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Mutagenesis by normal metabolites in *Escherichia coli*: phenylalanine mutagenesis is dependent on error-prone DNA repair

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Summary

In search of a model for the production of 'spontaneous' mutations induced by DNA damage produced during normal metabolism, 19 amino acids were tested for mutagenicity in *Escherichia coli* K-12 *uvrB*. Cystine, and, to a lesser extent, arginine and threonine were found to be antimutagenic; only phenylalanine was found to be mutagenic. At 2 mM, phenylalanine induced mutants at 1.5–2-fold above background [*lacZ53*(amber) → Lac$^+$, rifampicin resistance (missense), and bacteriophage T6 resistance]. Tyrosine and, to a lesser extent, tryptophan (each at 2 mM) inhibited the mutagenicity of phenylalanine.

Phenylalanine mutagenesis was detected in the *uvrB* strain, but not in the wild-type, *uvrB umuC* or *uvrB lexA* strains. Thus, phenylalanine seems to cause the production of excisable lesions ('UV-like'?) in DNA, which, if not excised, can induce mutations via error-prone DNA repair.

Mutagenesis in *Escherichia coli* by radiation and by certain chemicals is mediated by cellular functions that are also involved in the repair of DNA damage, hence such mutagenesis is assumed to result from the error-prone repair of DNA lesions (reviewed by Witkin, 1976; Walker, 1984). Genes that are required for error-prone repair include *lexA* (Witkin, 1967), *recA* (Kondo, 1968; Miura and Tomizawa, 1968), and *umuC* (Kato and Shinoura, 1977). The *uvrA* and *uvrB* genes function in the error-free excision repair of many bulky mutagenic lesions. Thus, *uvrA* and *uvrB* mutations cause more of this type of DNA damage to be processed by error-prone repair; the result being more mutagenesis (Witkin, 1966).

The effects of *recA*, *lexA*, *umuC* and *uvrA* or *uvrB* mutations on mutagenesis in *E. coli* by various agents have often been used to define the molecular basis of such mutagenesis, i.e., miscoding or misrepair mutagenesis, excisability of mutagenic lesions, etc. (e.g., Kondo et al., 1970). Similarly, the demonstration in *E. coli* that *uvrA* and *uvrB* mutants show increased levels of spontaneous mutagenesis, and that *recA*, *lexA* and *umuC* mutants show reduced levels of spontaneous mutagenesis allow the conclusions that (i) much of spontaneous mutagenesis causing base-substitution and frameshift mutations is due to error-prone DNA repair, and that (ii) lesions of unknown origin occur in DNA, which, if not excised, will induce mutations (Sargentini and Smith, 1981). The general importance of DNA damage and DNA repair in regulating the rate of spontaneous mutagenesis is substantiated in a recent review of the available data for several species (Sargentini and Smith, 1985).

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What could be the source of the 'spontaneous' DNA damage whose effect on spontaneous mutagenesis is regulated by the error-free and error-prone mechanisms of DNA repair? Spontaneous DNA damage conceivably is produced by a multitude of mechanisms involving normal DNA metabolism (reviewed by Drake et al., 1983), and DNA damage by environmental and dietary mutagens (reviewed by Ames, 1983). Also, endogenous mutagens may arise during normal cellular metabolism (reviewed in Sargentini and Smith, 1985). Since amino acid catabolism should be a common cellular occurrence, in this work we have tested amino acids for mutagenicity, specifically with the goal of finding a type of mutagenesis that is regulated by DNA repair genes.

A preliminary report of part of this work was presented at the UCLA Symposium on Cellular Responses to DNA Damage at Keystone, Colorado, 13 April, 1983 (Sargentini and Smith, J. Cell. Biochem., Suppl. 7B, 214, 1983).

Materials and methods

Bacteria

DNA repair-deficient strains SR250 (*uvrB5*), SR251 (*uvrB5 lexA101*), SR256 (*uvrB5 recA56*), and SR1034 (*uvrB5 umuC122*::Tn*5*) were all derived by transduction from the repair-proficient *E. coli* K-12 strain, SR248 (wild-type), and have been described (Sargentini and Smith, 1981, 1984).

