Proc. Natl. Acad. Sci. USA
Vol. 77, No. 8, pp. 4961–4965, August 1980
Medical Sciences

Fecalase: A model for activation of dietary glycosides to mutagens by intestinal flora

(red wine/tea/quercetin/carcinogenic glycosides/glycosidases)

GLEN TAMURA, CHERYL GOLD, ANNA FERRO-LUZZI*, AND BRUCE N. AMES

Department of Biochemistry, University of California, Berkeley, California 94720

Contributed by Bruce N. Ames, May 12, 1980

ABSTRACT **Many substances in the plant kingdom and in man's diet occur as glycosides. Recent studies have indicated that many glycosides that are not mutagenic in tests such as the *Salmonella* test become mutagenic upon hydrolysis of the glycosidic linkages. The *Salmonella* test utilizes a liver homogenate to approximate mammalian metabolism but does not provide a source of the enzymes present in intestinal bacterial flora that hydrolyze the wide variety of glycosides present in nature. We describe a stable cell-free extract of human feces, fecalase, which is shown to contain various glycosidases that allow the *in vitro* activation of many natural glycosides to mutagens in the *Salmonella*/liver homogenate test. Many beverages, such as red wine (but apparently not white wine) and tea, contain glycosides of the mutagen quercetin. Red wine, red grape juice, and tea were mutagenic in the test when fecalase was added, and red wine contained considerable direct mutagenic activity in the absence of fecalase. The implications of quercetin mutagenicity and carcinogenicity are discussed.**

Damage to DNA by environmental mutagens, both natural and man-made, is likely to be a major cause of cancer and other diseases (1, 2). The *Salmonella* test (3), along with other short-term tests (4), is being used to survey a wide variety of substances in our environment for mutagenicity. The test measures back mutation in several specially constructed mutants of *Salmonella* bacteria. A homogenate of rat liver (or other mammalian liver) is added to the (*Salmonella*) test as an approximation of mammalian metabolism (3). By using this system, approximately 85% ± 5% of the organic carcinogens tested have been detected as mutagens (5–9).

Recent studies indicate that many naturally occurring glycosides of mutagenic aglycones are not mutagenic in the *Salmonella* test (10–23). These glycosides are hydrolyzed by the bacteria in the human intestine where the mutagen is liberated. They are not cleaved by the liver or by liver homogenates used in the *Salmonella* test. For example, cycasin, a β-D-glucoside of the mutagen methylazoxymethanol, is not mutagenic in the *Salmonella* test unless β-glucosidase is added to the standard test (20, 21). Cycasin is a carcinogen in rats but is not a carcinogen when tested with germ-free rats (24, 25), which lack the microorganisms that cleave the sugar from the mutagenic moiety.

An enormous variety of substances are present as glycosides in the plant kingdom (10–27). It is desirable to have an enzyme preparation for use in mutagenicity tests that will hydrolyze the hundreds of sugars in these glycosides. Brown *et al.* (10–12) have developed a cell-free extract of rat cecal bacteria, cecalase, for use in mutagenicity testing. Sugimura *et al.* (14–16, 22) have used hesperidinase, a mold enzyme preparation, for this purpose. We developed a cell-free extract of human feces (fecalase) that contains a wide variety of glycosidase activities and that allows for *in vitro* activation of many natural glycosides to mutagens in the *Salmonella* test. Our work is based also on the observations by Chang *et al.* (28), who showed that homogenates of human feces contain different glycosidase activities. Bacterial cells make up approximately half of the human fecal solids (29). We compare fecalase activity with that of hesperidinase and cecalase.

MATERIALS AND METHODS

Materials. Chemicals were obtained as follows: franguloside (frangulin A), 8-hydroxyquinoline glucuronide, 8-hydroxyquinoline glucoside, and 9-aminoacridine from Sigma; robinin, quercitrin, and aloin from ICN; benzo[*a*]pyrene and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine from Aldrich; 4-nitroquinoline-1-oxide and 2-aminofluorene from Schuchardt (Munich); cycasin from Hiromu Matsumoto (25); neocycasin A and macrozamin from T. Nagahama (23); amoscanate from Ernest Bueding; hesperidinase (Tanabe Co. Ltd., 26 Sanbancho, Chiyoda-ku, Tokyo 102) from Takashi Sugimura (15); glusulase from Endo Laboratories (New York); carminic acid from J. P. Brown. Nitrophenol substrates were obtained from Sigma.

