Soy Isoflavone Conjugation Differs in Fed and Food-Deprived Rats

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ABSTRACT  An experiment clarifying the influence of food deprivation on the isoflavone conjugation pattern in rats was conducted. Food-deprived and fed rats were administered daidzein and genistein at 7.9 μmol/kg body, and changes in their plasma metabolites (i.e., free compounds, sulfates, glucuronides, sulfates/glucuronides) were measured quantitatively as a function of time. In the food-deprived group, total plasma daidzein and genistein reached maximum concentrations of 20.9 ± 4.4 and 11.4 ± 3.1 μmol/L, respectively, 10 min after administration, whereas in the fed group, the maxima were 2.4 ± 0.8 μmol/L for daidzein after 2 h and 1.8 ± 0.2 μmol/L for genistein after 4 h. In both groups, there were significantly more daidzein sulfates than genistein sulfates. Moreover, depriving rats of food before daidzein and genistein administration significantly increased plasma isoflavone sulfates with simultaneous significant decreases in plasma isoflavone glucuronides compared with fed rats. Additionally, nonconjugated daidzein and genistein appeared in plasma of food-deprived rats for 1 h after administration. Plasma concentrations of conjugates having both sulfate and glucuronide moieties were not significantly different between the groups. J. Nutr. 130: 1766–1771, 2000.

KEY WORDS: • rats • daidzein • genistein • sulfate • glucuronide

Epidemiologic studies have shown that the lower incidence of certain cancers and osteoporosis in populations that consume soy and soy products is associated with isoflavones, the inherent component of soy (Adlercreutz and Mazur 1997). Isoflavones influence steroid hormone metabolism (Loukovaara et al. 1995), inhibit cell proliferation and in vitro angiogenesis (Fotsis et al. 1995), exert antioxidant activity (Wei et al. 1995) and are potent inhibitors of tyrosine protein kinases (Akiyama et al. 1987). Nevertheless, some potential adverse effects of soy isoflavones have been reported. Neonatal exposure of rat male offspring to coumestrol through milk of rat dams fed a diet containing coumestrol produced significant deficits in their sexual behavior in adulthood (Whitten et al. 1995). The main soy isoflavones, daidzein and genistein, appear in soybeans in the form of glucosides, daidzin and genis- tin, respectively. Hutchins et al. (1995) showed that fermentation of soybeans increases the availability of isoflavones by releasing isoflavone aglycones from their glucosides as well as by increasing digestibility through the structural alteration of the food matrix. Recently published reports demonstrate the presence of β-glucosidase activity toward flavonoid and isoflavonoid glucosides in rat (Ioku et al. 1998) and human (Day et al. 1998) small intestine. Indeed, no differences in absorption between isoflavone glucosides and aglycones were found except that aglycones, but not their glucosides, were absorbed from the rat stomach (Piskula et al. 1999).

Despite considerable interest in soy isoflavones, their absorption and metabolism have not been well studied. In a number of papers, the total plasma concentration or amount of excreted compound was measured, but rarely as a function of time, and there is no information on the pharmacokinetics of isoflavonoids conjugates, i.e., glucuronides and/or sulfates. Although almost all isoflavones are glucuronized and/or sulfated after absorption (Yasuda et al. 1994 and 1996), most of the in vitro studies demonstrating beneficial action of isoflavones were done on nonconjugated aglycones. Moreover, the effective plasma concentrations of these compounds are still not known. Therefore, the aim of this study was to compare the absorption of daidzein and genistein in fed (F) and food-deprived (FD) rats with regard to their conjugation pattern. At present, there are still no definite recommendations concerning the amounts of isoflavones to be consumed to prevent diseases. On the basis of rat studies, the daily dose of isoflavones that humans would have to consume to suppress hepatocarcinogenesis would be 6–8 μmol/kg body (Hendrich et al. 1994), an amount rather difficult to reach without dietary supplementation of isoflavones. The average daily intake of isoflavones in high soy products consumed by the Japanese population is estimated to be ~2.3–2.5 μmol/kg body (Kimira et al. 1998). In this study, rats were administered daidzein and genistein at a dose of 7.9 μmol/kg body, and changes in their metabolites were measured quantitatively in blood plasma as a function of time.

