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Inhibition of Growth of *Pediococcus cerevisiae* by Polycyclic Hydrocarbon–Cysteine Conjugates[1] (37835)

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In previous reports from this laboratory, the amino acid analogues produced by conjugating polycyclic aromatic hydrocarbons with cysteine have been shown to be activated and incorporated into ribosomal proteins by the protein synthesizing system of rat liver (1, 2).

Bucovaz *et al.* (2) concluded that the amino acid analogues competed with the natural amino acids for incorporation into protein in a mammalian system. The particular amino acid involved varied with the nature of the aromatic moiety of the amino acid analogue. The findings suggested that CPcys[3] was competitive with arginine, THNcys with valine and isoleucine, DHPcys with glutamic acid, phenylalanine, and histidine, BMcys with methionine and leucine, and DHDcys with tyrosine and arginine.

This present study was designed to determine whether the growth of selected bacteria would be inhibited by the hydrocarbon conjugates and whether the effect could be counteracted by the natural amino acids which were competitive in the mammalian system.

Materials and Methods. The syntheses of the amino acid analogues have been described previously as follows: DHPcys and DHDcys (2), THNcys (3), CPcys (4), and BMcys (5).

Cultures were obtained from the American Type Culture Collection and maintained on salts–glucose–agar with appropriate amino acid supplements for mutants or on yeast–peptone–agar medium. *E. coli* strains were grown on a medium described by Anderson (6) with appropriate amino acid supplements. *P. cerevisiae* 8042 was grown on the amino acid medium of Steele *et al.* (7). Inoculation cultures were grown up in 10 ml of medium overnight. The cells were sedimented by centrifugation, and the pellet was washed with sterile saline twice. In preliminary studies, the effect of dimethyl sulfoxide (DMSO) on the growth of the microorganisms was tested. *P. cerevisiae* tolerated up to 2% DMSO in the medium without any effect on growth, but *E. coli* 9723F (phe-) tolerated only 0.4% (Table I).

TABLE I. Inhibition of Bacterial Growth by Dimethyl Sulfoxide.[a]

P. cerevisiae, ATCC 8042		*E. coli*, ATCC 9723F	
DMSO (%)	Inhibition (%)	DMSO (%)	Inhibition (%)
0	0	0	0
1	0	0.4	0
2	0	1	4
3	7	2	11
4	13	3	14
5	87	4	21

[a] Media and growth measurements are described in *Methods*. Incubation time was 5 hr.

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[3] Abbreviations for the hydrocarbon–cysteine analogues are: CPcys, *S*-(*p*-chlorophenyl)-L-cysteine; THNcys, *S*-(1,2,3,4-tetrahydro-2-hydroxy-1-naphthyl)-L-cysteine; DHPcys, *S*-(9-hydroxy-9,10-dihydrophenanthryl)-L-cysteine; BMcys, *S*-(7-benz(a)anthryl)-methyl-L-cysteine; DHDcys, *S*-(5,6-dihydro-6-hydroxy-5-dibenz(a,h)anthryl)-L-cysteine.

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Hence, DMSO was used to solubilize the conjugates only when *P. cerevisiae* was cultured.

For growth tests, the hydrocarbon–cysteine conjugates were dissolved in dilute sodium hydroxide or DMSO and this was added to 5 or 10 ml of a sterile test medium which was modified with respect to the concentration of an appropriate amino acid. A measured suspension of cells in saline was added for an inoculum and the culture was incubated at 37°. Growth was assayed by turbidimetry measured as optical density at 600 nm on a Spectronic 20 Colorimeter. Percentage inhibition was calculated from the difference in the net turbidity developed in the incubation mixture containing the hydrocarbon conjugate and in a control mixture grown simultaneously, divided by the net turbidity of the control times 100.

For studies on protein incorporation, conjugates labeled with ^{14}C were used. At intervals, a 1-ml aliquot of the growing culture was removed and precipitated by addition of 10 ml of 10% trichloroacetic acid (TCA). This mixture was heated at 95° for 20 min. The precipitate was collected on a 0.45-μm Millipore filter disk and washed with 10% TCA five times. Radioactivity measurements were made on the disks by the method of Bollum (8) using a Nuclear Chicago Mark II liquid scintillation counter.

Results and Discussion. E. coli was chosen for the first growth inhibition tests because this organism has been widely used in studies with amino acid analogues.