Feces. Six different fecalase preparations have been made over the course of a year. For each preparation, feces were obtained on the day of the preparation from two to four healthy adults (age 19–44 yr) on balanced Western diets. Donors were instructed to collect morning excretions in tared plastic containers and to bring them to the laboratory within the next one-half hour. Samples were weighed to the nearest 0.5 g and immediately processed. Each sample was treated separately in the successive steps through the centrifugation step and then assayed for β-galactosidase. The individual samples had roughly comparable activities; they were then combined to make a pooled fecalase preparation.

Homogenization. Feces were transferred to a glass beaker and diluted with 10 mM phosphate, pH 7.4/2 mM dithiothreitol. Dilution was between 1:1 and 3:1 (buffer:feces), depending on the consistency of the feces, to give a thick slurry as the final product. Homogenization was performed in a Sorvall (Newton, CT) or Polytron homogenizer (Brinkman) for approximately 1 min at room temperature.

Lysis of Bacterial Cells in Fecal Suspension. Fecal homogenates were treated by sonication in a Branson sonifier (Branson Sonic Power Company, Danbury, CT) or by high-pressure extrusion in a Manton–Gaulin homogenizer. For lysis by the homogenizer, the machine was first cooled by passing

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* Present address: Istituto Nazionale della Nutrizione, via Lancisi 29, Rome, Italy.

ice slush through it. Homogenates were passed through twice at a pressure of 9000 psi. For lysis by the sonicator, the homogenates were placed in a Branson rosette cooling cell immersed in a water/ice bath. For some preparations a glass beaker was used instead of the rosette cell, and the homogenate was stirred with a magnetic stirrer throughout the sonication. Optimal conditions for the breakage of the cells were 80 W for 4 min at <10°C.

Cellular debris was removed by centrifugation at 0°C for 20 min at 40,000 × *g* in a Sorvall ultracentrifuge. The supernatant was tested for glycosidase activity (see below), after which the retained samples were pooled.

Removal of Histidine and Dithiothreitol. Histidine and dithiothreitol, either of which would interfere with the mutagenicity assay, were removed from the preparation by gel chromatography. The supernatant material was passed through a column of Sephadex G-50 (medium) in 10 mM phosphate buffer (pH 7.4) at a rate of 4–5 ml/min. In one preparation, 2 mM dithiothreitol was used in the eluting buffer. Protein-containing fractions (those with absorbance >8 at 280 nm) were collected and pooled. The temperature was maintained at 4°C throughout the operation.

Sterilization and Storage. The pooled fractions were sterilized by passing them first through a type AP Millipore prefilter under vacuum and then successively through 0.8-μm and 0.45-μm Nalgene filters. The filtrate was then distributed in 2-ml plastic tubes and quick-frozen on dry ice prior to storing at −80°C in a Revco freezer. Samples were analyzed for protein (30) and tested for bacterial contamination on nutrient broth plates. Preparations were checked for phage (although we have not detected any) by plating fecalase with wild-type strain LT2 and strain TA100.

Assay of Enzyme Activities. Various glycosidase activities were determined by hydrolysis of nitrophenyl glycosidic substrates. Up to 100 μl of fecalase or hesperidinase and 0.3 ml of glycoside were diluted in 10 mM phosphate buffer (pH 7.4) to a total volume of 2 ml and incubated at 238°C. The reaction was arrested by addition of 1 ml of 1 M Na_2CO_3, and the increase in absorbance at 420 nm (*o*-nitrophenol) or 400 nm (*p*-nitrophenol) was measured in a Zeiss spectrophotometer. Assays were for variable time periods (<30 min), but results are reported from the linear portion of the time curve. Hesperidinase, an extract from *Aspergillus niger* (15, 22), also was assayed for glycosidic activities and its protein content was determined (30).

