Mutation Research, 156 (1985) 171–176
Elsevier

MTR 00971

Dietary factors affecting the urinary mutagenicity assay system

II. The absence of mutagenic activity in human urine following consumption of red wine or grape juice *

Judith Sousa [1,2], Joginder Nath [1] and Tong-man Ong [1,2]

[1] *Division of Respiratory Disease Studies, National Institute for Occupational Safety and Health, Morgantown, WV and* [2] *Division of Plant and Soil Sciences, West Virginia University, Morgantown, WV 26506 (U.S.A.)*

(Received 31 May 1984)
(Revision received 12 November 1984)
(Accepted 4 January 1985)

Summary

The mutagenic activity of urine samples from nonsmoking individuals before and after the consumption of either red wine or grape juice was determined. Urine samples collected from individuals on liquid or regular diets were concentrated using XAD-2 resin. No mutagenic activity of urine concentrates was detected with Salmonella tester strains TA98 or TA100 with or without microsomal activation. The addition of 1000 units of β-glucuronidase into the agar overlay did not show any mutagenic activity. The mutagens in red wine and grape juice, however, were extracted using the XAD-2 column. Concentrates of urine samples spiked with either of the two extracts exhibited mutagenic activity.

In a previous study (Sousa et al., 1984), mutagenic activity was detected in urine samples obtained from nonsmoking individuals who had consumed a fried beef meal. Urinary mutagenic activity was present up to 16–24 h following the meal. Baker et al. (1982) reported urinary mutagenic activity in urine samples obtained from individuals following either a fried pork or bacon meal. Mutagenic activity was reported to last as long as 24 h after the meal. these studies indicate that the consumption of mutagen-containing foods can lead to the excretion of mutagens in the urine. This would affect the urinary mutagenicity assay system used to determine whether human subjects have been exposed to mutagenic and/or potential carcinogenic compounds in occupational settings.

Several beverages, both alcoholic and non-alcoholic, have been shown to exhibit mutagenic activity in the Ames Salmonella/microsome assay (Lee and Fong, 1979; Loquet et al., 1981; Nagao et al., 1981; Stoltz et al., 1982). By screening 28 commonly consumed beverages, Stoltz et al. (1982) found that grape juice, red wine, and instant coffee exhibited marked mutagenic activity. In studies

* Results of cooperative investigation of NIOSH and West Virginia Agricultural and Forestry Experimental Station. Published with the approval of the Director of the West Virginia Agricultural and Forestry Experiment Station as Scientific Paper No. 1898.

Address correspondence to: Dr. Tong-man Ong, National Institute for Occupational Safety and Health, 944 Chestnut Ridge Road, Morgantown, WV 26505 (U.S.A.).

Abbreviations: 2AA, 2-aminoanthracene; βG, β-glucuronidase; DMSO, dimethyl sulfoxide; DCM, methylene chloride; ml-eq., milliliter equivalents of unconcentrated sample.

related to fecalase, Tamura et al. (1980) found that red wine and grape juice were strongly mutagenic when the XAD-2 concentrates of both beverages were tested with this enzyme. Without fecalase, the concentrate of grape juice was not mutagenic. In our laboratories, studies were conducted to determine (1) whether the extracts of red wine or grape juice from local stores are mutagenic, (2) whether mutagens can be recovered from urine spiked with red-wine and grape-juice extracts, and (3) if the urine from individuals who consumed red wine or grape juice is mutagenic.

Materials and methods

Extraction of mutagens from red wine and grape juice

Red wine and red grape juice were obtained from a local supermarket. Red wine (1500 ml) or grape juice (2360 ml) were extracted using washed Amberlite XAD-2 resin (Sulpelco, Inc., Sulpelco Park, Bellefonte, PA). Each bottle was passed through a 15-ml column of XAD-2 resin at a flow rate of 3–4 ml/min. After rinsing with 20 ml of distilled water, N_2 gas was forced through the column to remove the excess water and then eluted with 20 ml of DCM followed by 20 ml of acetone. The eluate was dried under N_2 gas at 45°C and redissolved in 0.8 ml of DMSO/100-ml sample. The extracts were stored at −20°C.

