Biochemical and Biophysical Research Communications **272,** 212–217 (2000)
doi:10.1006/bbrc.2000.2750, available online at http://www.idealibrary.com on IDEAL®

Resveratrol Is Absorbed in the Small Intestine as Resveratrol Glucuronide

Gunter Kuhnle,* Jeremy P. E. Spencer,† George Chowrimootoo,‡ Hagen Schroeter,†
Edward S. Debnam,‡ S. Kaila S. Srai,‡ Catherine Rice-Evans,*,†,[1] and Ulrich Hahn*
**Department of Biochemistry, University of Leipzig, Talstrasse 33, Leipzig, 04103, Germany; †Antioxidant Research Group, Wolfson Centre for Age-Related Diseases, GKT School of Biomedical Sciences, Hodgkin Building, King's College, Guy's Campus, London, SE1 9RT, United Kingdom; and ‡Royal Free and University College Medical School, Royal Free Campus, London NW3 2PF, United Kingdom*

Received April 26, 2000

We have studied the absorption and metabolism of resveratrol in the jejunum in an isolated rat small intestine model. Only small amounts of resveratrol were absorbed across the enterocytes of the jejunum and ileum unmetabolised. The major compound detected on the serosal side was the glucuronide conjugate of resveratrol (96.5% ± 4.6 of the amount absorbed) indicating the susceptibility of resveratrol to glucuronidation during transfer across the rat jejunum. The presence of the glucuronide was confirmed using HPLC-PDA and nanoES-MS/MS techniques. These findings suggest that resveratrol is most likely to be in the form of a glucuronide conjugate after crossing the small intestine and entering the blood circulation. This will have important implications for the biological functions of resveratrol *in vivo*. © 2000 Academic Press

Key Words: **resveratrol; absorption; glucuronide; small intestine; jejunum; ileum; *nano*ES-MS/MS.**

Resveratrol (trans-3,4′-5-trihydroxystilbene) (Fig. 1) is a naturally occurring phenolic compound of the stilbene family found in grapes and thus in red wine (1, 2). Its levels are often increased in plants during pathogen attack, UV irradiation, or exposure to ozone (3–5). Resveratrol has been promulgated as one of the most promising cancer chemopreventive agents and has been shown to inhibit cellular events associated with carcinogenesis (6, 7).

The wide range of biological effects exhibited by resveratrol are thought to be due to its powerful antioxidant properties (8) and its ability to interact with redox sensitive cell signalling pathways (6, 9). The ability of resveratrol to scavenge reactive oxygen species (ROS) *in vitro* has been well documented (8, 10–12). For example, resveratrol was observed to prevent oxidative DNA damage induced in the kidney by $KBrO_3$ (13) and acts as a potent scavenger of peroxyl radicals in an isolated rat heart ischaemia reperfusion model (14). Recently, the cancer chemopreventive activity of resveratrol has been related to its ability to trigger apoptosis (15, 16), a mechanism of programmed cell death which has been linked to ROS and lipid peroxides (17). In contrast, resveratrol has been shown to prevent apoptosis in K562 cells via the direct inhibition of arachidonate-metabolising enzymes such as lipoxygenase and cyclooxygenase (18). Resveratrol also possesses strong antiplatelet properties (19), may act as an agonist for the oestrogen receptor (20) and can inhibit phorbol ester-mediated activation of protein kinase C and AP-1-mediated gene expression in human mammary epithelial cells (21).

Although many studies have implicated a role in disease prevention, the mode of action *in vivo,* attributed to resveratrol itself, is still unclear. The extent of the potency of resveratrol *in vivo,* whether as an antioxidant or as a mediator of cellular events independently of antioxidant properties, will be dependent on its absorption and metabolism within the body after ingestion, the subsequent distribution and the rate of excretion. The purpose of this study was to investigate the fate of resveratrol in terms of its absorption and metabolism in the gastrointestinal tract using an isolated preparation of jejunum and ileum from the rat small intestine, and monitoring the resulting native compound, its conjugates and metabolites.

MATERIALS AND METHODS

Materials. Specialised chemicals used were: resveratrol from Extrasynthase (ZI Lyon Nord, Genay, France), β-Glucuronidase (type L-II from Limpets) from Sigma Chemical Company (Poole, Dorset, UK), β-glucosidase (from almonds) from ICN Biomedicals Ltd.

