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The presence of arginine may be a source of false positive results in the Ames test

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ABSTRACT

An increase in the number of revertant colonies in the Ames test is generally taken as a strong indication of mutagenic activity of a test compound. However, irrelevant positive findings may constitute a major problem in regulatory drug testing. In this study, mixtures containing only amino acids such as glycine, lysine, arginine and isoleucine, routinely used as peptide preservatives in polypeptide pharmaceutical products, were investigated for mutagenesis in the Ames *Salmonella typhimurium* test. The results demonstrated that in the presence of metabolic activation, all the solutions containing arginine induced an increase in the number of revertant colonies in strains TA98, TA100 and TA1535 compared with the solvent control. More specifically, for strain TA98, all arginine doses tested, *i.e.* from 0.4 to 8 mg/plate induced a statistically significant increase in the number of revertants. This increase was biologically significant from 1.2 to 8 mg/plate. For strain TA100, the five highest test doses, *i.e.* from 1.2 to 8 mg/plate, induced statistically and biologically significant increases in the number of revertants. A statistically significant increase in colony number was also observed in strain TA1535, but only at the maximal test dose of 8 mg/plate arginine. These increases were observed with arginine from two different sources, suggesting that the observed effect would not be due to the presence of potential impurities in the type of arginine used. Our findings show that a functional metabolic activation system was required to induce an increase in the number of colonies. The presence of vitamin C inhibited the arginine-induced increase in the number of revertant colonies in *S. typhimurium* strain TA98, suggesting a potential involvement of oxidative stress.

1. Introduction

The identification of potentially mutagenic substances is an important step in safety assessment. The Ames test in *Salmonella typhimurium* is part of the routine screening battery applied to all new drugs. *S. typhimurium* strains used in the Ames test are auxotroph for histidine due to mutations in different genes, and the bacteria are therefore unable to grow and to form colonies in its absence.

Chemical substances are tested routinely according to international guidelines for genotoxicity testing [1,2]. However, changes in the number of colonies per plate may involve other mechanisms than genotoxicity. In this respect, artefactual increases have been reported [3–5]. Furthermore, testing of proteinaceous substances in the basic Ames test may generate false positives due to the presence of growth-promoting constituents in the test sample, such as histidine, other amino acid or peptides [6–11]. These irrelevant positive findings may pose a major problem in regulatory drug testing, especially of biotechnology products, which are mainly based on proteins. However, there are few data describing the potential involvement of amino acids, which are commonly used in the formulation buffer of biotechnology products, in the generation of an artefactual increase in the number of colonies. Interestingly, using TA98 and TA100 *Salmonella* strains, Arimoto et al. [12] reported that the incubation period of the plate could be significantly reduced by supplementing the basic agar with a mixture of amino acids other than histidine. The results reported by Arimoto et al. [12] suggested that a mixture of amino acids was able to significantly promote the growth of *His*$^+$ revertants. The studies reported here were conducted in order to address the problem of generation of false positive Ames-test results and the possible misjudgement of mutagenic potential. In this respect, mixtures containing only amino acids such as glycine, lysine, arginine and isoleucine, which are routinely used as peptide preservatives in pharmaceutical preparations, were tested and their implication in the generation of false positives was discussed.

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2. Materials and methods

2.1. Test solutions

Six separate sterile solutions (pH 6.4) of amino acids were used in the present study. These solutions contained either one, two, three or all four of the following amino acids: glycine (2 g/L), lysine chlorhydrate (2 g/L), isoleucine (10 g/L) or arginine chlorhydrate (40 g/L).

Solution A complete solution, contains glycine, lysine, isoleucine, arginine.
Solution B contains glycine, lysine, isoleucine.
Solution C contains glycine, lysine, arginine.
Solution D contains glycine, lysine.
Solution E contains arginine.

All amino acids used in solutions A–E are from Sigma (St. Louis, MO, USA), with a purity ≥98.5% according to the European Pharmacopeia.

Solution F is identical to solution A except that arginine is from Merck (Darmstadt, Germany) with purity ≥98.5% according to the European Pharmacopeia.

The amino acids in solution A are commonly used in protein formulations as stabilizers, buffers and bulking agents.

2.2. Ames test

The plate-incorporation assay was carried out as described by Ames et al. [13], Maron and Ames [14] and OECD guidelines [15] on strains, TA98, TA100 and TA1535 of *S. typhimurium*. Briefly, bacterial cells from stock cultures were grown for 12 h in liquid medium at 37 °C. A volume of 0.1 mL bacterial suspension was added to 2 mL of top agar medium containing 0.6% agar, 0.6% NaCl and 20.96 μg of L-histidine and D-biotin. Test solution (or solvent control, or positive control agent) and the S9-mix (0.5 mL/plate; 10% S9 v/v) or potassium phosphate buffer (0.5 mL/plate; 0.2 M; pH 7.4) were also added to the top agar which was then mixed and poured onto the top of Vogel-Bonner minimal medium agar plates (3 replicates/dose for the treated and the positive control and 6 replicates for the solvent control). Colonies were counted 48 h after incubation at 37 °C. The highest volume of the test solution added per plate was initially limited to 100 μL, in agreement with OECD guidelines [15], and then increased up to 200 μL in confirmation studies.

The preincubation assay with metabolic activation was carried out as described by Bartsch et al. [16], Bridges et al. [17] and Yamanaka et al. [18]; briefly, 0.1 mL of test solution, 0.5 mL of S9-mix and 0.1 mL of cell suspension were mixed and put under agitation at 37 °C during 60 min. Then, 2 mL of soft agar were added and the final mixture was spread out in a Petri plate containing minimum medium agar.

