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Studies on the mutagenicity of nitrofurans in *Salmonella typhimurium*

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The *Salmonella* mutagenicity procedure developed by Ames and his associates [1] has found wide applicability in the detection and classification of mutagens. Results obtained with this assay also have a predictive value to identify carcinogens as a recent analysis reveals a remarkable correlation between mutagenicity in this assay and carcinogenicity in animals [2, 3]. Our laboratory has been concerned with an evaluation of short-term mutagenicity procedures and with gaining an understanding of the lack of reactivity in the *Salmonella* assay of substances reported to be mutagenic in other systems and to be carcinogenic as well [4]. In the present report we are concerned with nitrofurans which are mutagenic in *E. coli* and *Neurospora crassa* and carcinogenic in laboratory animals [5–11] but which were found to be non-mutagenic in the *Salmonella* assay when the original tester strains (TA1535, TA1538 etc.) were used [6, 8, 11]. More recently strain TA100, a derivative of TA1535, bearing a plasmid was constructed [12], this tester strain displays enhanced sensitivity to a number of mutagens and indeed nitrofurans exhibit mutagenicity in this strain [2, 12–15]. Still the question remains why nitrofurans are non-reactive in the original tester strains and why other known mutagens, carcinogens and DNA-modifiers are non-mutagenic in the original as well as the newer tester strains [4]. An elucidation of this lack of reactivity might help in the development of tester strains that are even more universal than TA100 (and TA 98 [12]).

Previous studies [4, 16–18] with strongly antimicrobial agents endowed with DNA-modifying activity have revealed that some of these agents are not detected as mutagens in the standard *Salmonella* assay because their antibacterial action which is exerted over a 48 hour period allows too few survivors to permit the scoring of mutants. Moreover, frequently the concentration for optimal mutagenicity is very close to or actually overlaps with the maximal antibacterial level. In this connection it must be remembered that in the standard *Salmonella* assay results are expressed as mutants per plate and not as mutants per survivors. We have shown that the mutagenic potential of some antimicrobial agents could be demonstrated when bacteria in liquid suspension are exposed for limited periods to the agent and then processed for the determination of the number of mutants and of survivors [4, 16–18]. In this report we show that nitrofurans are mutagenic for the original *Salmonella* tester strains when tested by this procedure.

For the present study two carcinogenic nitrofurans: [*N*(4-(5-nitro-2-furyl)-2-thiazolyl] formamide (NFTF) and 2-(2,2-dimethylhydrazino)-4-(5-nitro-2-furyl)thiazole) (DMNT) were chosen. Neither of these substances was mutagenic for *Salmonella* strains G46, TA1530, TA1535, TA1978 and TA1538 when tested by the two modifications of the Ames procedure [1, 19]: the spot test in which the bacteria are incorporated into the agar overlay and the test agent is placed onto the surface of the agar or the quantitative procedure in which the tester bacteria together with known amounts of the test agent are placed in the agar overlay (results not shown).

These findings confirm the results of other investigators [6, 8, 11]. When, however, the bacteria were exposed to the test agents in liquid suspension then the bacteria washed free of the chemical and processed for determination of the number of survivors and of mutants by plating on selected media, the mutagenicity of the two nitrofurans was readily demonstrable (Table 1). Moreover it was found that under these testing procedures the test agents induced mutations of both the base-substitution and the frameshift type. Agents with known mutagenic specificities were also tested, these retained their specificities, e.g. nitrofluorene induced mutations of the frameshift type only while ethylmethanesulfonate gave rise to base-substitution mutations (Table 1).

Neither of the two nitrofurans exhibited (Table 1) significant mutagenicity when tested with tester strains that are deficient in nitro reductase activity [20].