CPcys was slightly inhibitory to the growth of *E. coli* (wild) and more so to its amino acid-requiring mutants (Table II). *E. coli* mutants phe-, arg-, his-, and cys- were used to avoid the effects of an endogenously synthesized amino acid. The most growth inhibition was seen with the arg- mutant. This correlates with our earlier observation that CPcys competes with arginine in the protein-synthesizing system of mammalian liver (2). Moreover, when the medium was made 4×10^{-4} *M* in cysteine, there was no reversal of the inhibition of growth of the phe- or arg- mutant. Similarly, the lack of inhibition of growth of the cys- mutant is in accord with our previous observations that the cysteine-activating synthetase of liver does not utilize the hydrocarbon–cysteine conjugates. However, inhibition of the phe- and his- mutants by CPcys shows the effects were not specific. Similar lack of specificity has been noted by Shigeura *et al.* (9) who found L-*O*-ethyl threonine, an antagonist to isoleucine in *E. coli,* to be counteracted by leucine and valine. Also, Fenster and Anker (10) found incorporation of trifluoroleucine into peptides was inhibited by leucine, isoleucine, and phenylalanine.

None of the other hydrocarbon–cysteine conjugates, when incubated in media as described in Table II, inhibited the growth

TABLE II. Effect of Chlorophenylcysteine on Growth of *E. coli.*[a]

	Time (hr)				
	1	2	3	4	5
ATCC No.	Inhibition (%)				
9723F (phe-)	—	21	18	39	29
23790 (arg-)	—	27	52	61	—
15996 (his-)	27	23	16	26	29
23792 (cys-)	0	0	0	0	0
B (wild)	0	0	0	11	6

[a] The mutant media were supplemented with the dependent amino acid to 2×10^{-4} *M*. This was sufficient to permit growth in the control mixtures from 0.6 to 0.7 optical density units in 5 hr. The concentration of CPcys was 10^{-3} *M*. Media and inhibition calculations are described under *Methods*.

TABLE III. Inhibition of Growth of *P. cerevisiae* 8042 by Chlorophenylcysteine.

CPcys molarity	Inhibition (%)	Variation in growth medium	
		Amino acid	Molarity
1×10^{-3}	45	L-Phe[a]	6.1×10^{-4}
1×10^{-3}	25	L-Phe	1.8×10^{-6}
1×10^{-3}	10	L-Phe	1.2×10^{-3}
1.5×10^{-3}	54	L-Phe	1.2×10^{-3}
1×10^{-3}	22	L-Arg	8.0×10^{-6}
1.5×10^{-3}	49	L-Arg	8.0×10^{-6}

[a] The standard medium was 6.1×10^{-4} *M* in phenylalanine and 4×10^{-6} in L-arginine (7). These amino acids were reduced in the media to provide limiting concentrations. Incubation procedures are described under *Material and Methods.* Incubation time was 5 hr.

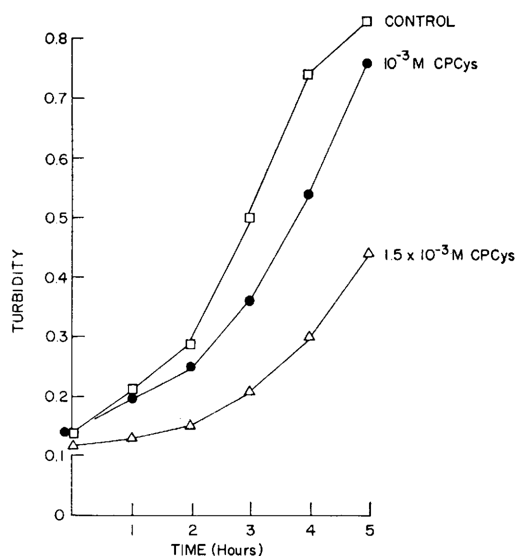

FIG. 1. Inhibition of growth of *P. cerevisiae* by CPcys. The standard medium was fortified by increasing the phenylalanine concentration to 1.2×10^{-3} *M*. Other amino acids were at the standard concentration (7). The effect of variation in phenylalanine and arginine concentration is shown in Table III.

of *E. coli* or its mutants. Also, DHPcys at 10^{-3} *M* had no effect on the 16-hr growth of *E. coli* (wild) or its phe- mutant under the same conditions that β-2-thienylalanine (2×10^{-3} *M*), an antagonist to phenylalanine, completely blocked growth. Similarly, THNcys and BMcys did not affect the growth of the phe- mutant.

The resistance of *E. coli* to growth inhibition by the larger polycyclic hydrocarbons may be related to the size of the residue substituted on the sulfur atom, i.e., the larger residues may be unable to fit enzymes sites in the cell. The solubility of the analogues, which decreases with size of the hydrocarbon moiety, must also be a factor since it limited the effective concentration of the analogue which could be attained in the medium. Moreover, DMSO could not be used with *E. coli.*

It seemed possible that the larger cysteine conjugates might not be entering the cells. However, in one experiment with DHPcys-^{14}C, the conjugate was readily taken up by *E. coli* (phe-) although there was no effect on growth. Moreover, Molinary and Wood have shown that DHPcys readily penetrated resting *E. coli* and was incorporated into β-galactosidase (11).