Collection of urine samples

All urine samples were obtained from nonsmoking individuals. For the spiking experiments, 1–2 day samples were collected and stored at 4°C between collections. For the liquid-diet studies, the subjects refrained from fried foods, alcoholic beverages, and grape juice for 24 h and then fasted for 12 h. During the fast, only coffee, tea, 2% lowfat white milk, fruit and vegetable juices, soft drinks, water and sugar were permitted. A control sample was obtained during the last 6 h of the fast. At the end of the 12-h fast, subjects consumed either red wine (750 ml) or grape juice (1180 ml) in 3 h. Urine samples were collected 0–3, 3–6, 6–10, 10–17 or 17–24 h beginning from the start of consumption of either the wine or juice. The liquid diet was continued. For the regular diet experiments, successive urine collections were obtained from individuals on a regular diet which omitted fried foods, red wine and grape juice for 24 h. Following collection of a 6-h control sample, each subject consumed either a 750-ml bottle of red wine or a 1180-ml bottle of grape juice in a 3-h period. All urine samples collected 0–5, 5–13 and 13–24 h after the start of consumption were stored separately at 4°C and were extracted within 2 days after collection.

Sample preparation of the spike urine

1–2 day urine samples were pooled, filtered through a Whatman No. 1 filter, and adjusted to pH 7.0. The pooled sample was divided into several 400-ml subsamples which were then spiked with DMSO or red-wine or grape-juice extracts equivalent to a 750-ml bottle or a 1180-ml bottle of red wine or grape juice, respectively. All extracts were prepared within 2 days prior to being 'spiked' into urine samples. The extract spiked-samples were then concentrated immediately.

Concentration of urine samples

Urine samples were extracted according to the procedure of Yamasaki and Ames (1977). Each sample was vacuum-filtered through a Whatman No. 1 filter, adjusted to pH 7.0, and applied to a 15-ml column of washed XAD-2 resin (approximately 4 g dry weight) at a flow rate of 3–4 ml/min. After washing with 20 ml of distilled water, N_2 gas was forced through the column to remove any excess water. The column was then eluted with 20 ml of DCM followed by 20 ml of acetone. The eluate was dried under N_2 gas at 45°C and redissolved in 0.4 ml of DMSO/100 ml of urine. All urine concentrates were stored at −20°C and tested within 3 days for mutagenic activity.

Mutagenesis assay

Extracts of the red wine, grape juice, and urine concentrates were tested for mutagenic activity with the Ames Salmonella/microsome assay system (Ames et al., 1975). In brief, 0.1 ml of tester cells from an overnight culture and 0.2 ml of test material or DMSO (control) were added to 2 ml molten soft agar containing biotin and a trace amount of histidine. The mixture was overlayed onto a VB minimal agar plate. For the metabolic

activation assay, 0.5 ml S9 mix was also added to the soft agar. The S9 was prepared from the liver of Aroclor-1254 (500 mg/kg body weight) pretreated male Wistar rats. For the deconjugation assay, 0.1 ml of a 10000 units/ml solution of *Escherichia coli* βG Type IX (Sigma Chemical Co., St. Louis, MO) was added to the soft agar. The number of revertant colonies was determined after the overlayed plates were incubated at 37°C for 2 days. Urine concentrates equivalent to 3.125, 6.25, 12.5, 25.0 and 50.0 ml of whole urine and wine and grape-juice extracts equivalent to 6.25, 12.5, 25.0 and 37.5 ml of sample were tested with the plate-incorporation assay using tester strains TA98 and TA100.

Results

The results of the XAD-2 extraction of mutagens from red wine are shown in Table 1. The red-wine extracts displayed toxic and mutagenic activities when tested with TA98. Although microsomal activation was not a requirement for activity, an increased (1.8-fold) response was observed in the presence of S9. When TA100 was plated with and without S9, an increase in the number of revertant colonies over that of the solvent control was observed. However, the increase was less than 2-fold and there was no dose-related response.

The red grape juice extract also displayed mutagenic activity when plated with TA98 (Table 2). As with the red-wine extract, microsomal activation was not required for activity, although an increased response was noted in its presence. When TA100 was tested with and without S9, no mutagenic response was noted for the grape-juice extract.