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[1] To whom correspondence should be addressed. Fax: 0207 848 6143. E-mail: catherine.rice-evans@kcl.ac.uk.

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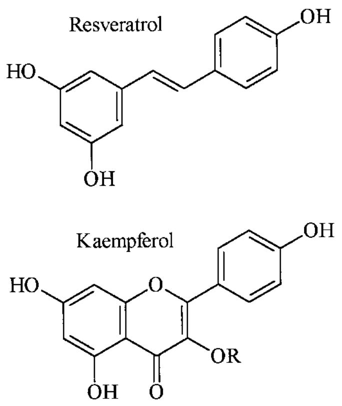

FIG. 1. The structures of resveratrol and kaempferol.

(Thame, Oxon, UK) and trifluoroacetatic acid from Fluka Chemical Company (Gillingham, Dorset, UK). For HPLC specialised solvents, acetonitrile and methanol, were purchased from Rathburn Limited (Walkerburn, UK) and HPLC columns from Waters (Watford, Herts., UK). Elgstat UHP double distilled water (18.2 $M\Omega$ grade) was used throughout the study. All other reagents used were of the analytical grade and obtained from Sigma Chemical Company (Poole, Dorset, UK).

Absorption studies. Absorption studies were conducted using the *in vitro* intestinal preparation of Fisher and Gardner (22) in which the lumen of isolated intestine was perfused with a segmental flow (defined as perfusion of buffer interspersed with bubbles of the gas mixture) of bicarbonate buffer (pH 7.4) equilibrated with 95% O_2:5% CO_2 and containing 28 mM glucose. The bicarbonate buffer consisted of Krebs bicarbonate saline solution (HCO_3^-: 25 mM; Na^+: 143 mM; Cl^-: 133.7 mM; K^+: 5.9 mM; HPO_4^-: 1.2 mM) containing Ca^{2+} (2 mM) and Mg^+ (1.2 mM) (23). Male Sprague-Dawley rats (230–260g) were anaesthetised with pentobarbitone sodium (90 mg/kg, i.p.) and sections of jejunum (15–30 cm long, beginning 10 cm from the ligament of Treitz) were cannulated and the lumen perfused. The segments of intestine were then removed from the animal and suspended in a chamber containing liquid paraffin at 37°C whilst maintaining the segmental flow of buffer. Before uptake experiments were conducted the segment was perfused for 40 min in order to flush blood from the vasculature and to allow fluid absorption to reach a steady state. Thereafter, resveratrol (200 μM) was added to the buffer and perfused through the system, in a single pass fashion, for up to 90 min. During perfusion, absorbed fluid dropped through the paraffin to the base of the chamber and was collected at timed intervals. Samples collected from both the jejunum and the ileum were immediately diluted 1:1 with methanol and stored at -70°C until analysis.

HPLC analysis. HPLC analysis to measure compound and metabolite concentrations was carried out using a Waters 626 pump and 600 controller system with an autoinjector 717 and a photodiode array detector 996 linked to the Millennium Software system. Samples were analysed by reverse-phase HPLC using a Nova-Pak C18 column (4.6 × 250 mm) with 4 μm particle size. The temperature of the column was maintained at 30°C. The mobile phase consisted of a mixture of aqueous methanol (20%) in hydrochloric acid (0.1%) and acetonitrile (MeCN) and was pumped through the column at 0.5 ml min^{-1}. The following gradient system was used (min/% MeCN): 0/5, 10/5, 40/50, 60/5 for detection of all flavonoids, hydroxycinnamates, and metabolites. The eluant was monitored by photodiode array detection at 280 and 320 nm and spectra of products obtained over the 220–600 nm range. Calibration curves of the compounds were constructed using an authentic standard of resveratrol (0–100 μM) and in each case were found to be linear with correlation coefficients of >0.995.

