Bull. Environm. Contam. Toxicol. 22,660-665 (1979)

Deactivation of Sodium Azide in the Salmonella/Microsome Test

Paul J. Dierickx
Instituut voor Hygiene en Epidemiologie, Wytsmanstraat 14, 1050 Brussel, Belgium

Mutagenic research has become of great importance in the last few years, mainly because of its suspected interest regarding carcinogenic consequences. A correlation as high as 90 % has indeed been found between carcinogenicity and mutagenicity (McCANN et al. 1975). For that purpose the Salmonella/microsome test provides a rapid and sensitive method for detecting mutagenic compounds (AMES et al. 1975). Moreover, using different strains of bacteria, one can distinguish between different kinds of mutations. So it has been shown that sodiumazide causes base-pair substitutions, but no frameshift mutations in Salmonella typhimurium (NILAN et al. 1973).

Recently DE FLORA (1978) has reported the metabolic deactivation of sodiumazide by rat liver extract, which is normally added to enhance the mutagenic response (AMES et al. 1975). We have found that some plant extracts also deactivate this mutagen in the Salmonella/microsome test. Because of the importance of the possibility to deactivate mutagenic responses, we further studied this deactivation phenomenon in detail.

MATERIAL AND METHODS

Male Wistar rats (200 g) were obtained from the animal house of the K.U.Leuven, Belgium. The vegetables were purchased from a local grocer.

Glucose-6-phosphate and NADP were obtained from Boehringer Mannheim Gmbh., Aroclor-1254 from Monsanto, glutathione from Sigma, and all other chemicals from Merck. They were of the purest form available.

Liver extracts (Aroclor-1254-induced or not, see results) were prepared according to AMES et al. (1975) to obtain the S-9. The S-9 mix contains per ml : 0.3 ml S-9, 8 µmoles magnesium-chloride, 33 µmoles KCl, 5 µmoles glucose-6-phosphate and 100 µmoles Na_2HPO_4.

The vegetables were washed in running tap water, cut, mixed in a Sorvall omni-mixer (3 x 1 min.) with an equal volume of sterile PBS and filtered through cheese cloth. The extracts were then centrifuged at 2°C (30 min., 3000 xg). Supernatants were stored at -20°C, before use.

The mutagenicity assay was thoroughly made with strain

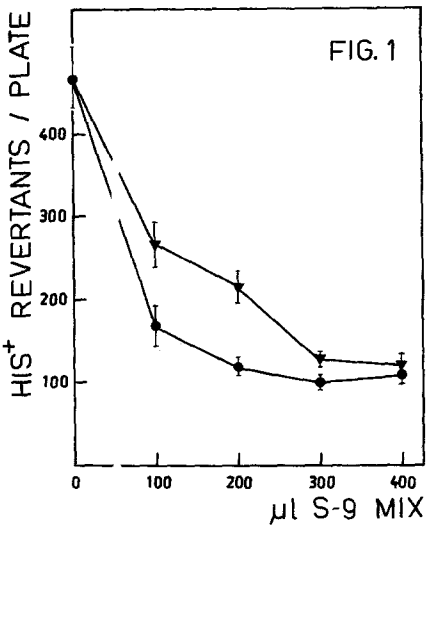
0007-4861/79/0022-0660 $01.20

TA100 of Salmonella typhimurium (kindly provided by Dr.Ames). NaN_3 was always applied in 100 µl water at 1 µg per petri dish (0.7^3 nM). Histidine reversions were measured by the standard method (AMES et al. 1975). Spontaneous revertants have not been substracted. All tests have been done at least twice in triplicate, with consistent results.

RESULTS

The metabolic deactivation of NaN_3 by rat liver extracts.