2.3. Rat liver S9

Rat liver S9 was prepared according to Ames et al. [14]. Enzymatic induction was provoked 5 days before sacrifice by a single intraperitoneal injection of Aroclor 1254 at a dose of 500 mg/kg in the form of a solution in corn oil at 200 mg/mL. The protein concentration of the different batches of S9 was in the range of 20–30 mg/mL, determined according to Lowry et al. [19]. The preparation was shown to be sterile. The complementary experiment on heat-inactivated S9 was performed by heating the S9 during 30 min at 56 °C prior to preparing the S9-mix.

2.4. S9-mix

Composition: One mL of S9-mix contained 0.1 mL of S9, 0.01 mL of 0.4 M $MgCl_2$, 0.01 mL of 1.65 M KCl, 0.5 mL of 0.2 M pH 7.4 phosphate buffer, 0.04 mL of 0.1 M NADP, 0.005 mL of 1 M glucose-6-phosphate and 0.335 mL of distilled water. All co-factors were filtered through a 0.45-μm sterilizing membrane before use. In the complementary experiment using co-factor free S9-mix, NADP and glucose-6-phosphate were replaced by distilled water

2.5. Vitamin C

In a complementary experiment, vitamin C (Fluka, purity >99.5%) was added at 5 mg/plate.

2.6. Treat and wash assay

The treat and wash assay was conducted according to the method described by Thompson et al. [11]. The protocol applied was as per the preincubation assay with the exception that the preincubation time was increased from 60 to 90 min. After the 90 min preincubation, 15 mL of a wash solution of Oxoid No. 2 nutrient broth in phosphate buffered saline (1:7 v/v) was added and the washed bacteria were collected after centrifugation at 2000 × *g* for 30 min. All but 0.7 mL of the supernatant was removed and discarded, and the bacteria were resuspended in the residual supernatant prior to plating mixed in top agar.

2.7. Evaluation and interpretation of results

The biological relevance of the results was considered first: an agent was considered as inducing a biologically significant mutagenic effect in the assay if it caused a dose-related increase in the mean number of revertant colonies for at least three doses with, for the highest increase, a value greater than or equal to three times the value for the solvent control for strain TA1535, and two times the value for the solvent control for strains TA98 and TA100 [15]. Statistical methods were used as an aid in evaluating the test results: data were analysed by means of Dunnett's method [20] allowing the comparison of the mean value obtained for each dose with the mean value observed for the corresponding solvent control. However, statistical significance was not the only determining factor for concluding a positive response.

3. Results and discussion

The results obtained in the first assay showed that the complete mixture containing glycine (2 g/L), lysine (2 g/L), isoleucine (10 g/L), and arginine (40 g/L) (solution A) induced a statistically significant and dose-related increase in the number of revertant colonies in strains TA98, TA100, and TA1535 in the treatment with S9-mix and with preincubation. At the two highest doses of 30 and 100 μL/plate, solution A induced in strains TA98 and TA100 statistically significant increases in the number of colonies compared with the solvent control; the induction ratios obtained at these doses were 1.6 and 1.7 for strain TA98 and 1.4 and 1.7 for TA100, respectively (Figs. 1a and 2a). A dose-related but non-statistically

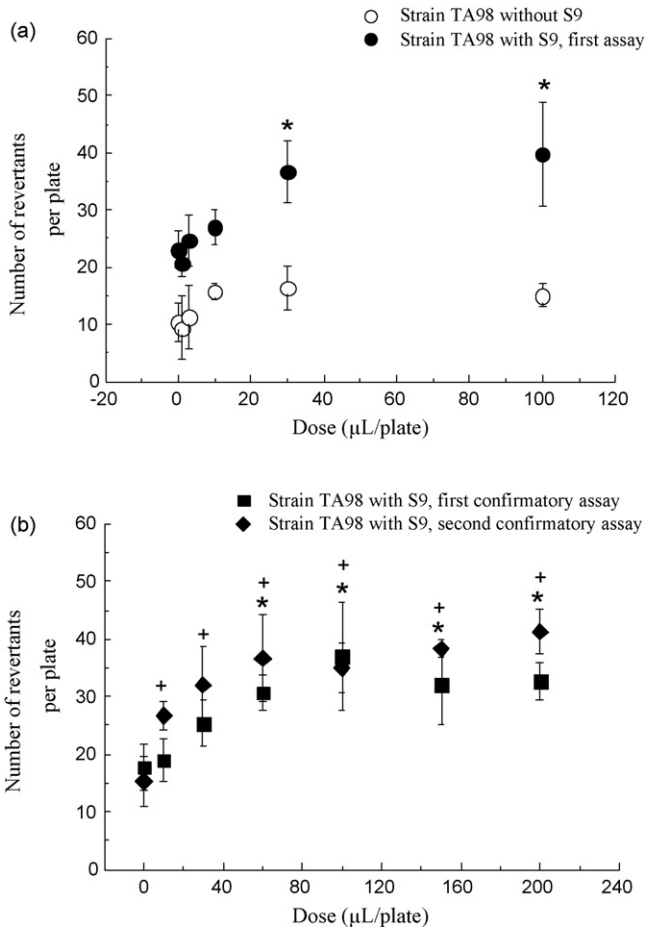

Fig. 1. Evaluation of the mutagenicity of complete solution (solution A: glycine 2 g/L + lysine 2 g/L + isoleucine 10 g/L + arginine 40 g/L) in strain TA98 with S9-mix with preincubation, in the first assay and in two confirmatory assays. Data are mean ± standard deviation of the mean values of triplicate samples. *,+ Significantly different from the mean value observed for the corresponding solvent control at $p < 0.01$ by Dunnett's test, in the first assay, first confirmatory assay and second confirmatory assay, respectively.