Media

The media (SMM1, PB, Glu-0, Glu-600, YENB plates, R-top and R-plates) have been described (Sargentini and Smith, 1984), except for the rifampicin plates, which were YENB plates containing rifampicin (Sigma) at 100 μg/ml and dimethyl sulfoxide at 1% (v/v), and the M-top agar which was NaCl at 0.8% and Bacto agar (Difco) at 1.8%. Compounds tested for mutagenicity were: L-arginine, L-glutamine, L-leucine, L-lysine · HCl, L-methionine, L-proline, L-threonine, L-tryptophan (Sigma); L-alanine, L-asparagine, L-aspartic acid, L-cystine (0.1 M in 0.3 N HCl), L-glutamic acid, L-serine, L-valine (Mann Res.); L-phenylalanine, L-histidine (Schwarz/Mann); L-glycine (Eastman Organic); L-isoleucine (Pierce), L-tyrosine (ICN Pharm.). Plates containing histidine or tryptophan were used within one week after preparation to prevent the marked reduction in growth yield observed with older plates.

Plate assay for mutagenesis

Logarithmic-phase cells were prepared by diluting overnight cultures 1:50 into warm SMM1, and shaking the cultures at 37°C until a concentration of 4×10^8 colony-forming units (CFU) per ml was attained. Cells were pelleted by centrifugation, washed once, and resuspended in PB (phosphate buffer) at 1.5×10^8 CFU/ml. Cells (0.1 ml) were spread on Glu-600 plates (lactose plates containing a growth-limiting amount of glucose, i.e., 600 μg/ml) $\pm$ the test compound at 2 mM and Glu-0 plates. Plates were incubated for 3 days at 37°C before scoring Lac^+ mutants. To determine the relative mutagenesis, the mean number of mutants on 5 Glu-0 plates (i.e., preexisting mutants) was subtracted from the mean number of mutants on 10 Glu-600 plates (i.e., plate mutants) and from the mean number of mutants on 5 Glu-600 plates containing the test compound (i.e., plate plus induced mutants). Then the ratio (test/control) was determined. The mean numbers of preexisting mutants per plate were 1(wild-type), 15(*uvrB*), and 0(*uvrB umuC* and *uvrB lexA*), while the mean number of plate mutants per control plate were 60(wild-type), 355(*uvrB*), 21(*uvrB umuC*), and 20(*uvrB lexA*). In experiments where the mutagenesis was normalized for constant growth (about 11 population doublings) on the different mutant-selection plates, the total cells per plate were counted after the glucose supply was exhausted by the Lac^- cells and when the Lac^+ cells still constituted less than 1% of the total cells (Sargentini and Smith, 1981). For Glu-600 control plates, the total Lac^- cells per plate was 3.4×10^{10} for both the *uvrB* and *uvrB umuC* strains.

Growth-normalization factors (total cells on test plates divided by total cells on control plates ± 1 SD) for the *uvrB* and *uvrB umuC* strains, respectively, were 0.93 ± 0.09 and 0.91 ± 0.13 for cystine, 1.03 ± 0.12 and 0.90 ± 0.07 for phenylalanine, 1.13 ± 0.07 and 1.19 ± 0.03 for arginine, and 1.30 ± 0.10 and 1.27 ± 0.03 for threonine. For other amino acids that were tested for mutagenicity, only the *uvrB umuC* strain was assayed for total

cells, and the derived growth normalization factor was also used for the isogenic *uvrB* strain.

Tube assay for mutagenesis

Cells were grown overnight in SMM1, diluted 10^{-5} with homologous medium ± phenylalanine at 2 mM, then dispensed into 9 tubes each (2-ml portions), and incubated with aeration for 48 h at 37°C. Then, cultures were assayed for mutants and viability. For mutants, the plating conditions were: Lac$^+$, 0.2 ml per Glu-0 plate; Rif^r, 1 ml plus 2.5 ml M-top agar per rifampicin plate; T6^r, 0.1 ml of 10-fold diluted cells plus 1 ml bacteriophage T6 lysate containing 10^{10} plaque-forming units (hold mixture 15 min at room temperature) plus 2.5 ml of R-top agar per R-plate. Viability was determined on YENB plates. All plates were incubated for 2 days at 37°C (R-plates and YENB plates, 1 day). Median values for mutants and CFU were used to determine the median mutant frequencies for each assay. The phenylalanine effect was the ratio (test/control) of the median mutant frequencies.

