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NONENZYMIC GLYCATION OF ALBUMIN BY ACYL GLUCURONIDES *IN VITRO*

COMPARISON OF REACTIONS WITH REDUCING SUGARS

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Abstract—Acyl glucuronides are ubiquitous metabolites formed from acidic xenobiotics and endogenous compounds, such as bilirubin. Previous studies indicated that the covalent binding of acyl glucuronides to proteins occurs via an imine intermediate in a manner analogous to the glycation of proteins via reducing sugars. When glucuronic acid was incubated in solution with albumin, it formed 10 and 4 times more fluorescent, Maillard reaction products with albumin after 25 days than did glucose or fructose, respectively. However, radiolabeled glucuronic acid exhibited less covalent binding to albumin than either glucose or fructose. Circular dichroism measurements indicate that glucuronic acid is about 0.02% open chain form with exposure of the reactive aldehyde, whereas fructose and glucose have 2 and 0.0026% present in solution as the open chain; thus, differences in reactivity of the reducing sugars were not correlated with exposure of the free aldehyde. Methyl glucuronate formed little fluorescent product with albumin, suggesting that the C-6 carboxylate of glucuronic acid may facilitate the reactions after covalent binding that lead to the formation of fluorescent products. When acyl glucuronide metabolites of two previously marketed acidic drugs, zomepirac and suprofen, were incubated with albumin at a concentration of 2.5 mM, more fluorescent product was formed than by 500 mM glucose. Reversible binding of the acyl glucuronides to albumin was 60–90%, but almost zero for the free reducing sugars, which indicates that reversible binding may explain the enhanced reactivity of the acyl glucuronides in forming fluorescent products with albumin. These results indicate that acyl glucuronides are reactive metabolites that may cause significant glycation of proteins with glucuronic acid *in vivo*.

In the last decade there have been extensive studies of the nonenzymic glycation of proteins by reducing sugars [1, 2]. Though initial interest focused on the hypothesis that glycation by glucose might prove to have an essential role in the sequelae of diabetes [3] and also aging [4], more recent studies have explored the potential role of fructose in diabetes [5, 6], especially for certain tissues which have an active sorbitol pathway, such as the eye. Because of the higher intrinsic reactivity of fructose compared to glucose when studied *in vitro* [5, 7], it is postulated that fructose could be relevant for glycation *in vivo* even though it may have lower concentrations than that of glucose in some tissues [5, 6]. Studies of glycation have been expanded to include additional carbohydrates such as galactose [8], fucose [9], mannose [9] and other reducing sugars [10], some of which have the potential to cause significant nonenzymic glycation *in vivo* [11]. Glucuronic acid is a reducing sugar of biological importance due to its role in the conjugative metabolism of xenobiotics and endogenous compounds [12] and its presence in glycoproteins, though its free concentration *in vivo* is normally negligible [13]. Recent studies of reactive acyl glucuronide metabolites of acidic compounds have indicated that these metabolites bind irreversibly to albumin and other proteins *in vivo* and *in vitro* and that some of the coupling to albumin is via the aldehyde of glucuronic acid in a manner analogous to nonenzymic glycation previously described for glucose and other reducing sugars [14, 15].

Acyl glucuronides are rather ubiquitous metabolites, formed by conjugation of glucuronic acid with carboxylic acids which are present in many drugs, endogenous compounds and other xenobiotics [12]. Acyl glucuronides were found to be reactive, binding irreversibly with albumin *in vitro* and *in vivo*, from studies first conducted with bilirubin glucuronides [16, 17]. Subsequent studies extended this observation to other acyl glucuronides, with several reports documenting covalent binding of acidic drugs to plasma proteins in humans after drug administration that resulted in exposure to acyl glucuronides [15, 18, 19]. Although acyl glucuronides were previously thought to be rapidly eliminated, more recent data have indicated that substantial concentrations of acyl glucuronides are present in plasma with levels of glucuronide metabolites reaching or exceeding that of the parent drug which are present in plasma at concentrations in the low micromolar level [19, 20]. The concentrations of reactive acyl glucuronides and reducing sugars (low millimolar) seen *in vivo* are much lower than those employed here *in vitro*; however, binding *in vivo* can be cumulative over many years for long-lived proteins [4]. Moreover, in disease states that

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compromise excretion, the reactive drug conjugates would be expected to accumulate and react more extensively with proteins, as shown for bilirubin glucuronides which can reach concentrations of up to 0.2 mM *in vivo* [21].