The results of the 'spiking' experiments are shown in Table 3. When the red-wine extract was spiked into the urine samples, mutagens could be

TABLE 1

MUTAGENICITY OF RED WINE IN *S. typhimurium* [a]

Concentration of wine extract plated (ml-eq. of wine)	Revertants/plate			
	TA98	TA98 S9 [b]	TA100	TA100 S9
Solvent control (0.3 ml DMSO)	21	25	174	185
6.25	76	169	251	285
12.5	116	210	246	261
25.0	104	255	186	269
37.5	72	229	155	269

[a] TA98 and TA100 were tested with and without S9. The results are the mean of 3 Expts. unless noted otherwise.
[b] The results are the mean of 4 Expts.

TABLE 2

MUTAGENICITY OF RED GRAPE JUICE IN *S. typhimurium* [a]

Concentration plated (ml-eq. of grape juice)	Revertants/plate			
	TA98	TA98 S9 [b]	TA100	TA100 S9
Solvent control (0.3 ml DMSO)	11	24	177	185
6.25	54	106	225	243
12.5	72	133	220	250
25.0	101	183	226	226
37.5	97	207	238	214

[a] TA98 and TA100 were tested with and without S9. The results are the mean of 3 Expts. unless noted otherwise.
[b] The results are the mean of 4 Expts.

TABLE 3

MUTAGENIC ACTIVITY OF URINE SAMPLES SPIKED WITH RED-WINE OR GRAPE-JUICE CONCENTRATES IN *S. typhimurium* TA98 [a]

Sample tested	Urine concentration plated (ml-eq.)	ml-eq. of red wine or grape juice	Revertants/plate
Control urine sample (0.2 ml DMSO)	50.0		24
Red wine	3.125	5.9	120
	6.25	11.7	158
	12.5	23.4	191
	25.0	46.9	217
Grape juice	6.25	7.8	150
	12.5	15.6	192
	25.0	31.3	257
	50.0	62.5	325
Solvent control (0.2 ml DMSO)			19

[a] TA98 was tested with S9. The results shown are the mean of 3 Expts.

TABLE 4

MUTAGENICITY ASSAY FOR URINE SAMPLES FROM 5 SUBJECTS ON A REGULAR DIET FOLLOWING THE CONSUMPTION OF RED WINE [a]

Subject	Sample collected [b]	Concentration plated (ml-eq.)	Revertants/plate		
			TA98	TA98S9βG [c]	TA98S9
A, male	Before wine	25.0	17	31	36
	0–5 h after	25.0	*	36	31
	5–13 h after	25.0	11	36	23
	13–21 h after	25.0	15	*	25
B, male	Before wine	25.0	*	31	25
	0–5 h after	25.0	*	35	23
	5–13 h after	25.0	7	33	26
	13–21 h after	25.0	*	*	*
C, female	Before wine	25.0	15	32	30
	0–5 h after	25.0	17	38	32
	5–13 h after	25.0	*	33	28
	13–21 h after	25.0	18	33	34
D, male	Before wine	25.0	18	38	32
	0–5 h after	25.0	16	36	32
	5–13 h after	25.0	15	30	34
	13–21 h after	25.0	8	30	36
E, female	Before wine	25.0	17	*	29
	0–5 h after	25.0	19	*	39
	5–13 h after	25.0	20	*	26
	13–21 h after	25.0	17	*	26
Solvent control	(0.1 ml DMSO)		16	25	26

[a] The results shown are the average of 2 plates.
[b] Numbers shown are hours after red-wine consumption.
[c] 1000 units of βG was added into the agar overlay.
* Not tested.

recovered by the urine-concentration procedure. The percent recovery averaged 59.4 for the highest concentration plated (data not shown). When the grape-juice extract was spiked into the urine specimen, the 'spiked' sample induced 50% more revertant colonies than the corresponding stock solution. The recovery was therefore over 100% for all concentrations tested (data not shown).

Studies of the urinary excretion of mutagenic activity in individuals on liquid diets did not show an increase in the number of TA98 or TA100 revertant colonies following the consumption of either red wine or grape juice (data not shown). At no point during the 24 h following the consumption of either the red wine (Table 4) or grape juice (Table 5) was there any indication of an increase in the number of revertant colonies or a dose-related response in the urine samples obtained from individuals on the regular diet. The addition of rat-liver S9 or βG did not show any activity.