Results

20 amino acids were individually tested for their ability to revert *lacZ53* cells to Lac$^+$. Serine was found to be toxic at the concentration (2 mM) that all the other amino acids were tested, so it was omitted from further consideration. Of the remaining 19 amino acids, none had any significant effect on Lac reversion in a *uvrB umuC* strain, while 4 amino acids did have a significant effect on the isogenic *uvrB* strain; arginine, cystine and threonine were, to varying degrees, antimutagenic, while phenylalanine was mutagenic (Table 1).

Since only phenylalanine was mutagenic in the *uvrB* strain and not in the isogenic *uvrB umuC* strain, this amino acid was selected for further study. A dose–response relationship was determined for phenylalanine mutagenesis in wild-type, *uvrB*, *uvrB umuC*, and *uvrB lexA* strains (Fig. 1). While the *uvrB* strain showed increasing mutagenesis with increasing phenylalanine concentration, phenylalanine mutagenesis was not detected in the wild-type, *uvrB umuC*, or *uvrB lexA* strains.

TABLE 1

EFFECT OF AMINO ACIDS ON SPONTANEOUS MUTAGENESIS IN *E. coli uvrB* AND *uvrB umuC* STRAINS

Amino acid tested	Relative Lac$^+$ mutagenesis in the presence of amino acids at 2 mM [a]	
	uvrB	*uvrB umuC*
Alanine	1.02	0.92
Arginine	0.74 [b]	1.14
Asparagine	0.93	0.96
Aspartic acid	0.84	0.92
Cystine	0.34 [b]	0.88
Glycine	1.05	1.16
Glutamic acid	0.88	0.96
Glutamine	0.86	0.90
Histidine	0.93	1.04
Isoleucine	0.94	0.99
Leucine	1.03	1.09
Lysine	1.16	1.11
Methionine	1.04	1.18
Phenylalanine	1.91 [b]	0.90
Proline	0.88	0.94
Threonine	0.69 [b]	1.08
Tryptophan	0.93	0.93
Tyrosine	0.96	0.93
Valine/isoleucine [c]	0.92	0.90

[a] Values are the means of relative mutants per total cells data (mutant frequency on test plates divided by mutant frequency on control plates) from triplicate plate-assay experiments (see Materials and Methods) for *lacZ53* strains.

[b] Values indicate significant effects on mutagenesis by either of two criteria. That is, the mean mutant frequency ±1 SD (range) for the amino-acid-supplemented plates did not overlap the range for the control plates, and the mean relative (test/control) range for the *uvrB* strain did not overlap the mean relative range for the *uvrB umuC* strain.

[c] Valine was tested in the presence of 2 mM isoleucine.

To exclude the possibility that this phenylalanine mutagenesis was caused by a contaminant in our sample of phenylalanine (Schwarz/Mann), two other sources were tested. The increase in Lac$^+$ mutagenesis due to the presence of 2 mM phenylalanine for the *uvrB* strain was about the same regardless of the source of phenylalanine. The mutagenesis enhancement factors (test/control) from duplicate experiments are: 1.68 and 1.62 for Schwarz/Mann, Lot AZ-1925; 1.79 and

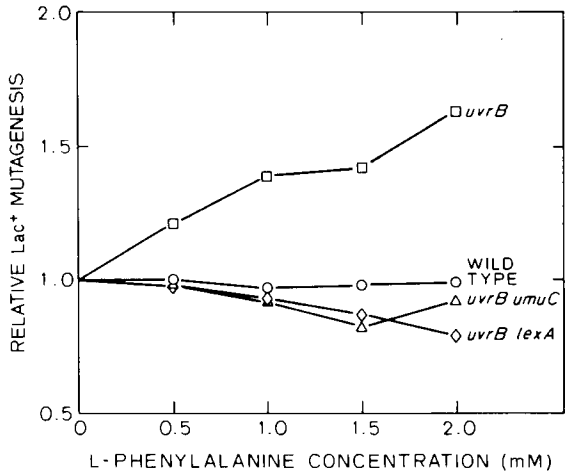

Fig. 1. Phenylalanine mutagenicity in *E. coli* strains deficient in DNA repair. Data for relative Lac^+ mutagenesis are the means from triplicate experiments, and are the numbers of Lac^+ mutants arising on mutant-selection plates containing phenylalanine divided by the numbers of Lac^+ mutants arising on control plates. See 'plate assay' in Materials and Methods.