The mechanism for the irreversible binding of acyl glucuronides to proteins is not completely understood, but is postulated to be by either of two mechanisms. The first mechanism proposed was direct nucleophilic displacement of glucuronic acid from the acyl glucuronide by reactive nucleophilic functional groups of proteins [22, 23]. An alternative mechanism was later proposed where acyl glucuronides undergo intramolecular based-catalyzed acyl migration [the β-1 acyl glucuronide rearranges to form isomeric conjugates with the acyl group on the 2-, 3- or 4-position of glucuronic acid], and then a Schiff's base (imine) is formed between the free aldehyde of glucuronic acid and the ϵ-amine of lysines present in proteins [14]. Although the exclusive existence of one mechanism has not been determined, studies with imine trapping reagents and the release of isomeric conjugates of acyl glucuronides from the protein adduct have shown conclusively that part of the irreversible binding of acyl glucuronides to albumin is attributable to Schiff's base formation in a manner analogous to the well described glycation of proteins via reducing sugars [14].

Because several of the documented reactive acyl glucuronides are conjugates of drugs that were withdrawn from the market due to toxicity [24] and bilirubin glucuronides lead to substantial concentrations of "biliprotein" in some patients with hepatic disease [21], present investigations have been directed toward understanding the chemical nature of the covalent adduct of acyl glucuronides with protein. Reports that glycation with reducing sugars can produce cross-linking of proteins [6], modify protein function [25, 26] or form advanced glycation end products (AGE)* which are immunogenic [27] suggested that the glycation of proteins *in vivo* by glucuronic acid present in reactive acyl glucuronides might provide a potential step in the mechanism for the toxicological properties of compounds metabolized to acyl glucuronides. Therefore, the studies presented here were designed to compare the glycation ability of glucuronic acid with that of glucose or fructose as measured by irreversible radiolabel incorporation and the generation of fluorescent products via the Maillard-like reaction. Moreover, acyl glucuronide metabolites were also examined to evaluate their potential for the formation of similar fluorescent products with albumin.

MATERIALS AND METHODS

Materials. Bovine serum albumin fraction V (BSA), human serum albumin fraction V (HSA), D-[U-^{14}C]glucose (256 mCi/mmol) and D-[U-^{14}C]-fructose (298 mCi/mmol) were purchased from the Sigma Chemical Co. (St. Louis, MO). D-[6-^{14}C]-Glucuronic acid was prepared as described [9] using sodium [^{14}C]cyanide (58 mCi/mmol), also obtained from Sigma. All radiolabeled sugars were purified to radiochemical purities > 98% by HPLC (Phenomenex Nucleosil SB, 100 Å 5 μm column, 0.7 N acetic acid as mobile phase at 40° and 4 mL/min) prior to use. Suprofen was isolated by ethyl acetate extraction and crystallization from Suprol® tablets (McNeil Pharmaceuticals, Spring House, PA). Glucuronic acid, sodium salt, was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Zomepirac-Na was supplied by McNeil Pharmaceutics. Spectropore® dialysis tubing was purchased from VWR Scientific (Houston, TX). Solvents for chromatography were HPLC grade and all other reagents were analytical grade.