Salicylic acid (final concentration: 10 μM) was added as an internal standard to samples (100 μl) before HPLC analysis. In all cases 30 μl were injected onto the column. The presence of glucuronide metabolites in the serosal fluid samples was established by treatment with β-glucuronidase. Incubation of samples with enzyme (1000 units/ml) was for 120 min at 37°C in a 0.1 M phosphate buffer, pH 3.8. The sulphatase activity which the enzyme possesses was effectively inhibited by 0.1 M phosphate buffer all (as detailed by Sigma protocol).

nanoES-MS/MS analysis of resveratrol metabolites. Samples of both crude serosal fluid as well as single peaks collected by semi-preparative HPLC were analysed by nanoES mass spectrometry to enable characterisation of compounds. nanoES mass spectrometry was performed using a Perkin-Elmer API 365 LC/MS/MS mass spectrometer (PE Sciex, Thornill, Canada) with a specially developed nanoES source (24) and metal coated capillaries (Protana, Denmark). To increase sensitivity, methanol and trifluoroacetic acid were added to the samples: water:methanol (1:1), 0.02% trifluoroacetic acid. Collision induced dissociation (CID) was performed using nitrogen as the collision gas and all spectra were recorded in positive ion mode. Lithium trifluoracetate was used in lithium ion exchange measurements to displace Na^+ and K^+ ions to confirm the presence of possible Na^+ and K^+ adducts.

RESULTS

The absorption of resveratrol across the jejunum and ileum in a rat intestinal model was investigated as a function of time up to 90 min, and the extent of its conjugation and metabolism examined. The components transferred were identified and quantified by HPLC and the identification confirmed by mass spectrometry. Figure 2 illustrates the time course for resveratrol transfer in the jejunum. Resveratrol was detected on the serosal side of the enterocytes but the amount which was transferred unmetabolised was very small (0.03 nmol/cm jejunum). In contrast, significant amounts (1.19 nmol/cm jejunum) of a more polar

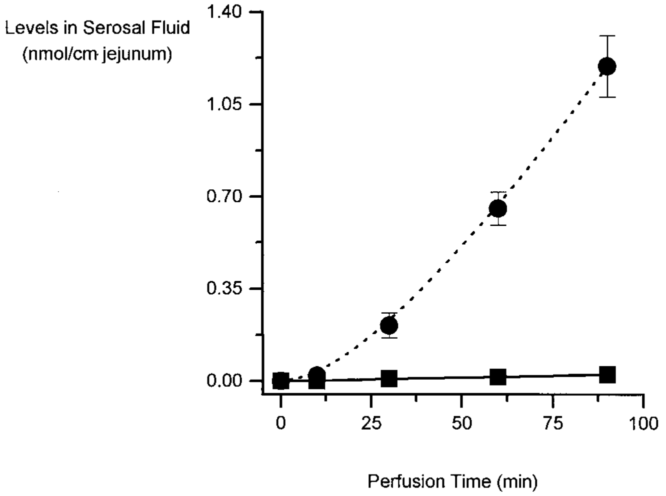

FIG. 2. Cumulative amounts of resveratrol and resveratrol glucuronide (nmol/cm gut) in serosal fluid over 90 min after perfusion with resveratrol (100 μM) through isolated rat jejunum. Experiments were performed as outlined under Materials and Methods. Data is plotted as the mean ± SD of three separate experiments each analysed in duplicate. (■) resveratrol, (●) resveratrol glucuronide.