1.64 for Mann Research Laboratories, Lot U-1726; and 1.62 and 1.56 for Sigma, Lot 11F-0198. The Sigma sample was prepared synthetically, and was characterized by the manufacturer as having no ninhydrin-reactive spots other than phenylalanine in two different thin-layer chromatography systems.

TABLE 2

EFFECT OF AMINO ACIDS ON CELL-DOUBLING TIME, AND MUTAGENESIS IN *E. coli uvrB*.

Growth medium [a]	Cell-doubling time (min) [b]	Relative Lac^+ mutagenesis [c]
SMM1	79	1.00
SMM1 + Phe	92	1.84
SMM1 + Trp	79	0.94
SMM1 + Tyr	76	1.01
SMM1 + Phe + Trp	78	1.41
SMM1 + Phe + Tyr	78	1.08
SMM1 + Phe + Trp + Tyr	80	1.04

[a] SMM1, defined minimal medium (Sargentini and Smith, 1984); Phe, phenylalanine; Trp, tryptophan; Tyr, tyrosine; all additives were at 2 mM.

[b] Values are the means of data from duplicate experiments for logarithmic-phase liquid cultures shaken at 37°C.

[c] Values are the means of data from duplicate plate-assay experiments (see Materials and Methods).

TABLE 3

MUTAGENICITY OF PHENYLALANINE MEASURED WITH VARIOUS MUTATION ASSAYS ON *E. coli uvrB*

Mutation assay [a]	Relative mutagenesis [b]	
	Individual experiments	Median
Lac^+	0.3, 1.4, 2.5, 1.4, 2.7, 3.5, 1.2, 0.4	1.4 (27)
Rif^r	2.5, 0.8, 3.5, 0.4, 1.3, 1.7, 1.0, 0.3	1.2 (10)
$T6^r$	2.8, 2.0, 1.3, 1.0, 1.9, 1.3	1.6 (130)

[a] Mutation assays are described in Materials and Methods, and measure the mutations to the ability to use lactose as the sole carbon source (Lac^+), or to resistance to rifampicin (Rif^r), or to bacteriophage T6 ($T6^r$).

[b] Values are the median mutant frequencies for cells grown in the presence of 2 mM phenylalanine divided by those values for cells grown in its absence. Values in parentheses are the median mutants per mutant-selection plate from control cultures.

Since phenylalanine, tyrosine and tryptophan share several enzymatic steps in their synthesis (reviewed in Gibson and Pittard, 1968), the fact that phenylalanine mutagenesis was prevented by the presence of tyrosine, and was reduced by about 50% in the presence of tryptophan (Table 2), further supports the notion that the observed mutagenesis was due to phenylalanine, and not to an impurity.

To insure that the observed mutagenicity of phenylalanine reflects a general phenomenon, other mutation assays were employed and their results are summarized in Table 3. Despite the more variable results with the tube assays, we concluded that phenylalanine induced rifampicin-resistant mutants, bacteriophage T6-resistant mutants, as well as Lac^+ mutants in the *uvrB* strain.

Discussion

Normal cellular metabolism has been proposed as a mechanism for producing excisable, mutagenic DNA damage (reviewed in Sargentini and Smith, 1985). In this work, 19 amino acids were tested to see if their catabolism was mutagenic. Only phenylalanine was mutagenic in *uvrB* cells, but not in wild-type, *uvrB umuC*, and *uvrB lexA* cells (Table 1, Fig. 1). Phenylalanine mutagenesis was detected with known base-substitution assays

(Table 3); *lacZ53*(amber) → Lac$^+$ (Sargentini and Smith, 1979), rifampicin resistance (missense) (e.g., Silengo et al., 1967; Austin et al., 1971), and also with bacteriophage T6 resistance.