Acyl glucuronides. Acyl glucuronide conjugates of suprofen and zomepirac were isolated from urine of a human volunteer after oral dosing. Urine was adjusted to pH 2–5 immediately upon collection, and then concentrated by passing urine at pH 2 through a Sep-Pak® C18 cartridge (Waters Associates, Milford, MA). Elution of the conjugate fraction with 80% acetonitrile/0.01 M trifluoroacetic acid (TFA) buffer was followed by removal of the acetonitrile under vacuum. The concentrated material was then purified by preparative C18 reversed phase chromatography (10 × 250 mm) using 4 mL/min methanol/0.01 M TFA as the eluent with monitoring of UV at 225 nm. The fraction of methanol eluent was adjusted to provide a retention time of 15–20 min for the respective acyl glucuronide. The acyl glucuronide fractions from preparative HPLC were combined and then lyophilized, and aliquots were analyzed by analytical HPLC for purity and for content of parent drug after hydrolysis with strong base. Purity was greater than 98%, present as the acyl glucuronide or its isomeric conjugates formed by acyl migration, though the isomers were less than 1% of the total conjugates for the acyl glucuronides of zomepirac and suprofen. Conjugates were characterized by analytical HPLC, susceptibility to cleavage by β-glucuronidase or strong base, and mass spectrometry as previously described [28].

Incubation of sugars with albumin. Bovine serum albumin at a concentration of 0.1 mM was incubated at 37° with 0.5 M sugars in 0.05 M sodium phosphate buffer (pH 7.0) containing 0.1 M sodium chloride (PBS). Experiments carried out with the solutions filtered through a 0.45 μm membrane prepared under sterile conditions were compared to solutions employing 0.015% sodium azide to maintain sterility. No microbial contamination was detected using either of these methods. At the indicated time intervals, aliquots were removed and dialyzed repeatedly against 250- to 1000-fold of PBS solution at 4° for 5–7 days. A similar experiment was conducted with HSA to provide a comparison to BSA. After the dialysis, a 0.15-mL sample was diluted to 4 mL with water and the intensities of fluorescence were measured (Ex: 350 nm; Em: 410 nm). Fluorescence measurements were carried out on an Aminco–Bowman spectrofluorometer calibrated with diflunisal (1 mg/mL in methanol). Incubation of BSA solutions served as a control.

* Abbreviations: AGE, advanced glycation end products; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HSA, human serum albumin; and TFA, trifluoroacetic acid.

For studies with radiolabeled sugars, the purified label was added to the incubation containing 0.5 M unlabeled sugar to a final concentration of 4 μCi/mL. Protein concentrations were assayed by the method of Bradford [29] using BSA as the standard (Bio-Rad Laboratories, Richmond, CA). UV absorbance was measured using an HP 8452A spectrophotometer.

Incubation of acyl glucuronides with albumin. The procedures were the same as for the incubations with sugars described above; however, only 2.5 mM suprofen glucuronide or zomepirac glucuronide was employed in contrast to a 500 mM concentration for the sugars. Acyl glucuronides hydrolyzed with strong base (5 M NaOH at 80° for 1 hr) and then neutralized, were incubated with albumin to serve as controls for the hydrolysis of acyl glucuronides that does occur slowly at pH 7.0. Zomepirac and suprofen are stable to the basic conditions employed. In separate similar experiments, 2.5 mM acyl glucuronides of suprofen and zomepirac were incubated with 0.1 mM BSA and the extent of covalent binding of the respective drug to the protein was determined over 72 hr using methods previously described [15].

Preparation of methyl-D-glucuronate. The methyl ester of glucuronic acid was prepared according to a literature method [30] by refluxing D-glucurono-3,6-lactone (5 g, 0.028 mol) with anhydrous methanol (50 mL) under nitrogen for 72 hr. The methanol was evaporated to yield a yellow syrup that was taken up with 30 mL absolute ethanol and allowed to stand in the refrigerator for 24 hr. Unreacted D-glucurono-3,6-lactone was recovered as a solid. The ethanol was removed *in vacuo* to yield a yellow syrup. The syrup was used for incubation with albumin without further purification. Proton NMR confirmed the methyl ester by the addition of a singlet at 3.66 ppm representing 3 protons and was compared to standards of glucuronic acid.