Discussion

Flavonoid compounds such as quercetin and kaempferol and their glycosides are commonly found in American and European grapes. Quercetin, kaempferol and other flavonoid compounds have been reported to exhibit mutagenic activity in Ames tester strains TA98 and TA100 (Brown and Dietrich, 1979; Brown et al., 1977; Hardigree and Epler, 1978; MacGregor and Jurd, 1978; Sugimura et al., 1977). The studies reported here indicate that XAD-2 resin columns can be used to extract the mutagenic components of red wine and grape

TABLE 5

MUTAGENICITY ASSAY FOR URINE SAMPLES FROM 5 SUBJECTS ON A REGULAR DIET FOLLOWING THE CONSUMPTION OF RED-GRAPE JUICE [a]

Subject	Sample collected [b]	Concentration plated (ml-eq.)	Revertants/plate	
			TA98	TA98S9
A, male	Before juice	50	18	17
	0–5 h after	50	10	20
	5–13 h after	50	15	24
	13–21 h after	50	14	22
E, female	Before juice	50	15	31
	0–5 h after	50	16	27
	5–13 h after	50	17	*
	13–21 h after	50	20	21
F, male	Before juice	25 [c]	*	30
	0–5 h after	25	*	21
	5–13 h after	25	*	30
	13–21 h after	25	*	27
G, female	Before juice	25 [c]	12	26
	0–5 h after	25	14	24
	5–13 h after	25	*	39
	13–21 h after	25	12	20
H, female	Before juice	50	14	21
	0–5 h after	50	14	20
	5–13 h after	50	17	21
	13–21 h after	50	22	23
Solvent control	(0.1 ml DMSO)		15	22

[a] The results shown are the average of 2 plates per dose.
[b] Numbers shown are hours after grape-juice consumption.
[c] Only 25 ml-eq. of urine was tested due to extract limitations.
* Not tested.

juice. Mutagenic activity towards Salmonella strain TA98 was observed in XAD-2 red-wine and grape-juice concentrates. Mutagenic activity was observed without metabolic activation, although metabolic activation markedly enhanced activity. A similar effect occurs with pure flavonoid compounds. Quercetin, myricetin, rhamnetin and 5,7-di-*O*-methylquercetin are active without microsomal activation, but they are more effective in the presence of rat-liver microsomes (MacGregor and Jurd, 1978; Hardigree and Epler, 1978). All other flavonoids require metabolic activation for activity. The percent mutagen recovery, based on mutagenicity data, indicate that the red-wine and grape-juice mutagens can be recovered from urine. The recovery was 59.4% and 144.6% for the red wine and grape juice, respectively. The results with the grape-juice extract seem to indicate that the urine can influence the activity of the concentrate. An increase in mutagenic activity was also observed by us (unpublished results) when aflatoxin B_1 was spiked into the urine of a nonsmoking individual.

The data reported here indicate that following the consumption of either red wine (750 ml) or grape juice (1180 ml), which presumably contain quercetin and kaempferol, there is no observable mutagenic activity in urine samples obtained up to 24 h following their consumption. This is in agreement with the findings of other investigators (Clark and Mackay, 1950; Booth et al., 1956; deEds, 1968; Gugler et al., 1975). Their studies indicate that following oral administration of quercetin and other flavonoid compounds, these compounds could not be recovered in the urine of the treated

animals or human subjects nor was mutagenic activity detected in the urine of these subjects. However, when quercetin was injected intravenously, quercetin and a conjugated metabolite were detected in human urine (Gugler et al., 1975). In a more recent study, MacGregor (1979) reported mutagenic actvity in rat urine after both intraperitoneal and oral administration of quercetin. These results, however, indicate that quercetin or its mutagenic metabolites are absorbed and excreted in the urine following ingestion. It should be noted that the rats in MacGregor's study were given a much greater dose per kg body weight than that used in human studies.

Although the results reported in the literature are contradictory, some studies have shown that an incorporation of βG into the assay system enhanced the mutagenic activity of urine from smokers (Russell and Krahn, 1981). In our study no effect was found when βG was incorporated. Since no proper control for the incorporation of βG in the human studies is available, our negative results cannot rule out the possibility that mutagens in a conjugate form were excreted by subjects who had consumed grape juice or red wine. Nevertheless, our data indicate that the ingestion of either 750 ml of red wine or 1180 ml of grape juice or less do not interfere with the urine mutagenicity assay system and therefore these beverages need not be restricted when implementing these studies.