Rat liver extracts inhibit the mutagenicity of NaN_3 in the Salmonella/microsome test, as DE FLORA (1978) has already reported. In order to investigate the nature of this inhibition we compared, in a first series of experiments, the inhibiting capacity of rat liver extracts obtained from normal and from Aroclor-



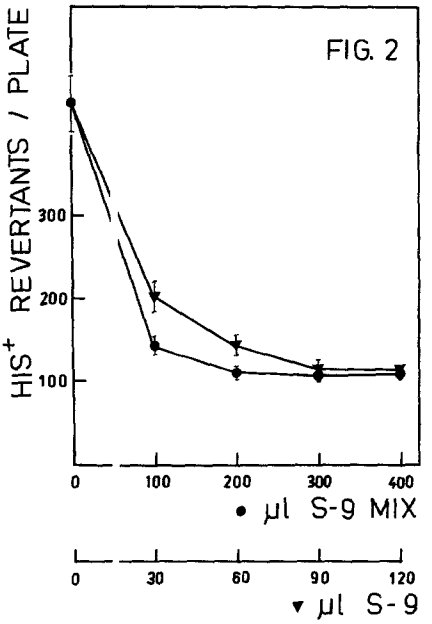


Fig. 1. Comparison of the deactivating action of the rat liver extracts, obtained from normal (▾) and from Aroclor-1254-induced (●) rats. NaN_3 added to the petri-dishes : 0.7 nM.

Fig. 2. The influence of the added NADPH generating system on the deactivation of the sodiumazide mutagenic action by liver extracts. Livers of Aroclor-1254-induced rats were used for these experiments. As usual the S-9 mix contained the NADPH generating system (●). Using solely the S-9 (▾), the volume was adjusted with 0.15 M KCl. NaN_3 added to the petri-dishes : 0.7 nM.

1254-induced rats. These results are shown in Fig. 1. Adding a relative small quantity of S-9 mix (100-200 µl), we observed that the rat liver extracts from Aroclor-1254-induced rats exhibit a greater inhibiting capacity than those from normal rats. When larger quantities (more than 300 µl) are added, this difference disappears.

Further investigating this phenomenon, we looked for the importance of the NADPH generating system, which is usually included in the S-9 mix (AMES et al. 1975). Therefore, some experiments were conducted, in which we compared the influence of the S-9 mix obtained from Aroclor-1254-induced rats with the influence of equal volumes of S-9 (without the NADPH generating system). The difference in volume between the S-9 mix and the S-9 was adjusted by adding 0.15 M KCl to the same volume. As can be seen in Fig. 2, the NADPH generating system stimulates the inhibiting effect of liver extracts on the mutagenicity of sodiumazide, providing that small quantities of extract are considered. Indeed, the NADPH enhanced inhibition disappears at higher concentrations (300 µl S-9 mix or more). However, it should be stressed that, at this moment, the level of spontaneous revertants is already reached.

When the liver extracts are boiled (10 min.) the inhibiting effect on the NaN_3-induced mutagenicity completely disappears.

Anti-NaN_3-mutagenic action of vegetable factors.

We now were interested to know if vegetable factors can inhibit the mutagenic effect of sodiumazide in S. typhimurium as well. With that purpose a series of vegetables were extracted as described in the methods. These included : potatoes, carrots,

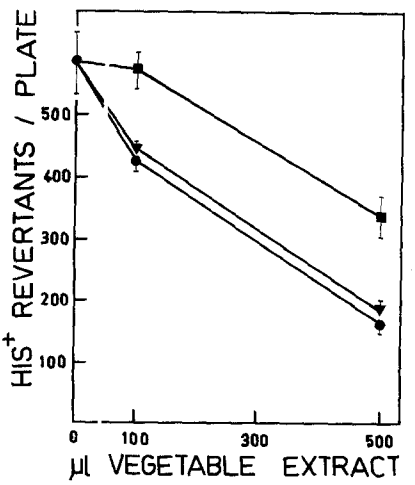


Fig. 3. The deactivating action of vegetable extracts on the mutagenic action of sodiumazide (0.7 nM). Cauliflower (■), leek (●), and spinach (▼).

parsley, celery, tomatoes, haricots, salad, endive, leek, spinach, and cauliflower. Testing these extracts in the Salmonella/microsome test, the three last mentioned appeared to have an antimutagenic effect (Fig. 3). The other extracts did not have any inhibiting capacity at all. Unlike the S-9 mix inhibition, we found a rectilinear correlation between the quantity of added extract and the antimutagenic effect, at least for leek and spinach. Cauliflower extract is clearly less inhibitive.