Incubation with methyl-D-glucuronate. The procedures were similar to those used for the incubation with sugars; however, since the reaction mixture produced precipitate after 24 hr, aliquots were centrifuged to separate the supernatant and precipitate at the indicated time. The supernatant containing soluble protein was dialyzed and analyzed as described above for incubations with sugars.

Reversible binding to albumin. Due to the inherent instability of acyl glucuronides in plasma, rapid ultrafiltration was employed to assess reversible binding to plasma proteins. Human serum albumin (0.5 mM) was mixed with various concentrations of reducing sugars or acyl glucuronides in PBS and equilibrated at 37° for 5 min. The 1.0-mL solution was then transferred to an MPS-1 ultrafiltration assembly (Amicon Co., Beverly, MA) equipped with a YMT semipermeable membrane. The samples were centrifuged at 500 *g* with a Beckman J2-21 centrifuge at room temperature. An aliquot of 100 μL was taken from each side of the filter membrane for analysis. For acyl glucuronides, the aliquots were added in 150 μL ethanol containing 0.5% phosphoric acid which stabilized the labile conjugates and precipitated albumin. The acidic solution was cooled on ice for 3 min; then 150 μL of acetonitrile was added with vortexing. After the addition of internal standards for HPLC and centrifugation, the mixture was decanted and analyzed by HPLC. For radiolabeled sugars, the aliquots were made basic with 0.2 M NaOH, heated at 40° overnight, then neutralized with phosphoric acid and made ready for scintillation counting (ReadySafe LSC fluid, Beckman). HPLC analysis was carried out with a reversed phase C18 column (Axxiom, 5 μm 4 × 150 mm) using a Hewlett Packard 1050 UV detector (San Jose, CA). For suprofen glucuronide, the detector was set at 280 nm and 40% methanol/0.01 M sodium acetate (pH 5.01) was employed as the eluent at 1 mL/min. For zomepirac glucuronide, the detector was set at 295 nm and 57% methanol/0.01 M TFA was used as the eluent at 1 mL/min. Peak area ratio measurements relative to internal standards provided acyl glucuronide concentrations in the protein free filtrate which were compared to concentrations in the albumin solution. The ratio [free concentration]/[total concentration] represents the free fraction, i.e. percent unbound, of the acyl glucuronide in the albumin solution. There was very little hydrolysis of the acyl glucuronides in the albumin solution during the short period of exposure to albumin at physiological pH during equilibration and centrifugation.

Circular dichroism measurement. The JASCO J-20 spectropolarimeter was calibrated with ammonium *d*-camphor-10-sulfonate [31]. Fructose (0.1 M), glucose (0.5 M) and glucuronic acid (0.5 M) were dissolved in 0.15 M sodium phosphate buffer at pH 7.4 and scanned using a 10 mm cell.

RESULTS

The incubation of the three reducing sugars with BSA produced fluorescent products (Fig. 1) with

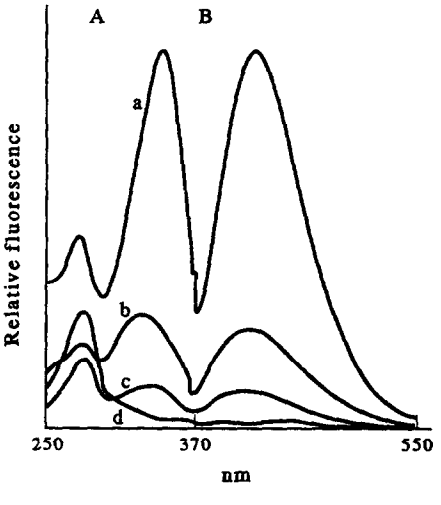

Fig. 1. Fluorescence excitation (A) and emission (B) scans of glycated BSA. BSA (0.1 mM) was incubated with 500 mM glucuronic acid (a), fructose (b), or glucose (c) for 25 days (other conditions are described in the text). A control incubation with only BSA (d) is also shown. Excitation and emission held at 350 and 410 nm, respectively.