The present findings indicate that under *modified conditions* the mutagenicity of nitrofurans for the original *S. typhimurium* tester strains can be demonstrated. This is not unexpected in view of the demonstrated mutagenicity of these substances (see above). It was, however, puzzling to find that the two nitrofurans tested were capable of inducing mutations of the base-substitution as well as of the frameshift type. This in turn suggests that nitrofurans are capable of interacting with cellular DNA in at least two manners. The chemical basis of this dual effect and its possible relevance to the carcinogenic process remain to be elucidated. It is noteworthy that both events are dependent upon a metabolic conversion involving the nitro function (see also refs. 15 and 20), as they are both abolished when nitro reductase deficient tester strains are used. It should be noted that evidence of a dual mutagenic action of nitrofuran derivatives exists in the literature. Thus the *E. coli* strain used by McCalla *et al.* [6] and others [8] for demonstrating the mutagenic activity of nitrofurans is one which responds to base-substitution mutations [6] while the earlier report of Zampieri and Greenberg [9] which demonstrated the mutagenicity of nitrofurazone for *E. coli*, showed that this chemical was capable of reversing mutations induced by proflavin, which indicates that this nitrofuran is a frameshift mutagen.

The present findings together with the fact that these nitrofurans are endowed with DNA-modify activity in *E. coli* and *Salmonella* systems as evidenced by their ability to preferentially inhibit the growth of DNA repair-deficient bacteria [11] and unpublished results), suggest that in routine testing the *Salmonella* mutagenicity assay should be used in tandem with bacterial systems capable of detecting DNA-modifying activity. Substances that are positive in a DNA-modifying assay but negative by the Ames procedure may then be tested in liquid suspension. It is further noteworthy that certain agents (e.g. 1,1,2,2-tetrabromethane, sodium hypochlorite) that are DNA-modifiers [16, 17], but non-mutagenic in the standard Ames assay using TA1535 and TA1538 are also non-mutagenic when tested under standard conditions with TA100 (unpublished results). These substances are, however, mutagenic for TA1530, TA1535 and TA100, when tested in liquid suspension ([16, 17] and unpublished results). These findings suggest that additional tester strains be developed.

Table 1. Mutagenicity of nitrofurans

Time (Min)	Agent	Amount	TA1535 viable cells/ml	TA1535 mutants per 10^8	TA1538 viable cells/ml	TA1538 mutants per 10^8	TA1535-FR-1 viable cells/ml	TA1535-FR-1 mutants per 10^8	TA1538-FR-2 viable cells/ml	TA1538-FR-2 mutants per 10^8
0	DMNT	0.5 μg/ml	3.8×10^8	9	4.1×10^8	12	2.2×10^8	7.3	2.9×10^8	14.1
20			2.4×10^8	18	3.3×10^8	35				
40			1.5×10^8	33	2.0×10^8	55	5.1×10^8	6.9	5.4×10^8	14.3
60			3.3×10^7	176	1.3×10^8	69				
80			1.8×10^7	395	1.1×10^8	91				
0	DMNT	25 μg/ml	1.8×10^8	9	1.8×10^8	11.7	2.8×10^8	9.4	1.7×10^8	11.8
20			1.5×10^6	—	2.6×10^5	—	3.3×10^8	11.8	2.3×10^8	13.5
0	NFTF	0.5 μg/ml	2.4×10^8	10.0	1.9×10^8	11.6	1.5×10^8	9.3	2.6×10^8	10.4
20			1.9×10^8	17	1.5×10^8	39.3				
40			1.2×10^8	26	1.3×10^8	54.6	2.7×10^8	8.9	3.7×10^8	10.5
60			1.0×10^8	35	1.1×10^8	67.3				
80			7.6×10^7	50	9.7×10^7	81.4				
0	NFTF	25 μg/ml	2.5×10^8	10	1.1×10^8	11.0	2.9×10^8	8.3	4.1×10^8	12.0
20			7.4×10^6	—	7.3×10^4	—	3.6×10^8	10.6	4.9×10^8	13.3
0	Ethyl methanesulfonate	0.005 M	3.5×10^8	9	4.4×10^8	11.6				
20			8.7×10^7	28	1.3×10^8	9.2				
0	2-Nitrofluorene	25 μg/ml	2.8×10^8	8.2	3.1×10^8	10.3				
20			2.7×10^8	8.1	2.8×10^8	28.9				