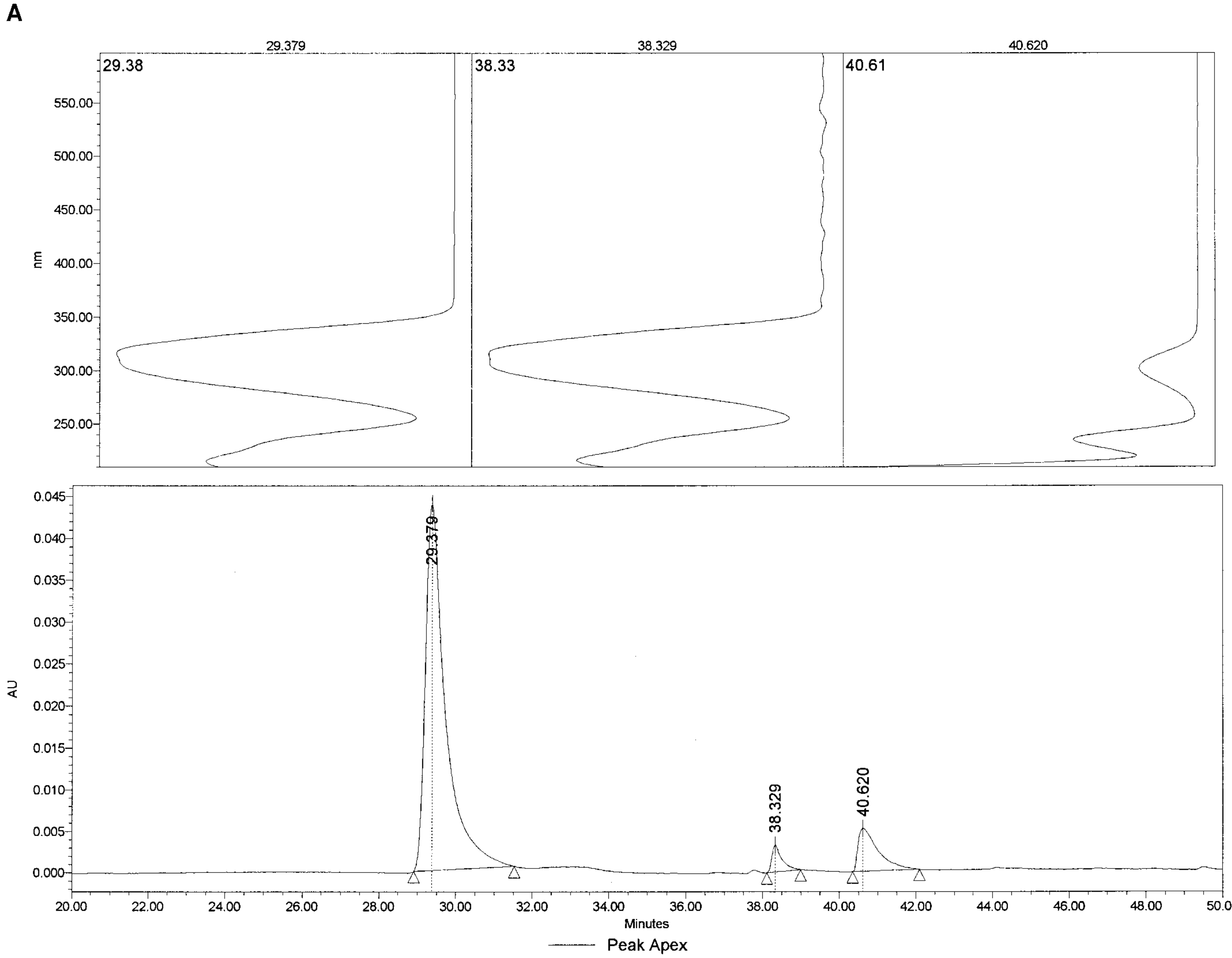

FIG. 3. Representative HPLC chromatogram after perfusion of isolated rat jejunum with resveratrol (100 μM). A) no enzyme treatment; B) treatment with β-glucuronidase (1000 units/ml) for 2 h at 37°C. Resveratrol and salicylic acid have retention times of ~38.25 min and 40.55 min, respectively.

product with similar spectral characteristics were detected on HPLC analysis of the serosal fluid samples (Fig. 3A). The presence of a glucuronide conjugate was first investigated by treatment with β-glucuronidase. Figure 3 shows representative HPLC traces before (Plot A) and after (Plot B) β-glucuronidase treatment. Treatment with the enzyme for 120 min at 37°C resulted in complete loss of the peak at 30.3 min and recovery of a peak at 38.5 min which coelutes with resveratrol standards (Fig. 3) suggesting the glucuronidation of resveratrol as it crosses the jejunum. To eliminate the unlikely possibility that the new resveratrol conjugate was a glucoside, the serosal samples were also treated with β-glucosidase since certain β-glucuronidases can possess some glucosidase activity. There was no reduction of the peak at 30.3 min after treatment with β-glucosidase. The stability of resveratrol during the course of the 90 min perfusion was assessed by analysis of both pre- and post-experimental perfusion buffers and no significant loss of resveratrol occurred over the 90 min duration of perfusion at 37°C.

The cumulative transfer of both resveratrol and resveratrol glucuronide was lower across the ileum compared to that across the jejunum. Levels of resveratrol glucuronide in the serosal fluid after perfusion of the ileum, with the same concentration of resveratrol, were ~38% of that transferred across the jejunum. Absorption of the native non-glucuronidated resveratrol across the ileum could not be quantified as the amounts detected were too low to quantify.

