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MUTAGENICITY OF CYSTEINE AND PENICILLAMINE AND ITS ENANTIOMERIC SELECTIVITY

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Abstract—We previously observed that postmitochondrial supernatant (S9) from rat liver and kidney homogenates transforms L-cysteine into a mutagen that reverts bacteria of the strain *Salmonella typhimurium* TA100 to histidine independence. In the present study the enantiomers of cysteine and penicillamine (β,β-dimethylcysteine) have been investigated for mutagenicity. The *Salmonella typhimurium* strain TA92 was found to be more sensitive than TA100 to the mutagenic action of L-cysteine and was therefore also included. This strain allowed the unambiguous realization of a (weak) mutagenic effect of L-cysteine even in the absence of mammalian enzyme preparations. D-cysteine did not show mutagenicity under any experimental conditions. However, it was strongly bacteriotoxic. On the other hand, both enantiomers of penicillamine exerted clear mutagenic effects. Qualitatively, their mutagenicity was similar to that of L-cysteine in the following respects: (i) the penicillamines were directly mutagenic, (ii) their mutagenicity was enhanced by S9, (iii) kidney S9 enhanced the mutagenicity more than did liver S9, (iv) TA92 was more sensitive than TA100. Thereby it is noteworthy that the ratios of the specific mutagenicities in the two strains were virtually identical in the direct, kidney-S9-mediated and liver-S9-mediated tests suggesting that the ultimate mutagens under these different metabolic conditions were identical.

On the other hand, substantial quantitative differences in the mutagenicity between the β-thiol amino acids were observed. L-penicillamine was about eight times more mutagenic than the clinically used enantiomer, D-penicillamine. In the direct tests, the mutagenic potency of L-cysteine was equal to that of D-penicillamine. In the S9-mediated experiments, the mutagenic potency of L-cysteine was intermediate between those of L- and D-penicillamine.

The cysteine derivative D-penicillamine (D-β,β-dimethylcysteine) is used in the treatment of rheumatoid arthritis, metal poisonings, cystinuria and the associated nephrolithiasis. We previously observed that L-cysteine is transformed by postmitochondrial supernatant from rat liver and kidney homogenates into a mutagen that reverts bacteria of the strain *Salmonella typhimurium* TA100 to histidine prototrophy [1]. The exact molecular mechanisms of this activation and the toxicological significance are not yet known. Nevertheless, it appeared reasonable to investigate D-penicillamine for mutagenicity under the same experimental conditions due to its structural similarities with L-cysteine. The structure of D-penicillamine differs from L-cysteine in two respects, the stereochemistry and the substitution of the two hydrogens on the β-carbon atom by methyl groups. Therefore, D-cysteine and L-penicillamine were included in this study.

MATERIALS AND METHODS

L-cysteine and D-cysteine were purchased from Sigma Chemie GmbH, Taufkirchen (Federal Republic of Germany). L-penicillamine (purity >98%) and D-penicillamine (purity >99%) were from Fluka AG, Buchs (Switzerland). The test compounds were dissolved in water immediately before use. The solutions were adjusted to pH 7.0 with dilute NaOH solution.

Bacteria. Among the bacterial strains used in our initial study on the mutagenicity of glutathione and L-cysteine in the Ames test, *Salmonella typhimurium* TA100 was most responsive. In the mean-time we found a second bacterial strain which detects the mutagenicity of L-cysteine and glutathione with high sensitivity. This strain TA92, carries the same substitution mutation (hisG46) in the histidine operon as TA100. Both strains contain the episome pKM101. They differ in that TA92 has an intact polysaccharide layer and a functional DNA excision repair system while TA100 is deficient in these functions (*rfa*, *uvrB*). Both strains were used in the present study. They were generously provided by Dr. B. N. Ames, Berkeley (California).