Mutagenicity Assays. Mutagens were assayed for mutagenic activity (up to 200 μl of a solution in dimethyl sulfoxide) by using the requisite *Salmonella typhimurium* tester strains (3). When required for the metabolic activation of specific compounds, the S9 liver homogenate mix containing 20–100 μl of S9 from Aroclor 1254-induced rats (3) was added. Fecalase was added in amounts ranging from 100 to 500 μl per plate. Both the standard test (3) and a preincubation modification (31) were employed. The preincubation technique was used to expose the compound to the fecalase in a relatively small volume to allow for more complete hydrolysis. To the mutagen were added the fecalase, S9 liver homogenate mix when necessary, and the tester strain. The mixture was incubated in a sterile test tube for 20 min to 2.5 h at 37°C. Then 2 ml of top agar was added, and the mixture was plated and incubated as usual (3).

RESULTS

Fecalase Activity. A wide variety of glycoside-splitting activities are present in fecalase. Activities for one preparation of fecalase are shown in Table 1 and are compared with those from hesperidinase, an extract of *Aspergillus niger* used by Sugimura *et al.* in mutagenicity assays of glycosidic compounds (15, 16, 22) and rat cecalase, a cell-free extract of rat cecal contents (10–12, 32). Fecalase showed no significant decrease in activity (<15%) when stored at −80°C for 9 months, as measured with a rutin (a rutinoside of quercetin) mutagenicity assay.

Table 1. Activities of glycosidases of fecalase, hesperidinase, and cecalase

Glycosides*	mM	Activity, nmol/min/mg of protein: Fecalase	Hesperidinase	Cecalase†
ONP-β-D-Galactoside	1.3	740	60	59
ONP-α-D-Galactoside	2.5	220	480	180
ONP-β-D-Xyloside	5	180	350	38
PNP-α-D-Glucoside	5	85	10	184
PNP-β-D-Glucuronide	2.5	40	20	7
PNP-β-D-Glucoside	5	30	110	29
PNP-α-L-Fucoside	1.5	6	<0.05	5

* Conditions were saturating for all glycosides except the glucuronide. ONP, *o*-nitrophenyl; PNP, *p*-nitrophenyl.

† Data from Prizont *et al.* (32) and J. P. Brown (personal communication). Preparation methods and enzyme assay conditions for rat cecalase varied and are not exactly the same as the fecalase assay conditions, and the values should only be used for a rough comparison. The glucosides were *ortho* rather than *para*, and the glucuronide was of phenolphthalein.

Variations in procedure for making the fecalase preparation had relatively little effect on the activity of the preparation. The Manton–Gaulin homogenizer, which lyses cells by extruding the mixture through small holes at high pressure, and the sonicator were equally satisfactory in rupturing cells and resulted in the release of similar levels of enzymatic activity. The Manton–Gaulin homogenizer is preferable when volumes exceed 2 liters. In one fecalase preparation, 2 mM dithiothreitol was added to the eluting buffer for the gel chromatography to prevent possible oxidative damage to the enzymes in this step, but it had no significant effect on the activity of the preparation as measured by a rutin mutagenicity assay or by splitting *o*-nitrophenyl-β-D-galactoside (data not shown).

The reproducibility of enzyme specific activity in fecalase preparations from different individuals and at different times is difficult to define exactly. We prepared six different pooled preparations, each from two to four different individuals, but some of these preparations were made while we were still developing the preparation method. The total variation was within a 3-fold range as measured by the splitting of *o*-nitrophenyl-β-D-galactoside and within a 2-fold range as measured by a mutagenicity assay of rutin. Our last two preparations were made with our standardized method and were within 50% of each other.

Activation of Mutagens by Fecalase. Fig. 1 shows the mutagenic activity released from the splitting by fecalase at 37°C of a variety of glycosides, freeing the mutagenic aglycone. None of the glycosides tested were mutagenic without fecalase. Rutin and quercitrin were activated to mutagens with a 20-min incubation and 100 μl of fecalase. Incubation times up to 2.5 hr and volumes of fecalase up to 500 μl were necessary to activate robinin, cycasin, 8-hydroxyquinoline-β-D-glucoside, franguloside, and neocycasin A to mutagens, and these conditions gave greater activity with rutin and quercitrin. Two *C*-glucosides of anthraquinones were tested: aloin, the aglycone of which is weakly mutagenic (11), and carminic acid, the aglycone of which has not (to our knowledge) been tested. Both were negative in the test (<0.03 revertants per nmol).