MATERIALS AND METHODS

Chemicals. Daidzein and genistein were purchased from Fujicco (Kobe, Japan), sulfatase type H-5 and VIII, β-glucuronidase type VII-A and α-saccharic acid 1.4 lactone were purchased from Sigma Chemical (St. Louis, MO). Other chemicals were of analytical or HPLC grade.
Animals, diets and treatments. Wistar male rats (n = 10; Charles River Japan, Atsugi, Japan) were supplied at the age of 8 wk and kept in a temperature- (23 ± 1°C) and light- (07:00–19:00 h) controlled room in the institute animal facility for ~1 wk before the experiments, with free access to tap water and diet. Feeding the rats nonpurified diet (standard MF, Oriental Bioservice, Chiba, Japan) containing some soy components resulted in a concentration of isoflavones in blood plasma of ~0.8 μmol/L. Therefore it was necessary to switch rats to a phenolic-free powdered diet (Oriental Bio-service) (Okushio et al. 1996) for ~1 wk before the experiments to eliminate isoflavones from plasma to levels below the detection limit (30 nmol/L).

The comparison of daidzein and genistein absorption and conjugation was performed on two groups of 5 rats, one of which was denied access to food at 0900 h, i.e., 24 h before the experiment. On the day of the experiment, daidzein and genistein freshly dissolved in water/methanol/acetic acid, 58.8 mg; inositol, 235 mg.

Plasma daidzein or genistein glucuronide concentration was determined by HPLC analysis. Rat plasma (50 μL) was mixed with 50 μL of sulfate type H-5 solution in 0.2 mol/L acetate buffer, pH 5, and the mixture was incubated at 37°C in shaking water bath for 2 h. Plasma daidzein or genistein were determined by HPLC after extraction from blood plasma. To 50 μL of plasma, 50 μL of 0.2 mol/L sodium acetate buffer, pH 5, and 900 μL of methanol/acetic acid (100:5, v/v) were added. The mixture was vortexed for 30 s, sonicated for 30 s, again vortexed for 30 s and centrifuged for 5 min at 4°C and 5000 × g. The supernatant was diluted with 100 mmol/L of lithium acetate (1:1, v/v), centrifuged for 2 min at 4°C and 5000 × g, and 20 μL was injected onto a HPLC column (TSKgel ODS-80TS, 5 μm, 150 × 4.6 mm, TOSOH, Tokyo, Japan). The flow of the mobile phase consisted of water/methanol/acetic acid (58:40:2, v/v/v) containing 50 mmol/L Na2CO3 were administered orally to rats at 7.9 μmol/kg body. Before (control) and after administration, blood samples (~400 μL) were collected from the tail vein at certain time intervals into heparinized tubes, plasma was then obtained by centrifugation for 20 min at 4°C and 1000 × g. Experiments were made after the experimental protocol was approved by the Institute Ethic Committee; rats received humane care consistent with institutional guidelines.

Determination of isoflavones and their conjugates in rat plasma. Identification and plasma concentration of isoflavones and their conjugates were studied by the method described previously (Piskula and Terao 1998a, Piskula et al. 1999). Nonconjugated daidzein and genistein were determined by HPLC after extraction from blood plasma. To 50 μL of plasma, 50 μL of 0.2 mol/L sodium acetate buffer, pH 5, and 900 μL of methanol/acetic acid (100:5, v/v) were added. The mixture was vortexed for 30 s, sonicated for 30 s, again vortexed for 30 s and centrifuged for 5 min at 4°C and 5000 × g. The supernatant was diluted with 100 mmol/L of lithium acetate (1:1, v/v), centrifuged for 2 min at 4°C and 5000 × g, and 20 μL was injected onto a HPLC column (TSKgel ODS-80TS, 5 μm, 150 × 4.6 mm, TOSOH, Tokyo, Japan). The flow of the mobile phase consisted of water/methanol/acetic acid (58:40:2, v/v/v) containing 50 mmol/L Na2CO3 were administered orally to rats at 7.9 μmol/kg body. Before (control) and after administration, blood samples (~400 μL) were collected from the tail vein at certain time intervals into heparinized tubes, plasma was then obtained by centrifugation for 20 min at 4°C and 1000 × g. Experiments were made after the experimental protocol was approved by the Institute Ethic Committee; rats received humane care consistent with institutional guidelines.

