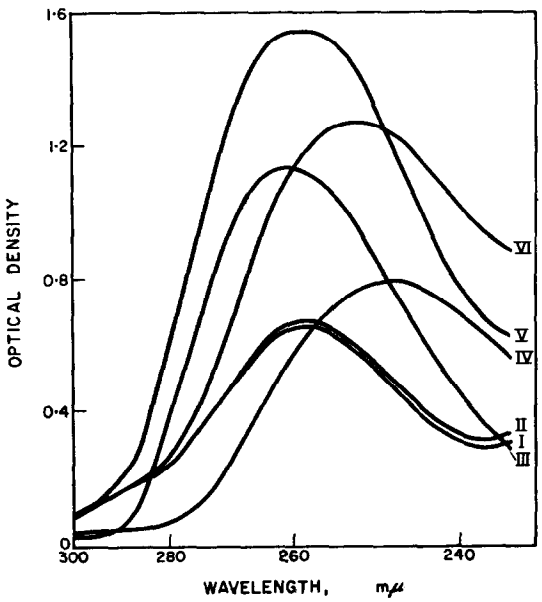

FIG. 4. Spectral curves of *C. utilis* broths containing 4-APP, adenine, or a mixture thereof. Curves I and II represent broths containing 4-APP measured spectrophotometrically before and after incubation with *C. utilis*, respectively. Curves III and IV are corresponding vessels but containing adenine. Curves V and VI contain both adenine and 4-APP.

thiamine, pyridoxine, adenosine, and hypoxanthine did not cause immediate reversal of the inhibition. These data suggest that the primary site of action of 4-APP in *C. utilis* is on purine metabolism, and that inhibition that can be overcome by thiamine or pyridoxine is of a secondary nature. That multiple sites are involved in purine metabolism has been demonstrated by Booth and Sartorelli[9] who observed one locus of inhibition before the biosynthesis of 4-amino-5-imidazolecarboxamide ribotide and another after the synthesis of the latter, but before the synthesis of polynucleotide guanine without affecting polynucleotide adenine in ascites cells. These results in part support earlier observations of Zimmerman *et al.*,[6] who noted that *E. coli* would convert guanine to adenine in the presence of 4-APP at a three-fold rate, whereas the rate of synthesis of guanylic acid from guanine was unchanged. In

contrast to the results of Booth and Sartorelli are those of Bennett *et al.*,[10] who reported significant inhibition of incorporation of adenine into polynucleotide purines by 4-APP and a less significant effect on the synthesis of soluble purines from formate. The effect on synthesis of polynucleotide purine may be due to the formation of 4-APP ribonucleoside, and the mono-, di-, and triphosphates which were observed in neoplastic tissues by Henderson and Junga,[11] who have suggested that the action of the drug may be at the nucleotide stage of the purines, exercising an effect on the nucleotide pool sizes.[12]

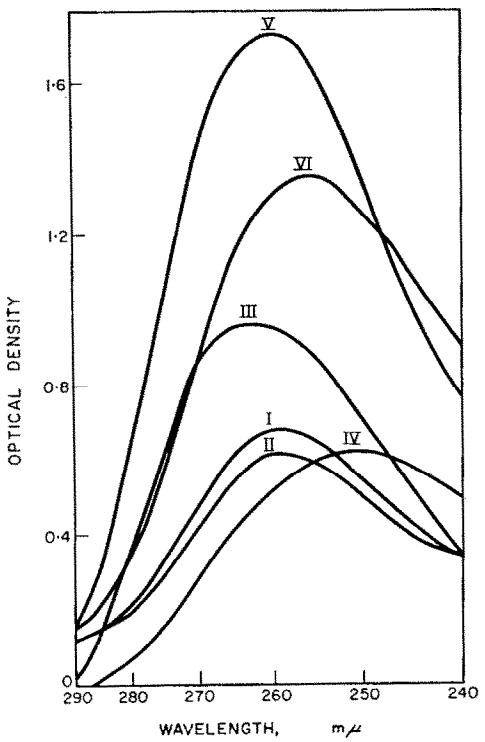

FIG. 5. Spectral curves of *E. coli* broths containing 4-APP, adenine, or a mixture thereof. Curves I and II represent broths containing 4-APP measured spectrophotometrically before and after incubation with *E. coli*, respectively. Curves III and IV are corresponding vessels but containing adenine. Curves V and VI contain both adenine and 4-APP.

A comparison of the response in *C. utilis* with other microorganisms by other investigators reveals a number of interesting points. (1) Purines such as adenine, guanine hypoxanthine, and their ribosides overcome inhibition in *L. arabinosus*, as does 4-amino-5-imidazolecarboxamide.[4] *C. utilis* does not have this broad response to purine derivatives. (2) Thiamine is more effective than adenine in overcoming inhibition of Neurospora.[13] The reverse is true in *C. utilis*. (3) Thiamine has no effect on the inhibition of *E. coli* by 4-APP.[6] Such is not the case with *C. utilis*. (4) Neurospora responds only to adenine of the purines tested,[3] whereas *C. utilis* is capable of eventually utilizing hypoxanthine and adenosine. The reasons for the lag phases in the response to thiamine, pyridoxine, adenosine, and hypoxanthine are as yet unknown. Utilization

of these compounds might involve such possibilities as mutation or adaptation of microorganisms.

Since Feigelson and Davidson[8] observed urinary excretion of 4-hydroxypyrazolo-(3,4-*d*)pyrimidine, 4,6-dihydroxypyrazolo(3,4-*d*)pyrimidine, and 4-amino-6-hydroxy-pyrazolo(3,4-*d*)pyrimidine upon intraperitoneal injection of 4-APP into rats, an investigation into the metabolic fate of 4-APP in *C. utilis* and *E. coli* was undertaken in order to determine whether the 4-APP is metabolized by these microorganisms. It was of interest to note that neither *C. utilis* nor *E. coli* produces these compounds in any detectable quantity. The pathway mediating the conversion of adenine to hypoxanthine in these microorganisms does not act upon 4-APP.

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