Bacteria were grown overnight in nutrient broth (8 g Bacto nutrient broth (DIFCO) + 5 g NaCl/l). For inoculation, stock cultures that were stored at −70° were used. Before the experiment, bacteria were centrifuged, resuspended in resuspension medium (1.6 g Bacto nutrient broth + 5 g NaCl/l) and adjusted nephelometrically to a titer of about 2.4×10^9 bacteria (colony-forming units) per ml. In each experiment the presence of the R-factor pKM101 was ascertained by growing diluted cultures on ampicilline-containing and ampicilline-free complete agar plates. The numbers of colonies were always virtually identical under the two culture conditions.

Tissue preparations. Male Sprague–Dawley rats

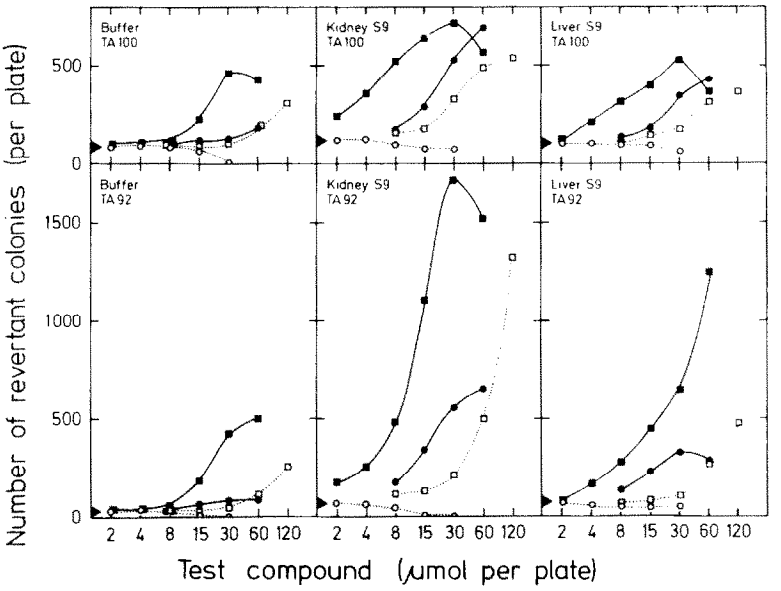

Fig. 1. Effect of L-cysteine (●), D-cysteine (○), L-penicillamine (■) and D-penicillamine (□) on the reversion of histidine-dependent *S. typhimurium* TA100 (upper panels) and TA92 (lower panels) directly (left panels) and in the presence of kidney (middle panels) or liver (right panels) postmitochondrial supernatant. The results of one of two experiments are shown. The results of the other experiment were very similar. Values are means from two replicate incubations. The individual values always deviated from the means by less than 10%. The triangles (▶) denote the number of spontaneous mutants (mean of four replicate plates) under the respective experimental conditions.

(200–300 g) were obtained from WIGA, Sulzfeld (Federal Republic of Germany). The livers and kidneys of animals which had not been treated with an inducing agent were homogenized in three volumes of sterile, cold KCl (150 mM, buffered with 10 mM sodium phosphate, pH 7.4). The homogenates were centrifuged at 10,000 *g* for 10 min. One volume of the resulting supernatants was mixed with one volume of a solution containing 12.5 mM $MgCl_2$, 50 mM KCl, 6 mM NADP and 7.5 mM glucose-6-phosphate in 75 mM sodium phosphate buffer, pH 7.4. These preparations were termed liver and kidney S9, respectively. For all experiments, fresh tissue preparations were used from animals that were killed on the day of the experiment.

Mutagenicity experiments. Reversion of his$^-$ *Salmonella typhimurium* strains was used for studying mutagenic activity. A minor modification of the assay described by Ames *et al.* [2] was used. The test compound (in 200 μl neutralized aqueous solution), 667 μl S9 (or 150 mM KCl in the experiments for direct mutagenicity), 100 μl of the bacterial suspension (2.4×10^8 colony forming units) and 2 ml of 45° warm soft agar (0.55% agar, 0.55% NaCl, 50 μM histidine, 50 μM biotin, 25 mM sodium phosphate buffer, pH 7.4) were mixed in a test-tube and poured onto a petri dish containing 24 ml minimal agar (1.5% agar in Vogel–Bonner E medium with 2% glucose). After incubation for 3 days in the dark, colonies (his$^+$ revertants) were counted.