Enzymatic hydrolysis of daidzein and genistein conjugates and determination of their concentration in plasma. Rat plasma (50 μL) was mixed with 50 μL of sulfate type H-5 solution in 0.2 mol/L acetate buffer, pH 5 (the preparation contained 500 U of β-glucuronidase and 1 μL of sulfatase), and the mixture was incubated at 37°C in a shaking water bath for 1 h. Daidzein and genistein released during the incubation and their nonconjugated forms present in plasma before the hydrolysis were extracted with 900 μL of methanol/acetic acid (100:5, v/v) and determined as described above. The result was the total plasma concentration of daidzein and genistein.

To determine daidzein and genistein glucuronides, only 50 μL of plasma was mixed with 50 μL of β-glucuronidase (type VII-A) solution containing 50 U of the enzyme in 0.2 mol/L acetate buffer (pH 5), and the mixture was incubated at 37°C in shaking water bath for 2 h. Plasma daidzein or genistein glucuronide concentration was calculated as the difference between the plasma concentration before and after hydrolysis with β-glucuronidase.

For determination of daidzein and genistein sulfate conjugates only, 50 μL of plasma was incubated with 50 μL of H-5 sulfate solution (same as above), containing in addition 100 mmol/L of d-saccharic acid 1,4 lactone as a β-glucuronidase inhibitor. Plasma daidzein or genistein sulfate concentration was calculated as the difference between the plasma concentration before and after hydrolysis with sulfatase.

The results of subtraction of plasma concentrations of glucuronides, sulfates and nonconjugated forms of daidzein or genistein from their respective total plasma concentrations were attributed to the concentration of sulfate/glucuronide conjugates, the conjugates with both sulfate and glucuronide moieties.

Statistical analyses. Reported values represent means ± SD (n = 5). Comparisons were made at each time point using paired or unpaired Student’s t test, with P ≤ 0.05 considered to be significant.

RESULTS

Daidzein and genistein were administered orally to FD and F rats in 25 mmol/L Na2CO3 at 7.9 μmol/kg body (~2 mg/kg body), which resulted in plasma isoflavone concentrations in the micromolar range. Absorption profiles of daidzein and genistein in the F and FD groups are presented in Figure 1. After absorption, isoflavones are metabolized, forming glucuronide and/or sulfate conjugates. Enzymatic hydrolysis (β-glucuronidase and/or sulfatase) of blood plasma taken from rats after administration of daidzein and genistein resulted in two peaks in the HPLC chromatogram (compared with the chromatogram of hydrolyzed control plasma); these were identified as daidzein and genistein (Piskula et al. 1999). Using a combination of plasma enzymatic hydrolysis with β-glucuronidase and/or sulfatase, the concentration of total plasma isoflavones,
and the concentration of their glucuronides, sulfates and sulfates/glucuronides were measured. For determination of sulfates, sulfatase type H-5 was used. Because it also contains 500 U of β-glucuronidase per 25 U of sulfatase, it was necessary to add a β-glucuronidase inhibitor, 100 mmol/L of D-saccharic acid 1.4 lactone, to the enzyme solution. The amount of inhibitor was significantly higher than in a previous experiment, in which sulfatase type VIII (containing <3 U of β-glucuronidase per 25 U of sulfatase) was used (Piskula and Terao 1998a). The effectiveness of inhibition was confirmed when the same amounts of isoflavones were released after hydrolysis of plasma from isoflavone-administered rats with type H-5 and VIII sulfatases, both containing β-glucuronidase inhibitor. Moreover, when the same plasma was hydrolyzed with 500 U of β-glucuronidase type VII-A with 100 mmol/L of D-saccharic acid 1.4 lactone, no released daidzein or genistein was found; this is in contrast to the control hydrolysis without the inhibitor added. For each kind of enzymatic reaction, the hydrolysis time was set as the time at which further extension of hydrolysis did not influence the amount of isoflavones released.

Blood samples were collected throughout the 24-h experiment, and pharmacokinetic profiles of isoflavones and their conjugates were obtained for each rat. However, individual differences in the extent of absorption resulted in a high variation of results, and prolonged blood sampling caused significant blood loss. Therefore, at the end of the experiment, rats developed symptoms of hypoxia.