Investigating the NADPH dependence of the vegetable antimutagenic factors, we compared leek and spinach extracts as such with the same extracts, to which an external NADPH generating system was added. In no case, however, we found a NADPH dependent stimulation of the antimutagenic vegetable extracts. When the extracts were boiled during 10 min., their antimutagenic activity was completely lost.

We should like to point out that the antimutagenic vegetable factors are also lost when the extracts are stored at -20°C. Adding 500 µl freshly prepared extract of spinach or cauliflower we found an inhibiting effect of 42 % or 69 % with regard to the control. When the same extract had been stored during 19 weeks at -20°C, these values were respectively diminished to 0 % and 7 %. In the same way we found for freshly prepared leek extract an inhibiting effect of 72 %, which was diminished to 48 % 13 weeks thereafter.

Anti-NaN_3-mutagenicity of sulfhydryl amino acids.

Since the mutagenic activity of several carcinogens is inhibited by cysteine (ROSIN and STICH 1978), we looked for the effect of this and some other sulfhydryl compounds in our test system. As shown in Fig. 4, cysteine behaves as a potent anti-

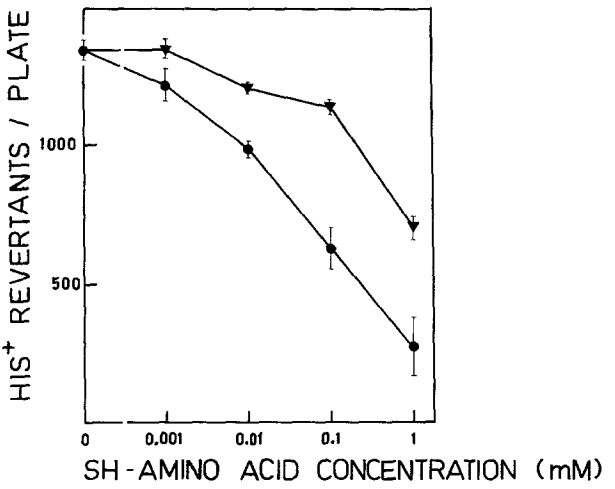


Fig. 4. The antimutagenic action of SH-amino acids on the mutagenic effect of sodiumazide (0.7 nM) : methionine (▼) and cysteine (●).

mutagen for sodiumazide in S. typhimurium TA100. To a less extent the same holds true for methionine : only higher concentrations of this SH-amino acid have a deactivating effect. Reduced glutathione (γ-glutamyl-cysteinyl-glycine), tested in the same concentrations, did not have any inhibiting effect at all, nor did cysteamine. Because the latter can be considered as decarboxylated cysteine, we may conclude that the acid group of cysteine is of vital importance for the antimutagenicity of cysteine.

Our attempts at the deactivation of cysteine as an antimutagen, by using the well known sulfhydryl inhibitors iodoacetate and p-hydroxy-mercuribenzoate, completely failed, mainly because of their high toxicity. So we could not use this method for checking the presence of SH-amino acids in our rat liver and vegetable extracts.

Using thin-layer chromatography, however, (silica gel 60F-254 precoated plates, solvent : 2-propanol/water 70/30 v/v, detection with freshly prepared iodo-azide and subsequently with 1 % starch solution), we could demonstrate that none of our extracts contained cysteine or methionine, or at least not in a concentration which could explain their antimutagenic effect.