A number of beverages reported to contain glycosides were

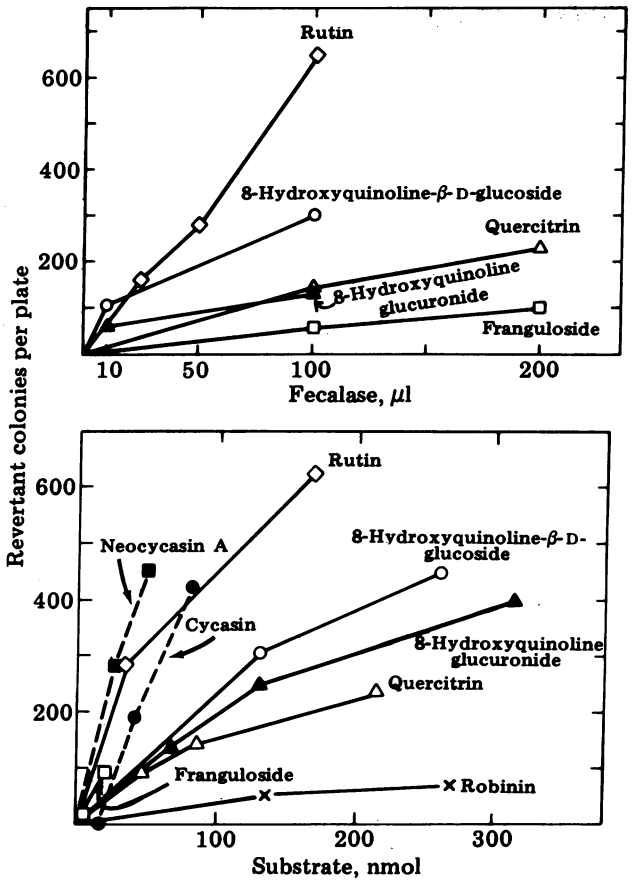

FIG. 1. Mutagenicity of glycosidic substrates with fecalase. (*Upper*) Constant substrate with various amounts of fecalase. (*Lower*) Constant fecalase with various amounts of substrate. The conditions of assay—nanomoles of glycoside (*Upper* only), *Salmonella* tester strain, preincubation time, and volume of S9 liver homogenate mix—were as follows: rutin —165 nmol, TA98, 20 min, 20 μl; 8-hydroxyquinoline glucuronide —130 nmol, TA100, 0, 100 μl; 8-hydroxyquinoline-β-D-glucoside —130 nmol, TA100, 90 min, 100 μl; quercitrin —86 nmol, TA98, 20 min, 20 μl; franguloside —20 nmol, TA1537, 120 min, 20 μl; robinin —TA98, 90 min, 20 μl; cycasin —*his*G46, 90 min, 0; macrozamin—*his*G46, 150 min, 0; neocycasin A —*his*G46, 150 min, 0; methylazoxymethanolglucosiduronic acid—*his*G46, 90 min, 0. Macrozamin and methylazoxymethanolglucosiduronic acid were negative on all strains and are not plotted (<0.01 revertants per nmol: 240 min; 500 μl of fecalase). *Lower:* 500 μl of fecalase was used with all compounds except that 200 μl was used with franguloside and 100 μl was used for 8-hydroxyquinoline glycosides, rutin, and quercitrin. Both axes should be multiplied by 50 for cycasin and neocycasin (both, - - -). Control values were subtracted (e.g., TA98 = 40, TA100 = 180, TA1537 = 20, *his*G46 = 2).

also tested. Black tea, an herb tea (containing 24 herbs), a red wine (California Burgundy), and grape juice were strongly mutagenic in the test when fecalase was added, with 54,000, 12,000, 51,000, and 39,000 revertants per cup (200 ml), respectively (Fig. 2). The teas were mutagenic on TA98 only in the presence of fecalase, whereas the red wine had about half the mutagenic activity even in the absence of fecalase. White wine (California Chablis) and coffee were not mutagenic on TA98. Earlier experiments (with glusulase) had shown comparable mutagenicity with tea and red wine (California zinfandel and a different Burgundy) and a lack of mutagenicity with a white wine (California Riesling). XAD-2 columns were used to adsorb flavonol glycosides to prevent interference of histidine in the test (33). We have shown that these flavonoids are efficiently adsorbed onto the XAD-2 column, as determined by experiments using rutin and quercitrin (>85% adsorption at 5 mg of glycoside).