Further characterisation of the peak at 30.3 min attributed from the HPLC analysis to resveratrol glucuronide was achieved by the use of nano electrospray

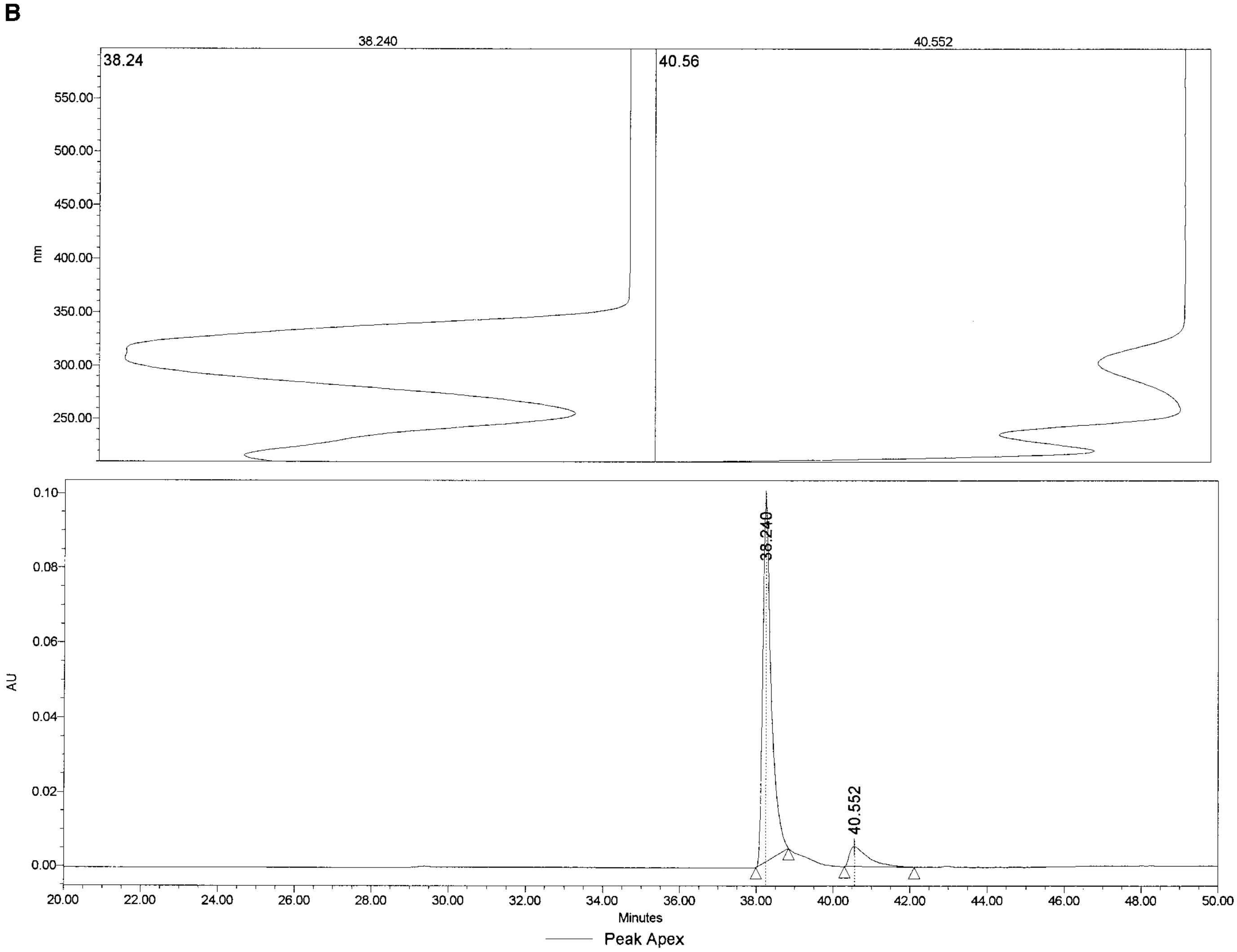

FIG. 3—*Continued*

mass spectrometry (nanoES-MS/MS). Analysis of the peak gave a m/z of 405 [resveratrol glucuronide + H]$^+$; m/z of 428 [resveratrol glucuronide + Na + H]$^+$; m/z of 444 [resveratrol glucuronide + K + H]$^+$. The sodium and potassium adducts were confirmed by lithium ion exchange measurements using lithium trifluoracetate which resulted in the appearance of a new peak at m/z 412 [resveratrol glucuronide + Li + H]$^+$ and the disappearance of the peaks at m/z 428 and 444. The product ion scan of the major peak at m/z 405 using collision induced dissociation (CID) resulted in fragmentation to form a major peak at m/z 229 which corresponds to resveratrol + H.