Acknowledgements

The authors wish to thank Professor Bruce N. Ames for the gift of the tester strains, Messrs. John Stewart and Bryon Burchell for their technical assistance and Ms. Kay Kennedy for typing the manuscript.

References

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test, Mutation Res., 31, 347–364.

Baker, R., A. Arlauskas, A. Bonin and D. Angus (1982) Detection of mutagenic activity in human ruine following fried pork or bacon meals. Cancer Lett., 16, 81–89.

Booth, A.N., C.W. Murray, F.T. Jones and F. deEds (1956) The metabolic fate of rutin and quercetin in the animal body, J. Biol. Chem., 233, 251–257.

Brown, J.P., and P.S. Dietrich (1979) Mutagenicity of plant flavonols in the Salmonella/mammalian microsome test, Activation of flavonol glycosides by mixed glycoside from rat fecal bacteria and other sources, Mutation Res., 66, 223–240.

Brown, J.P., P.S. Dietrich and R.J. Brown (1977) Frameshift mutagenicity of certain naturally occurring phenolic compounds in the 'Salmonella/microsome' test: Activation of anthraquinones and flavonol glycoside by gut bacterial enzymes, Biochem. Soc. Trans., 5, 1489–1492.

Clark, W.G., and E.M. MacKay (1950) The absorption and excretion of rutin and related flavonoid substances, J. Am. Med. Ass., 143, 1411–1415.

deEds, F., Flavonoid metabolism, in: M. Florkin and E.H. Stotz (Eds.), Comprehensive Biochemistry, Vol. 20, Elsevier, Amsterdam, 1968, Chapter II.

Gugler, R., M. Leschik and H.J. Dengler (1975) Disposition of quercetin in man after single oral and intravenous doses, Eur. J. Clin. Pharmacol., 109, 229–234.

Hardigree, A.A., and J.L. Epler (1978) Comparative mutagenesis of plant flavonoids in microbial systems, Mutation Res., 58, 231–239.

Lee, J.S.K., and L.Y.Y. Fong (1979) Mutagenicity of Chinese alcoholic spirits, Fd. Cosmet. Toxicol., 17, 575–578.

Loquet, C., G. Toussaint and J.Y. LeTalaer (1981) Studies on mutagenic constituents of apple brandy and various alcoholic beverages collected in western France, a high incidence area for oesophageal cancer, Mutation Res., 88, 155–164.

MacGregor, J.T. (1979) Mutagenicity studies of flavonoids in vivo and in vitro, Toxicol. Appl. Pharmacol., 48, A47.

MacGregor, J.T., and L. Jurd (1978) Mutagenicity of plant flavonoids: Structural requirements for mutagenic activity in *Salmonella typhimurium*, Mutation Res., 54, 297–309.

Nagao, M., Y. Takahashi, K. Wakabayashi and T. Sugimura (1981) Mutagenicity of alcoholic beverages, Mutation Res., 88, 147–154.

Russell, J.F., and D.F. Krahn (1981) Development of protocol for evaluating the mutagenic activity of human urine in the *Salmonella typhimurium* suspension assay, Environ. Mutagen., 3, 352.

Sousa, J., J. Nath and T. Ong. (1984) Detection of urinary mutagenic activity following a fried beef meal, Environ. Mutagen., 6, 476.

Stoltz, D.R., B. Stavric, D. Krewski, R. Klassen, R. Bendall and B. Junkins (1982) Mutagenicity screening of foods, I. Results with beverages, Environ. Mutagen., 4, 477–492.

Sugimura, T., M. Nagao, T. Matsushima, T. Yahagi, Y., Seino, S. Shirai, M. Sawamura, S. Natori, K. Yodhihira, M. Fukuoka and M. Kuroyanagi (1977) Mutagenicity of flavone derivatives, Proc. Jpn. Acad., 53, Ser. B, 194–197.

Tamura, G., C. Gold, A. Ferro-Luzzi and B.N. Ames (1980) Fecalase: A model for activation of dietary glycosides to mutagens by intestinal flora, Proc. Natl. Acad. Sci. (U.S.A.) 77, 4961–4965.

Yamasaki, E., and B.N. Ames (1977) Concentration of mutagens from urine by adsorption with the non-polar resin XAD-2: Cigarette smokers have mutagenic urine, Proc. Natl. Acad. Sci. (U.S.A.), 74, 3555–3559.