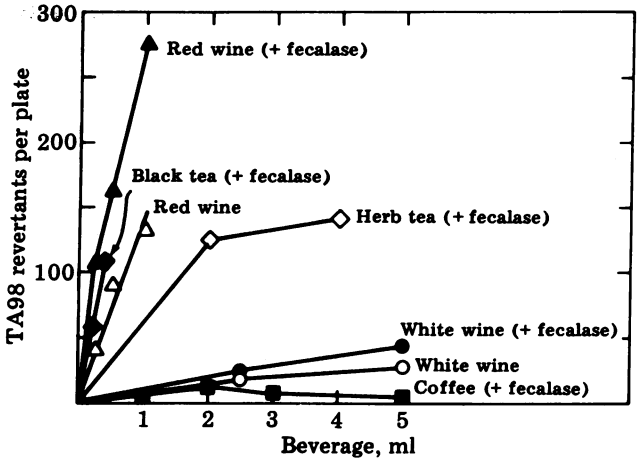

FIG. 2. Mutagenicity of beverages on *Salmonella* strain TA98 with fecalase. The teas and coffee were not mutagenic on TA98 without fecalase. Standard conditions (20-min preincubation at 37°C with 20 μl of S9 liver homogenate, 100 μl of fecalase, and tester strain) were employed. Control values (see Fig. 1) were subtracted. The beverages were concentrated with XAD-2 prior to testing as described for urine (33): 50 ml of beverage per 4-ml column of resin. Grape juice (Concord, red) was also tested and was mutagenic with fecalase (194 revertants per ml) and not mutagenic without fecalase (<10 revertants per ml).

DISCUSSION

Many recent studies have pointed out the importance of gut flora in transforming a wide variety of naturally occurring glycosides of mutagens to active forms (10–22, 24–26). These studies indicate that the rat liver homogenate used in the *Salmonella* test at present should be complemented by the addition of microbial enzymes to the test to allow for a more complete assay of potential carcinogens in the plant world.

Many compounds eaten by man exist as glycosides that are cleaved by bacteria in the gut where the aglycone may be absorbed by the intestine (10–16, 22–26). Many of these aglycones have been shown to be mutagenic in *Salmonella* tests (10–19) or to transform or mutate mammalian cells (34, 35), and at least one naturally occurring glycoside (cycasin) has been shown to be carcinogenic in normal rats but not in germ-free rats (24, 25). Many of the mutagens are commonly found in foods, and Nagao *et al.* have shown that the mutagenic activity of tea on *Salmonella* strain TA98 requires glycosidase activity (14). Brown and Dietrich have shown that many flavonol glycosides, a class of compounds that is ubiquitous among the higher plants, are activated to a mutagenic form by mixed glycosidases from rat cecal bacteria and other sources (10–13).

Various mixed glycosidases for use in the test have been employed in previous investigations, including cell-free extracts from rat cecal bacteria (10–12, 32), glusulase from the snail *Helix pomatia* (33), and hesperidinase from the mold *Aspergillus niger* (14–16, 22). The latter two enzyme mixtures have an acid pH optimum. Fecalase, an enzyme extract from human feces, is a simple and physiologically relevant model for metabolism by human gut bacteria to be used in conjunction with the *Salmonella* test.