* *Procedure*: Bacteria (*S. typhimurium*) in Columbia-base broth (BBL) were grown to the middle of the exponential growth phase at which time portions of the cultures were distributed into sterile centrifuge tubes which were chilled in an ice bath (4°). Each tube was supplemented with a known amount of the test agent. At intervals tubes were withdrawn, the bacteria harvested by centrifugation, washed twice with Columbia-base broth, resuspended in their original volume and processed for determination of the number of total viable cells by plating 0.1 ml-dilutions in duplicate on Columbia agar. Revertants to histidine prototrophy (mutants) were determined by plating 0.2 ml-portions of undiluted cultures on each of ten plates consisting of minimal media containing biotin and a trace of histidine [1, 19], respectively. Mutation frequency was calculated on the basis of a minimal count of 50 revertants per 10 plates. The plates were incubated in the dark [21] at 37° for 2 days and colonies enumerated.

TA1535-FR-1 and TA1538-FR-2 are derivatives of TA1535 and TA1538 which are deficient in nitro reductase [20].

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The effect of salicylate on the activity of acetyl-CoA carboxylase in rat liver

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One of the characteristics of the widely used pharmacological drug—salicylate—is its effect on lipid metabolism, particularly, on lipid synthesis. It has been established that salicylate administered intraperitoneally to rats in doses of 200–500 mg/kg inhibits the incorporation of the labelled acetate into fatty acids of the liver [1]. Goldman[2] has shown that salicylate in concentrations of 10^{-4} and 10^{-3} M inhibits by 30 and 90 per cent respectively the synthesis of fatty acids in the soluble fraction of rat liver.

In our laboratory it has been shown that salicylate inhibits the incorporation of [1-^{14}C]acetate into the total unsaponifiable lipids and fatty acids but does not affect the incorporation of [2-^{14}C]mevalonate into cholesterol by the supernatant fraction (700 *g*) of rat liver homogenate [3]. Later it was shown that salicylate in concentration of 10^{-2} M almost completely inhibits the incorporation of [1-^{14}C]acetyl-CoA into unsaponifiable lipids and decreases markedly (by 65–79 per cent) incorporation of this substrate into fatty acids. However, the incorporation of [2-^{14}C]malonyl-CoA into unsaponifiable lipids and fatty acids is not inhibited by salicylate [4, 5]. It was assumed that salicylate acts upon the stage of carboxylation of acetyl-CoA, inhibiting the key enzyme of fatty acid synthesis—acetyl-CoA carboxylase (Acetyl-CoA: CO_2 ligase (ADP), E.C.6.4.1.2).

The present research concerns the effect of salicylate on the activity of acetyl-CoA carboxylase in the partially purified soluble fraction of rat liver. In the experiments *in vitro* salicylate was added directly to the soluble fraction; in the experiments *in vivo* salicylate was administered intraperitoneally.

METHODS

Wistar male rats (150–200 g) were used and kept on the usual laboratory ration. To study the possible inhibition of carboxylase activity by salicylate *in vivo* potassium salicylate was administered intraperitoneally twice, 14 and 2 hr prior to decapitation, in a dose of 250 mg per 1 kg of body wt. The control animals received saline twice. After decapitation of the animals the blood in the liver was washed out with physiological saline through *V. portae* and the pooled 4–8 livers passed through a tissue press to remove the connective tissue and then homogenized in phosphate buffer solution, pH 7.4, containing EDTA and 2-mercaptoethanol [6]. All procedures were conducted at 0°. The homogenate was centrifuged at 700 *g* for 10 min. The resulting supernatant was centrifuged twice at 12,000 *g* for 15 min and once at 140,000 *g* for 60 min, and then subjected to gel filtration in a Sephadex G-25 column to remove