DISCUSSION

The path of the curve which reflects the S-9 mix deactivation of sodiumazide (controls in Fig. 1 and 2) is in good agreement with the results of DE FLORA (1978); the path as such gives a first indication that this interaction is an enzymatic one. The fact that the S-9 mix from uninduced rats is less inhibitive gives further support to this hypothesis. Finally, the NADPH dependence also confirms the same statement. One could argue that this NADPH effect is small, but it should be stressed that the substrate forms a limiting factor in our experiments (only 1 µg per plate). On the basis of analogous results, LÖFROTH (1978) assumes an enzymatic interaction in the S-9 mix inhibition of the mutagenicity of hexavalent chromium. MORIYA et al. (1978), on the contrary, studying the inhibition of mutagenic pesticides, suggest, that the mutagenic activities of these pesticides are destroyed by compounds that contain the sulfhydryl group, rather than by metabolism. However, they overlooked, that, in the first place they too had found a greater inhibition with the S-9 mix than with the S-9 solely, and secondly, they had used a cysteine concentration as high as 20 mM. Add to this that, in our hands, sulfhydryl compounds as glutathione and cysteamine do not have any antimutagenic effect at all.

That azide is not a carcinogen (AMES et al. 1975), could be explained, in our opinion, by the fact that it is easely deactivated by liver enzyme(s).

Regarding the vegetable antimutagenic factors, we are only informed about the report from KADA et al. (1978), who found an antimutagenic action of vegetable factor(s) obtained from cabbage, radish, turnip, and ginger on the mutagenic principle of tryptophan pyrolysate. Previously KADA (1977) had shown that cauli-

flower, pumpkin, and cabbage contain antimutagenic factor(s) for N-butyl-N-(acetoxymethyl)nitrosamine. Unlike the S-9 mix, we found a linear correlation between the antimutagenic effect and the quantity of added spinach- and leek extract. This means, together with the fact that we could not observe any NADPH dependence, that the active antimutagenic factor from vegetables is a non-enzymatic one. As follows from our chromatographic investigations this factor is not identical with cysteine nor with methionine. Our active vegetable extracts are not only heat sensitive, as these from KADA et al. (1978), but they are also fairly labile, when stored at -20°C.

The antimutagenic properties of cysteine have recently been well documented for chemically very different mutagens (ABRAMOVSKY et al. 1978; ROSIN and STICH 1978; MORIYA et al. 1978). The same does not apply to methionine. From our observation that cysteamine has no anti-sodiumazide-mutagenic properties at all, it follows that antimutagens have to fulfil well defined molecular conditions, which can of course vary for different mutagens. As conjugation with glutathione is generally considered to be a very important detoxication pathway (JAKOBY 1976), while glutathione in our experimental conditions has no antimutagenic effect, it is questionable whether cysteine in natural conditions could behave as an antimutagen.

In any case, it is fortunate that we have antimutagenic factors in our natural environment. The results reported here indicate that at least two inactivation pathways for the mutagen sodiumazide are possible. Research is in progress to elucidate each of them.

ACKNOWLEDGMENT

I thank Gerda Du Buisson-Jenar for her excellent technical assistance.

REFERENCES

ABRAMOVSKY, I., G. VORSANGER, and K. HIRSCHHORN : Mut. Res. 50, 93 (1978).
AMES, B. N., J. McCANN, and E. YAMASAKI : Mut. Res. 31, 347 (1975).
DE FLORA, S. : Nature 271, 455 (1978).
JAKOBY, W. B. : p. 207. In F. J. DE SERRES, J. R. FOUTS, J.R. BEND, and R. M. PHILPOT (Eds.) : In vitro metabolic activation in mutagenesis testing. Elsevier/North-Holland, Amsterdam, 1976.
KADA, T. : Proc. Ann. Meeting Agr. Chem. Soc. Japan 444 (1977).
KADA, T., K. MORITA, and T. INOUE : Mut. Res. 53, 351 (1978).
LÖFROTH, G. : Naturwissenschaften 65, 207 (1978).
McCANN, J., E. CHOI, E. YAMASAKI, and B. N. AMES : Proc. Nat. Acad. Sci. USA 72, 5135 (1978).
MORIYA, M., K. KATO, and Y. SHIRASU : Mut. Res. 57, 259 (1978).
NILAN, R. A., E. G. SIDERIS, A. KLEINHOFS, C. SANDER, and C. F. KONZAK : Mut. Res. 17, 142 (1973).
ROSIN, M. P., and H. F. STICH : Mut. Res. 54, 73 (1978).