An enormous variety of glycosides (as to both the sugar and the aglycone moieties) are present in edible plants in nature (10–16, 23, 25–27). Among the glycosides shown here to be split by fecalase (in enzyme or mutagenicity assays) are α- and β-D-glucosides, α- and β-D-galactosides, β-D-glucuronide, β-D-xyloside, α-L-fucoside, α-L-rhamnoside (in quercitrin), 3-β-D-glucosyl-D-glucoside (in neocycasin A), 6-rhamnosyl-β-D-glucoside (in rutin), and 6-α-L-rhamnosyl-D-galactoside (in robinin). Fecalase, hesperidinase, and cecalase also hydro-

lyzed *N*-acetyl-β-D-glucosaminide, and *N*-acetyl-β-D-galactosaminide glycosides (data not shown). Only the two *C*-glucosides (aloin and carminic acid) of the sugar types tested were not split by fecalase. Fecalase also has been effective in activating nonmutagenic glycosides to mutagenic aglycones with the various natural substrates tested, including several flavonols (quercetin, kaempferol), an anthraquinone (franguloside), and two methylazoxymethanol derivatives (cycasin and neocycasin A). Fecalase was unable to activate two other methylazoxymethanol derivatives (macrozamin and methylazoxymethanol-glucuronide) under the conditions used. The nonlinear character of the dose–response curve for methylazoxymethanol and derivatives (5) requires release of micromolar quantities of methylazoxymethanol before mutagenesis is observed, and this may be the reason for the negatives in the test; the particular sugar linkages were split in other substrates. Limitations were imposed by the small quantity of these derivatives, and further study is indicated.

Fecalase, rat cecalase, and hesperidinase all seem to be satisfactory in splitting a wide variety of glycosides (Table 1). Any of them would appear to be suitable for use in the *Salmonella* test when testing natural substances that might contain glycosides. The advantages of fecalase are: (*i*) it is most relevant to the human condition; (*ii*) people eat a much more varied diet than rats, and it is possible that fecalase might contain some glycosidase activity not present in rat cecalase if induction of bacterial flora is important for some of the minor glycosidase activities; and (*iii*) it is easily prepared on a large scale and can be frozen so that one has to make a preparation infrequently. Cecalase would probably be more reproducible from batch to batch than fecalase, because rats eat a more defined diet, though we have found fecalase to be reasonably reproducible. Hesperidinase has the advantage of being easy to use and of being commercially available. It had good activity on all of the glycosides tested except for the fucoside.

Fecalase was effective in activating some naturally occurring complex mixtures containing mutagenic flavonol glycosides. Flavonol glycosides of mutagens such as quercetrin are known to occur in large quantities in tea leaves (26, 27), and we show in Fig. 2 that tea is mutagenic in the presence of fecalase. Nagao *et al.* (14) independently have shown the mutagenicity of green tea and black tea and the requirement of hesperidinase for this mutagenic activity. We show (Fig. 2) that a red, but not a white, wine is mutagenic; this presumably is due to the presence of glycosides of mutagenic flavonols, such as quercetin, known to be present in grape skins (26) that are used in making red, but not white, wine. The considerable direct mutagenic activity of the red wine in the absence of fecalase may be due to free quercetin formed in the wine during fermentation. The absence of direct-acting activity in grape juice is consistent with this hypothesis, though the type of grape used in the wine and the grape juice differed. We also show that coffee is negative on tester strain TA98 with fecalase, indicating an absence of mutagenic flavonols or their glycosides. Coffee had previously been shown by Nagao *et al.* to be mutagenic in tester strain TA100 without glycosidase activation, which indicated the presence of nonglycoside mutagens of a different type. We present a simple method for analyzing the mutagenicity of flavonols and their glycosides in complex mixtures containing histidine (which interferes in the mutagenicity test) by using XAD-2 resin.

The *Salmonella*/microsome test in the past has been shown to be about 85% ± 5% effective in detecting carcinogens as mutagens (5–9), but compounds tested in previous studies were usually synthetic substances to which man had not been exposed in evolution. The ubiquity of the flavonols in edible plants in nature raises the possibility that during mammalian evolution methods for dealing with these mutagens may have evolved. Quercetin and kaempferol are among the most common—and among the most mutagenic (12, 17–19, 22)— of the flavonols. They also have been shown to be activated by human (autopsy) liver to a form mutagenic to *Salmonella* (36), to transform (34) and to mutate (35) animal cells in culture, and to cause sister chromatid exchange in human cells in culture (22). Brown (13) has discussed the significance of mutagenic flavonoids (see also Sugimura, ref. 22) and anthraquinones in detail.