Because daidzein and genistein are absorbed from the rat stomach (Piskula et al. 1999), the compounds and their conjugates administered were found in plasma 3 min after administration. Moreover, 7 min later, in the FD group, plasma total daidzein and genistein reached maximum concentrations of 20.9 ± 4.4 and 11.4 ± 3.1 μmol/L, respectively (Fig. 1B). In the F group, the maxima for daidzein and genistein were found at 2 and 4 h after administration, i.e., 2.4 ± 0.8 and 1.8 ± 0.2 μmol/L, respectively (Fig. 1A). In the F group, isoflavones were found exclusively in the conjugated form, whereas in the FD group, nonconjugated daidzein and genistein were noted 5 min after administration at maximum concentrations of 0.85 ± 0.35 and 0.70 ± 0.30 μmol/L, respectively, and their plasma concentrations dropped below the detection limit within 1 h. In both groups, despite the same molar dose administered, total plasma concentration of daidzein was higher than that of genistein, opposite to the results after isoflavones were administered to rats with the absorption site restricted to the stomach (Piskula et al. 1999).

In the FD group (Fig. 1B), a rapid increase in plasma isoflavone concentration was followed by its rapid decrease, which indicates that very fast absorption is followed by efficient elimination and/or distribution of the compounds administered. Fed rats were also absorbing isoflavones very rapidly (Fig. 1A). However, the extent of absorption in this group during the first 2 h was significantly lower and, in contrast to the FD group, no strong fluctuations in plasma isoflavone concentration were observed.

Because of the several-fold differences in the extent of absorption between the F and FD groups as well as high individual variation among rats, the comparison of conjugation patterns of daidzein (Fig. 2) and genistein (Fig. 3) between the groups was possible only for the relative content of each type of metabolite (i.e., glucuronides, sulfates and sulfate/glucuronides) compared within the groups, more daidzein sulfates than genistein sulfates were found in both groups. The last type of isoflavone conjugates measured was the sulfate/glucuronides. Similar to the glucuronides, feeding was also the factor increasing the more glucuronides of daidzein and genistein compared with the FD group (Fig. 2A, Fig. 3A). Furthermore, when glucuronides of daidzein and genistein were compared within the group, in both groups, there were always more glucuronides of genistein than of daidzein. Also, glucuronides were the group of isoflavone conjugates whose concentration first dropped below the detection limit, i.e., 12 h after administration. Unlike glucuronides, sulfates of both daidzein (Fig. 2B) and genistein (Fig. 3B) decreased in the F group. When concentrations of plasma sulfates of daidzein and genistein were compared within groups, more daidzein sulfates than genistein sulfates were found in both groups. The last type of isoflavone conjugates measured was the sulfate/glucuronides.
paired Student’s HPLC. Values are means (100%) genistein concentration and its conjugates were determined by daidzein and genistein (Fig. 2C). Plasma concentration of sulfate/glucuronide conjugates of (rats orally administered genistein at 7.9 mmol/kg body. Plasma total (100%) genistein concentration and its conjugates were determined by HPLC. Values are means ± SD, n = 5. Significantly different (P < 0.05, paired Student’s t test) means at a time are marked (*).

plasma concentration of sulfate/glucuronide conjugates of daidzein and genistein (Fig. 2C, Fig. 3C); however, this increase was found to be significant only for a limited period after genistein administration (Fig. 3C). Of all the plasma genistein and daidzein conjugates determined, genistein conjugated both with sulfate and glucuronide moieties (Fig. 3C) and sulfated daidzein (Fig. 2B) were the metabolites that lasted in rat plasma for the longest time, and this was observed in both the F and FD groups.

DISCUSSION

Before administration, isoflavones were dissolved in 25 mmol/L Na₂CO₃. This is similar to another study (King et al. 1996) in which oral genistein administration at 79 μmol/kg body to fed rats resulted in the maximum total plasma genistein concentration of 11 μmol/L; this is significantly higher than results in this study, probably reflecting the 10-fold higher dosage. Because the first measurement of plasma genistein concentration in the study of King et al. (1996) was made 2 h after administration and was the highest in that experiment, it is difficult to state whether it was the maximum. The differences in the extent and profile of absorption between the fed and food-deprived groups (Fig. 1) can be attributed to several factors. At acidic pH, isoflavone solubility is low; therefore, isoflavones were administered completely solubilized in 25 mmol/L Na₂CO₃ at pH 10.6. Because the stomach produces acidic secreta, there was likely a decrease in solubility of administered isoflavones there, which strongly influenced the extent of absorption (Piskula and Terao 1998b, Piskula et al. 1999). Unlike in the FD group, rats in the FD group had stomachs with low pH contents which, despite provoking rapid precipitation of administered isoflavones, also delayed the dose transition and limited its surface contact with the digestive tract. In addition, interactions of the administered compounds with food particles are among the factors influencing their absorption (Welling 1996).