From the data obtained the percentage of total resveratrol equivalents (resveratrol + metabolites) which has been taken up by the section of jejunum and transferred to the serosal side has been calculated. This approximates to 6% of the amount available for transfer. The calculation is based on the assumption that the volume of the jejunal segment is 5 ml ($r = 0.25$ cm; $l = 25$ cm) and that the amount of resveratrol therein throughout the duration of the experiment is 500 nmol. Approximately 99% of the resveratrol equivalents recovered on the serosal side of the gut has been identified as resveratrol glucuronide.

DISCUSSION

Resveratrol does not have a wide distribution in the plant world, however, consumption by humans of this compound can be significant due to its presence in red wine (1) and peanuts (25). Many studies have suggested that resveratrol may be beneficial to human health (5, 14, 26–28). For example, it is thought that the reduced risk of cardiovascular disease associated with moderate consumption of red wine may be due to resveratrol (5) and is believed to possess cancer chemopreventative activity (6). Although resveratrol has been observed to exhibit powerful ROS scavenging effects (13) and influence the intracellular redox balance (6) *in vitro*, the effects *in vivo* are not well understood. The nature of the bioactive compound and its accessibility to the tissues will clearly be affected by absorption and metabolic events in the small intestine. The purpose of this work was to study the absorption of resveratrol in the small intestine and to determine the form in which it might circulate *in vivo*.

Our data clearly show that the major product transferred across the intestinal epithelium is a glucuronide of the parent resveratrol aglycone (~99%). Nano electrospray mass spectroscopy provided unambiguous evidence that resveratrol glucuronide is the predominant compound in the serosal fluid. Furthermore, the mass spectroscopy data suggest that the 4′-hydroxyl group might be the preferential glucuronidation site, although further studies are required to confirm this assignment.

Our previous studies have demonstrated the presence of a UDP-glucuronosyl transferase in the small intestine through evidence that glucuronidation of polyphenolic compounds such as flavonoids and hydroxycinnamates appear to be the most prevalent mode of metabolism in the small intestine (23). Particularly relevant here is kaempferol, a flavonol, which was also extensively glucuronidated under these conditions (>99%). Kaempferol shows structural similarities to the stilbene resveratrol, with a 4′-hydroxyl group in the B-ring, a 2,3-double bond in the C-ring allowing conjugation across to the A-ring containing meta dihydroxy structure (Fig. 1). However, such flavonols in general are present in edible plants in the form of glycosides and their hydrolysis to the aglycone has also been demonstrated in gastrointestinal studies (23, 29).

Glucuronides of phenolic compounds have generally been assumed to be rapidly excreted *in vivo* and to be pharmacologically inactive. However, several studies are demonstrating that some drug glucuronides may be pharmacologically active (30, 31). For example, the ability of morphine glucuronide to cross the blood-brain-barrier (32) and the analgesic action of morphine-6-glucuronide have been reported (33). If resveratrol glucuronide is the major circulating form of resveratrol, based on these rat intestinal model studies, the question arises as to the nature of its bioactive form which hitherto has been attributed to the native form. β-glucuronidases have been found in a variety of organs and body fluids, such as macrophages, blood cells, liver, lung and serum (31, 34). Consequently, it is conceivable that resveratrol glucuronide might be cleaved back to aglycone *in vivo* via the action of β-glucuronidases present in a variety of different cell populations, for example, macrophages at sites of inflammation or by other cells under oxidative stress. Thus, it is possible that β-glucuronidase might modulate the rate of resveratrol glucuronidation which occurs in the small intestine and liberate active parent aglycone in tissues and body fluids with a high activity of β-glucuronidase.

ACKNOWLEDGMENTS

Financial support from the Biotechnology and Biological Sciences Research Council (Grant No. F06405), the European Union (Grant No. QL K4-CT-1999–01590), the Deutsche Forschungsgemeinschaft and the Deutsche Fonds der Chemischen Industrie is gratefully acknowledged.

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