Very recent evidence shows that quercetin is a carcinogen for the rat (confirming the evidence from the short-term tests) but suggests that mice may not be affected even at considerably higher doses. Of 25 rats fed quercetin at 1000 ppm in the diet for 14 months, 20 developed multiple intestinal tumors of the ileal section and 5 developed bladder transitional cell carcinoma; none of the 19 control rats showed any such tumors (37). Mice fed quercetin at 20,000 ppm in the diet for 17 months showed no extraordinary differences in tumor incidence when compared with controls, though this study is not yet complete (22).

Two factors may reduce the mutagenicity and carcinogenicity of these compounds in humans. First, quercetin does not seem to be absorbed. A study on quercetin (38) found that after oral dosage, free quercetin was present in the intestines but no quercetin, either free or conjugated, was detected in the bloodstream. The authors concluded that less than 1% of the dose was absorbed; however, if quercetin reacted with tissues or was otherwise transformed, this estimate could be too low. Second, microbial degradation of flavonoids is extensive. The quercetin study (38) found that although less than 1% of the quercetin was absorbed by the intestines, only about half of an oral dose of 70 mg/kg of body weight was recovered from the feces, indicating extensive microbial degradation. Supporting evidence is found in a study on hesperetin (39), a flavanone which, though nonmutagenic, is structurally similar to quercetin and kaempferol. Hesperetin is completely degraded by anaerobic incubation with gut bacteria at the rate of about 4 mg/g of cecal dry weight per day when the incubation was carried out at low doses. In addition, in other work (40, 41) when rats were fed low doses (<30 mg/kg of body weight) of hesperetin or three other flavanones, no detectable quantities of any of these compounds were found in the bile or urine, although high doses (>150 mg/kg) resulted in small quantities (less than 12%) of all of these compounds being found in the bile.

In spite of this evidence, flavonoids still may conceivably play a role in the etiology of cancer of the stomach and other areas of the gastrointestinal tract, and they remain an area of potential interest. Mutagenic flavonol aglycones, such as quercetin, are present in some foods, such as pickles (22, 42) and red wine (Fig. 2), so that the stomach and intestine are exposed to these agents in addition to the exposure of the colon by the hydrolysis of flavonol glycosides. If flavonols do turn out to have some mutagenic effect *in vivo*, plant breeding has been suggested (13) to reduce the amount of flavonoids present in the food we eat. The case of cycasin indicates that at least some naturally occurring glycosides may be quite toxic to man. Fecalase should aid in the detection of potentially dangerous natural substances.

Bacterial enzymes also may activate mutagens through other modes of action. Benzidine-based dyes may be reduced by bacterial flora to release benzidine (43–46), and other azo dyes may be reduced to release other carcinogenic and mutagenic aromatic amines. Bueding and colleagues (47, 48) have shown that amoscanate, an isothiocyanate antibiotic, can be reduced by the bacterial flora *in vivo* to an aromatic amine that is mu-

tagenic in *Salmonella*. In one experiment not reported here, fecalase did not enhance the observed chemical reduction of the azo dye Ponceau 3R (Red #1) by NADPH, though we have not explored this area in detail. However, Brown *et al.* (49) have evidence that rat cecal enzymes can mediate the chemical reduction of azo dyes. The chemical reduction appears to be rapid relative to the enzymatic reduction. Simple chemical reductions of azo dyes by dithionite (50) or by reduced cofactors such as NADPH, FMN, and riboflavin may serve as a model for reduction of azo dyes by gut bacteria. It has been shown that a variety of azo dyes with riboflavin yield mutagenic amine cleavage products (51).

We are indebted to Diane Burgess and Brian Schmidt for some of the earlier experiments with beverages and to Glenn Jaffe for the azo dye experiments. We would like to thank J. P. Brown, George Chang, Lynne Haroun, J. MacGregor, and C. Ballou for discussions and help, T. Sugimura for hesperidinase, and H. Matsumoto for cycasin and related chemicals. This work was supported by U.S. Department of Energy Contract DE-AM03-76SF00034 to B.N.A., by National Institute of Environmental Health Sciences Center Grant ES01896, and by a President's Undergraduate Fellowship to G.S.T.

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