Given that phenylalanine mutagenesis was not observed in excision repair-proficient cells (wild-type, Fig. 1), it seems likely that excisable, mutagenic DNA lesions were involved. However, we have thus far been unable to detect such lesions with a variety of indirect assays. A 'repairless' strain, *uvrB recA*, showed no greater slowing of growth in the presence of phenylalanine at 2 mM than was observed in the isogenic wild-type and *uvrB* strains, i.e., about a 15% increase in cell-doubling time (data not shown). Also, we could not demonstrate any phenylalanine-induction of free bacteriophage in a λ-lysogen of the *uvrB* strain (data not shown). Furthermore, the *uvrB* and *uvrB recA* strains were not sensitized by spreading UV-irradiated cells on plates containing phenylalanine at 2 mM, nor did phenylalanine at 2 mM increase UV or γ radiation mutagenesis in the *uvrB* strain (data not shown). Thus, we concluded from these assays for lethality and sensitization, that the putative phenylalanine-induced DNA lesions are too rare to be easily detected.

The antimutagenic effect of tyrosine and tryptophan on the mutagenicity of phenylalanine (Table 2) may have multiple explanations. Tyrosine and tryptophan can reduce the uptake of phenylalanine (Whipp and Pittard, 1977), and they can reverse the tyrosine and tryptophan 'starvation' that occurs in the presence of excess phenylalanine due to the feedback inhibition and repression of enzymes that are common to the biosynthesis of these three amino acids (reviewed in Gibson and Pittard, 1968). The 15% reduction in growth rate produced by 2 mM phenylalanine was reversed by adding either tyrosine or tryptophan at 2 mM (Table 2).

Since the presence of excess phenylalanine seems to produce excisable lesions in DNA (i.e., the mutation yield is higher in cells deficient in the first step of excision repair; Fig. 1), it suggest that the mutagenic damage is 'UV-like' as regards the excision process that usually repairs them. Since UV-radiation produces excited-state molecules that produce excisable DNA damage, it suggests that phenylalanine, or one of its metabolites, may be raised to its excited state enzymatically, and thus produces 'UV-like' damage in DNA. The enzymatic production of excited states is well known (Cilento, 1984).

In proteins the excited-state energy of phenylalanine can be transferred to tyrosine, which in turn can transfer its energy to tryptophan (e.g., Longworth, 1971). Therefore, tyrosine and tryptophan may be antimutagenic for phenylalanine mutagenesis by virtue of their being quenchers of the excited state of a metabolic product of phenylalanine. Consistent with this idea of the quenching of excited states are the observations that cobaltous chloride is antimutagenic (e.g., Inoue et al., 1981), Co(II) is a quencher of triplet states (page 302 of Calvert and Pitts, 1966), and at 20 μg/ml, cobaltous chloride also inhibits the mutagenicity of phenylalanine (data not shown).

In contrast to the mutagenic effect of phenylalanine, antimutagenic effects on spontaneous mutagenesis in the *uvrB* strain were found for cystine, arginine and threonine, with cystine showing the most significant effect (Table 1). We believe that this is the first report that these 3 amino acids are antimutagenic, however, we detected no antimutagenic effects for histidine and methionine as have been detected in fungi (reviewed in Clarke and Shankel, 1975).

Spontaneous mutations arise through errors made both during DNA replication and DNA repair (Sargentini and Smith, 1985). The errors made during DNA replication can be due to the inherent inaccuracy of the replication machinery on an undamaged template, or they can be due to the metabolic production of damage that alters the coding properties of the purines and pyrimidines. The metabolic production of noncoding lesions in the DNA would initiate repair processes.

In the absence of environmental factors, the overall spontaneous mutation rate of a cell should be the resultant of all of its normal metabolic activities that are either mutagenic or antimutagenic. Phenylalanine metabolism is offered as just one of the presumably numerous metabolic activities that contribute to spontaneous mutagenesis, and the metabolism of cystine, arginine and threonine are offered as normal metabolic activities that contribute to antimutagenesis.

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