In both groups, daidzein was present in plasma at a higher concentration than genistein (Fig. 1). Similar observations were made after daidzein and genistein glucosides were administered to rats as a soy extract (King 1998). Additional measurement of urinary isoflavones in that study revealed a significantly higher content of daidzein than of genistein metabolites; the same result was reported in a human study by Xu et al. (1994) in which isoflavones were administered to adult women in the form of soy milk. In both cases, it was concluded that daidzein was more bioavailable than genistein.

Here, from the comparison of absorption profiles of daidzein and genistein, it is clear that daidzein plasma concentration dropped below the detection limit before genistein (20 vs. 24 h, respectively), suggesting that daidzein is eliminated preferentially from the common blood circulation or is further metabolized to derivatives other than simple conjugates. Because flavonoids enter the enterohepatic circulation after metabolic conjugation (Hackett 1986), it is possible that genistein is eliminated preferentially to daidzein with bile and subsequently reabsorbed, which results in its extended presence in blood. Extensive secretion of genistein conjugates with bile and its enterohepatic circulation were confirmed by Sfakianos et al. (1997) who showed that in female Sprague-Dawley rats, >70% of the genistein dose infused into the duodenum was excreted with bile as genistein 7-O-β-glucuronide within 4 h after infusion. The genistein metabolites collected with bile and reinfused into the mid-small intestine were again recovered at 70% from bile. The only genistein metabolite found in that study was genistein 7-O-β-glucuronide. That result differs from those of this and other studies in which genistein was found as a mixture of glucuronide and/or sulfate conjugates after its metabolic conversion. The discrepancy might have resulted from using animals that had had no exposure to genistein, beginning from gestation, or because genistein was infused into rats in a manner that bypassed the stomach. In experiments with rats, Manach et al. (1997) showed that the conjugation pattern of quercetin that was consumed with diet changed after an adaptation period. Rats not adapted to quercetin produced isorhamnetin and tamarixetin, two derivatives of quercitin, but after adaptation for 10 d to a quercetin-rich diet, no tamarixetin was detected. Moreover, because genistein is absorbed from the rat stomach, conjugates that differ from those produced by intestinal absorption may be
formed. After oral administration of genistein to rats, several genistein metabolites were isolated and identified from urine and bile; the main ones were 4′-O-sulfate and 4′-O-sulfate/7-O-β-glucuronide (Yasuda et al. 1996). In this study, the majority of daidzein and genistein conjugates had a sulfate moiety (sulfates and sulfates/glucuronides). However, it must be pointed out that the identity of conjugates here was based on the specificity of the enzymes used. A number of papers report sulfation of flavonoids or other phenolic compounds in rats. A large proportion of plasma quercetin was sulfated (36%) after in situ perfusion of quercetin through the rat jejunum plus ileum (Crespy et al. 1999). Moreover, when isolated rat liver was perfused with quercetin and catechin, three sulfated metabolites were formed from each flavonoid (Shali et al. 1991), i.e., quercetin gave two glucuronide/sulfate conjugates and one sulfate, whereas catechin yielded one such double conjugate and two sulfates. More than 98% of p-nitrophenol infused to male Sprague-Dawley rats was excreted with urine at the same rate as sulfates and glucuronides (Tremaine et al. 1984).