P. cerevisiae was selected for further tests because of its dependence on a large number of amino acids in the medium. As might be expected, this microorganism was more sensitive to the effects of the hydrocarbon–cysteine conjugates.

Chlorophenylcysteine (CPcys) inhibited the growth of *P. cerevisiae* 45% in the standard medium in which the concentration of arginine was 4×10^{-6} *M* and phenylalanine 1.8×10^{-6} *M*. Figure 1 shows the time course of growth. Table III shows that at double the standard concentration of arginine, the percentage inhibition was reduced from 45 to 22%, but increases in the CPcys level nullified the effect. Reducing the phenylalanine content reduced the apparent percentage growth inhibition because of concommitant reduced growth in the controls. When the phenylalanine content was doubled, there was a reduction of the inhibition from 45 to 10%. Increasing the CPcys to 1.5×10^{-3} *M* restored the inhibition to 54%. Since arginine but not phenylalanine competed with CPcys in the mammalian liver protein incorporation system (2), there is apparently a lack of similar specificity in the bacterial system.

TABLE IV. Uptake of Phenylalanine-^{14}C and DHPcys-^{14}C by *P. cerevisiae* 8042 with Time.[a]

			Time (hr)			
			0.5	1	2	5
Phe	DHPcys	Growth inhibition[b]	% Uptake of radioactive compound			
+^{14}C	—	0	4.4	8.6	12	12
+^{14}C	+	14	4.2	8.1	12	12
—[c]	+^{14}C	35	8.5	12	23	25

[a] The phenylalanine concentration in the medium was 2.2×10^{-5} *M* with a specific radioactivity of 3.8×10^{10} cpm/mole. DHPcys was 10^{-3} *M*. Its radioactivity was 5.2×10^{8} cpm/mole. The optical density of the medium at zero time was adjusted to 0.3 by a heavy inoculation.

[b] Incubation time was 5 hr.

[c] No phenylalanine was provided excepting by the cellular pool. Minimal growth occurred relative to the medium containing 2.2×10^{-5} *M* phenylalanine.

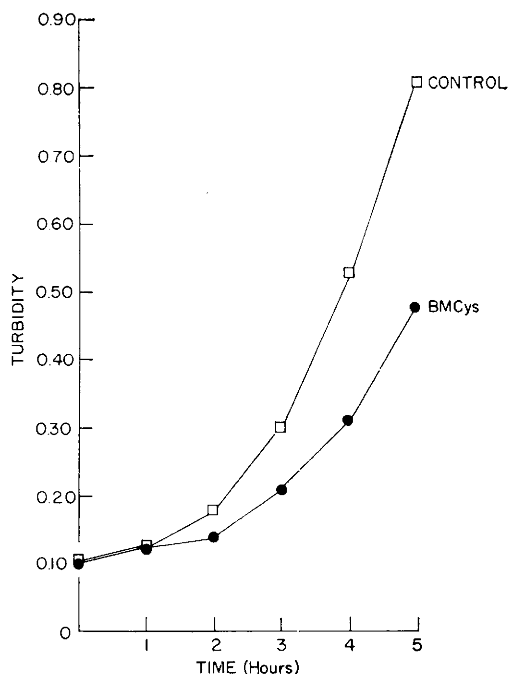

FIG. 2. Inhibition of growth of *P. cerevisiae* by BMcys. The methionine content of the test medium was reduced from the regular $6.8 \times 10^{-4} M$ (7) to $2.7 \times 10^{-4} M$ making it the limiting amino acid. Other amino acids were at the standard concentration (7). The BMcys was $10^{-3} M$ and was solubilized by making the medium 2% in DMSO.