RESULTS

The detailed results of a mutagenicity experiment on the thiol amino acids are presented in Fig. 1. As a measure of the mutagenic potencies, specific mutagenicities were calculated from the initial part of the dose–response curves (Table 1).

L-Cysteine was mutagenic under all three metabolic conditions studied. For each metabolic condition and each dose of L-cysteine, the number of revertants induced from the strain TA92 was very similar to (usually marginally higher than) the number of revertants induced from the strain TA100. The main difference between the two strains was that TA92 showed only approximately half of the number of revertant colonies on solvent control plates as compared to TA100. Hence, TA92 was more sensitive than TA100. This increased sensitivity became useful in the direct test, where the effect of L-cysteine was very weak. At the optimal concentration, L-cysteine increased the number of revertants to twice the spontaneous level with the strain TA100 and to three times the spontaneous level with the strain TA92. Addition of liver or kidney S9 strongly enhanced the effect of L-cysteine. In the liver S9-mediated test, L-cysteine increased the number of revertants up to 4-fold above solvent control. The specific mutagenicities were increased by a factor of 5, as compared to those in the direct test. Kidney S9 was even more effective than liver S9 in enhancing the effects of L-cysteine. The number of revertants in the presence of L-cysteine was then up to 10-fold above the solvent control value. The specific mutagenicities of L-cysteine were increased by a factor of 9, as compared to those found in the direct test.

D-Cysteine did not lead to a measurable increase in the number of revertants above solvent control under any experimental conditions. In contrast, even

Table 1. Mutagenic potency of the enantiomers of cysteine and penicillamine under various experimental conditions*

	Specific mutagenicity (Revertants per μmol compound)					
	Salmonella typhimurium TA100			*Salmonella typhimurium* TA92		
Test compound	Buffer	Kidney S9	Liver S9	Buffer	Kidney S9	Liver S9
L-cysteine	1.5	13	8	2	18	10
D-cysteine	<10†	<10†	<10†	<5†	<5†	<5†
L-penicillamine	12	55	27	13	70	28
D-penicillamine	1.5	7	2.3	2	11	3.3

* The slopes in the non-toxic part of the dose-mutagenicity (specific mutagenicity) were calculated from the data shown in Fig. 1.

† D-Cysteine did not show any mutagenicity, but exhibited relatively strong toxicity. The indicated sensitivity limits of the test are estimated values.

at low doses of D-cysteine, the number of revertant colonies per plate was decreased. This effect was probably due to bacteriotoxicity, as the density of the his⁻ background lawn was concomitantly reduced. Taking into account the highest dose at which D-cysteine could be tested adequately (4 μmole) and the minimal effect that normally would be recognized (20 colonies above control with TA92, and 40 colonies above control with TA100) it is clear that mutagenicity, comparable in its potency with that of L-cysteine in the direct test, would not have been detectable. On the other hand, mutagenicities, comparable to those of L-cysteine in the S9-mediated experiments, should have been recognized, particularly with the strain TA92.

L-Penicillamine was the strongest mutagen among the four thiol amino acids tested in both bacterial strains and under each of the three metabolic conditions used. Its mutagenicity, like that of L-cysteine, was potentiated by liver S9 and (more effectively) by kidney S9. At low concentrations of L-penicillamine, the number of revertants induced from TA92 was similar to that induced from TA100. At high concentrations, the effects in TA100 were weaker than those observed in TA92. (We suspect that the bacteriotoxicity was stronger to TA100 than to TA92.)