Sulfotransferases, located in the cytoplasm, often compete with glucuronyltransferases for substrates. The balance between sulfation and glucuronidation of various phenolic substances may be affected by the dose administered (Koster et al. 1981, Mehta et al. 1978). Generally, the capacity to conjugate with sulfate is limited compared with glucuronidation, and at a large dose, there is a shift from sulfation toward glucuronidation. Despite the use of the same methodology as in this study, results of a study on (-)-epicatechin absorption in food-deprived rats showed >85% of plasma (-)-epicatechin was glucuronized 4 h after oral administration of a high dose of 172 μmol of (-)-epicatechin per kg body (Piskula and Terao 1998a). This is clearly different from the results of this study in which only 20 and 38% of total isoflavone metabolites were glucuronized 4 h after daidzein and genistein administration, respectively, to food-deprived rats. In mice, the major urinary metabolites of orally administered phenol were phenol sulfate and phenol glucuronide; sulfation was the dominant pathway at all dose levels but decreased as the dose increased in favor of glucuronidation (Kenyon et al. 1995). Furthermore, comparing glucuronidation and sulfation of phenolic compounds, species differences must be considered. In ddY mice, during metabolism of troglitazone, an antiabetic agent, hepatic glucuronidation clearance was 170-fold higher than sulfation clearance, whereas in rats, sulfation was sixfold higher than glucuronidation (Izumi et al. 1997). Using the liquid chromatography-mass spectrometry technique for analysis of urine from humans and rats administered genistein, 4 and 26%, respectively, of genistein metabolites were identified as sulfate conjugates (Cimino et al. 1999).

Apart from species differences, sex difference also has a strong effect on the balance between sulfation and glucuronidation. Oral administration of isoflavones to male Wistar rats resulted in high plasma concentrations of their sulfated conjugates (Fig. 2B, C and Fig. 3B, C). In a report on the metabolic fate of acetaminophen in rats, Kane et al. (1990) showed that males excreted more acetaminophen sulfate than females and that these differences persisted in cultured hepatocytes. Moreover, after the acetaminophen dose was increased, a dose-related shift from sulfation toward glucuronidation was observed in both genders. Similarly, a study focused on sex differences in the excretion of glucuronide conjugates showed that female rats administered p-nitrophenol excreted more p-nitrophenolglucuronide in urine than males (Rush et al. 1983).

One of the conclusions of this study is that food deprivation increases plasma sulfate conjugates of orally administered isoflavones, with a simultaneous decline of glucuronide conjugates. This is likely the result of decreased production of uridine diphosphate glucuronic acid, which in turn is a consequence of a decrease in its precursor UDP-glucose (Price and Jollow 1998). It was also shown that food deprivation did not influence the rate of harmol sulfation in isolated rat hepatocytes but inhibited the glucuronidation rate by 50% (Sundheimer and Brendel 1984). There was also a suggestion that starvation can induce a shift from conjugation toward hydroxylation, which in turn can give rise to the formation of hazardous metabolites (Banhegyi et al. 1988). On the other hand, food deprivation increases plasma concentration of sulfate conjugates (Fig. 2B and Fig. 3B), especially of daidzein. This might be important in the protective action of dietary daidzein because daidzein sulfocojugates, at a micromolar concentration, were reported to be potent inhibitors of steroid sulfatase, an enzyme involved in the evolution of breast cancer (Wong and Keung 1997).

The formation of isoflavone glucuronides or sulfates can affect their biological activity profoundly; in many cases, this was demonstrated for their nonmetabolized forms. Nonconjugated (free) and sulfated forms of isoflavones were regarded as biologically “active,” whereas their glucuronides and sulfates/glucuronides were considered biologically “inactive” (Adlercreutz et al. 1993). Recently published data demonstrate that the glucuronide derivatives of daidzein and genistein also exhibit some biological activities. Although conjugation with glucuronic acid decreased the ability of isoflavones to compete with the binding of estradiol to estrogen receptors, their 7-O-glucuronides, at nutritionally relevant concentrations, were shown to activate human natural killer cells even better than their parental compounds and were weakly estrogenic (Zhang et al. 1999).

For populations with a low consumption of soy and soy products, dietary supplementation with isoflavones might be beneficial. Conjugation enzymes such as sulfotransferase or glucuronyltransferase convert isoflavones to more water-soluble products to enhance their excretion. The balance of these conjugation reactions determines the rate of metabolism and clearance of xenobiotic agents. It is clear from this study that food deprivation is a factor altering this balance. Therefore, understanding the effect of nutritional conditions on the conjugation profile of potentially beneficial soy isoflavones might be of both fundamental and practical value. Nevertheless, to eliminate the influence of interspecies differences in metabolism, a similar study in humans is required.

**LITERATURE CITED**