Dihydrophenanthrylcysteine (DHPcys) was only slightly effective in inhibiting the growth of *P. cerevisiae*. In order to determine whether the compound was being taken up by the bacteria, carbon-labeled DHPcys was placed in a phenylalanine-deficient medium. A heavy inoculum was added (Table IV). The phenylalanine pool came into equilibrium within 2 hr, as reflected by radioactivity measurements on the bacterial protein although growth persisted. When DHPcys was added at 45 times the phenylalanine concentration of $2.2 \times 10^{-5} M$, growth was inhibited 14%, but the uptake of phenylalanine was not affected. When the only phenylalanine available in the medium was provided by the bacterial pool, growth was inhibited 35%, but DHPcys was incorporated into protein in twice the amount. Since DHPcys is known to incorporate into proteins of *E. coli* (11), rabbit reticulocytes (12), and rat liver (1, 2), the findings here may be interpreted as similar. In similar experiments, increasing the amount of phenylalanine, up to a $10^{-2} M$ level, did not reverse the growth inhibition. Histidine which is competitive with DHPcys in the liver system, did not reverse the slight growth inhibitory effects of DHPcys on *P. cerevisiae.*

Benzanthrylmethylcysteine was a better inhibitor of growth than DHPcys, but the effect was not reversed by methionine, the competitor in mammalian systems (Fig. 2). Similarly, dihydrodibenzanthrylcysteine (DHDcys) produced a marked inhibition of *P. cerevisiae* when added to the medium (Fig. 3). In each of the last two cases, the solubility of the analogue was a limiting factor in producing the most effective concentration.

It has been noted by Richmond (13) that the characteristic of slow "linear" growth reflects the incorporation of amino acid analogues into bacterial protein rather than an interference with amino acid synthesis (Figs. 1–3). This interpretation of our present results is in keeping with our previous demonstration that hydrocarbon–cysteine analogues are incorporated into ribosomal

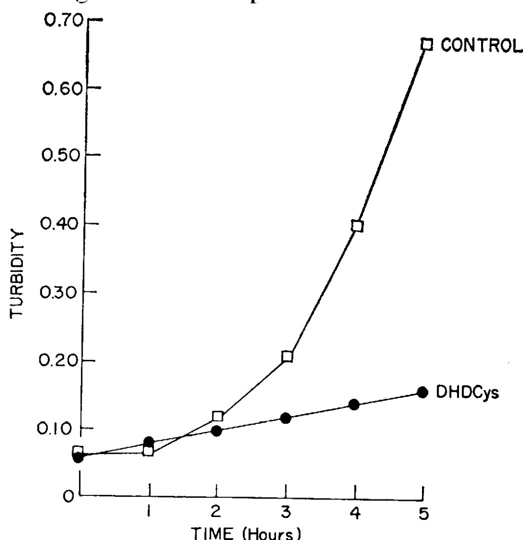

FIG. 3. Inhibition of growth of *P. cerevisiae* by DHDcys. The concentration of DHDcys in the medium was $10^{-3} M$. All amino acids were at the standard concentration (7). The concentration of DMSO was 2%.

protein (2). The analogues used in these studies probably do not interfere with endogenous amino acid synthesis to any significant extent. The lack of complete inhibition in the bacterial system suggests the hydrocarbon conjugates are poor competitors for enzyme systems.

Summary. Polycyclic aromatic hydrocarbons conjugated with cysteine are analogues of natural amino acids and inhibit the growth of *P. cerevisiae*. The inhibitory effects observed were partially reversed by amino acids previously shown to be competitive with the analogues in a rat liver system but with less specificity.

The inhibition of growth of *E. coli* and its mutants was limited to only one analogue, *p*-chlorophenylcysteine.

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1. Bucovaz, E. T., and Wood, J. L., J. Biol. Chem. **239**, 1151 (1964).

2. Bucovaz, E. T., Morrison, J. C., James, H. L., Dais, C. F., and Wood, J. L., Cancer Res. **30**, 155 (1970).

3. Boyland, E., and Sims, P., Biochem. J. **77**, 175 (1960).

4. du Vigneaud, V., Wood, J. L., and Binkley, F., J. Biol. Chem. **138**, 369 (1941).

5. Wood, J. L., and Fieser, L. F., J. Amer. Chem. Soc. **62**, 2674 (1940).

6. Anderson, E. H., Proc. Nat. Acad. Sci. USA **32**, 120 (1946).

7. Steele, B. F., Sauberlich, H. E., Reynolds, M. S., and Bauman, C. A., J. Biol. Chem. **177**, 533 (1959).

8. Bollum, F. J., J. Biol. Chem. **234**, 2733 (1959).

9. Shigeura, H. T., Hen, A. C., Hiremath, C. B., and Maag, T. A., Arch. Biochim. Biophys. **135**, 90 (1969).

10. Fenster, E. D., and Anker, H. S., Biochemistry **8**, 269 (1969).

11. Molinary, S. V., and Wood, J. L., Biochem. Biophys. Res. Commun. **43**, 899 (1971).

12. Frendo, J., and Wood, J. L., Proc. Soc. Exp. Biol. Med. **139**, 173 (1972).

13. Richmond, M. H., Bacteriol. Rev. **26**, 398 (1962).

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