D-Penicillamine induced virtually the same pattern of mutagenic effects as did L-penicillamine. However, the mutagenic potency of the D-enantiomer was weaker. Its specific mutagenicity was 9–16% of that of the L-enantiomer under the same conditions. Nonetheless, relatively strong effects of D-penicillamine could be demonstrated, since the compound could be adequately tested up to very high concentrations due to its low bacteriotoxicity and good solubility.

DISCUSSION

In this study we extended our previous finding that L-cysteine is mutagenic in the Ames test [1] in two respects: (i) We found a *Salmonella typhimurium* strain, TA92, that is more sensitive to the mutagenic action of L-cysteine than the originally used strain TA100. In TA92 the L-cysteine-induced effects were slightly stronger than in TA100, while the spontaneous mutation rate was lower. (ii) The resulting increased test sensitivity was useful for clearly delineating mutagenicity of L-cysteine even in the absence of mammalian enzyme preparations. This mutagenicity, however, was much weaker than that observed in the S9-mediated experiments.

D-cysteine showed no mutagenic effect. The sensitivity of the test, however, was limited by strong bacteriotoxicity. D- and L-penicillamine elicited mutagenic effects which were qualitatively similar to those observed with L-cysteine. The penicillamines were directly mutagenic and their effects were potentiated in the presence of S9 preparations. As with L-cysteine, kidney S9 exhibited a stronger activating effect than liver S9. Furthermore, it is noteworthy that the clinically used D-enantiomer of penicillamine was substantially less mutagenic than the L-enantiomer. Under most experimental conditions it was even less mutagenic than the natural amino acid L-cysteine. The only exception was in the direct test in which L-cysteine and D-penicillamine were about equipotent mutagens.

For each of the three mutagenic amino acids the ratios of the specific mutagenicities in the two bacterial strains were virtually identical in the direct, liver S9-mediated and kidney S9-mediated experiments. This favours the notion that the ultimate mutagens under the different metabolic conditions were chemically identical.

At first glance it may be surprising that an important endogenous compound which is found in all organisms, L-cysteine, can exert mutagenic effects. On the other hand, it became evident after the development of sensitive test systems that mutagenicity is not a rare property of compounds. For example, among 639 industrial compounds (most of them synthesized as potential pharmaceuticals) tested in our laboratory 207 showed a positive response in the Ames test [3]. With these considerations it would be surprising if organisms could synthesize their many compounds, required for all kinds of purposes, without forming potential mutagens. Naturally, when an endogenous chemical with harmful properties has to be formed by an organism, one would expect the evolution of protection mechanisms, e.g. in the form of barriers around endangered cellular structures, detoxifying

enzymes or repair systems. Precedents of specific protection systems are superoxide dismutase, catalase and glutathione peroxidase against reactive oxygen species [4], O^6-methylguanine-DNA methyltransferase and DNA 7-methylguanine-DNA glycosylase against nonphysiological DNA methylations by *S*-adenosylmethionine [5, 6] and cholesterol 5,6-oxide hydrolase [7] against the endogenous mutagen cholesterol 5,6-oxide [8]. One may speculate that protection systems against potentially harmful endogenous compounds may not necessarily be effective against foreign, structurally related chemicals.

D-Penicillamine is considered a relatively nontoxic drug. The adverse effect most often observed is acute allergic reactions. Furthermore several cases of nephrotoxicity in patients treated with D-penicillamine were reported [9–12]. Concerning the nephrotoxicity, the effective activation of D-penicillamine by kidney S9 observed in this study is noteworthy. Whether mutagenicity, nephrotoxicity and allergies are attributable to a single or different reactive metabolites, however, remains to be investigated.

In this study we showed that not only the natural amino acid L-cysteine, but also structurally related, nonphysiological compounds can exert mutagenic effects, particularly after activation by kidney subcellular preparations. The L-enantiomers were more effectively activated than the D-enantiomers and L-penicillamine was more mutagenic than L-cysteine.

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