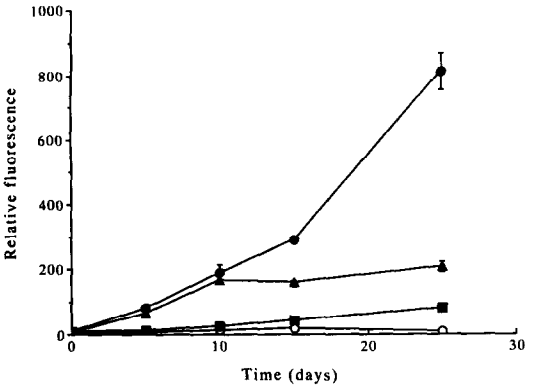

Fig. 2. Time course of fluorescence generated by sugars and BSA. BSA (0.1 mM) was incubated with 500 mM glucose (■), fructose (▲) or glucuronic acid (●) under the conditions described in the text. Fluorescence values were normalized with respect to protein concentrations. A control of BSA solution (○) was incubated in the absence of sugars. Values are means ± SD, N = 3.

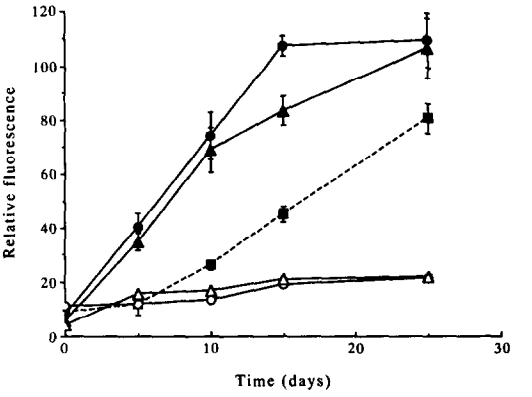

Fig. 3. Time course of fluorescence generated by acyl glucuronides and BSA compared to glucose. BSA (0.1 mM) was incubated with 2.5 mM suprofen glucuronide (●) or zomepirac glucuronide (▲) under the conditions described in the text. Fluorescence values were normalized with respect to protein concentrations. Acyl glucuronide solutions hydrolyzed with base [(○) for suprofen glucuronide, (△) for zomepirac glucuronide] served as controls. Glucose [(■) 500 mM] incubated with BSA is shown for comparison. Values are means ± SD, N = 3.

similar maximum excitation/emission at 350/410 nm. These fluorescent products were formed in a time-dependent manner with relative intensities of 1:3.9:10.1 for glucose, fructose, and glucuronic acid when measured at 25 days as shown in Fig. 2. A marked increase in fluorescent product occurred between days 15 and 25 for glucuronic acid and at the conclusion of the experiment there was no apparent plateau observed for incubations of albumin with the uronic acid. Similar results were obtained by using HSA rather than BSA (data not shown). The control incubation of only BSA produced no fluorescent product (Fig. 1). Acyl glucuronides, although incubated at a much lower concentration (2.5 mM) than that of the reducing sugars (500 mM), provided fluorescent intensities 40% higher than that of glucose on day 25 and also exhibited time-dependence for the product formation (Fig. 3). The fluorescence spectra of the product formed by incubations of BSA with the acyl glucuronides were similar to that obtained with the reducing sugars. Control incubations with hydrolyzed acyl glucuronides which contained 2.5 mM drug and free glucuronic acid had no apparent increase in fluorescence over time.

The time-dependent binding of radiolabeled glucose, fructose and glucuronic acid showed a relative covalent binding to BSA at day 25 of 1.00:1.68:0.77 (Fig. 4) when the specific activity of the label and the concentration of the sugars were the same. In the first 24 hr, the rate of covalent binding of glucuronic acid was higher than that of glucose, though lower than seen for fructose (see inset to Fig. 4); however, at later times the amount of glucuronic acid bound was less than that of glucose. All three reducing sugars reached an apparent plateau after 5 days (120 hr) and the stoichiometry of the binding at day 25 was 1.2:1.0 for glucuronic acid relative to BSA which indicates

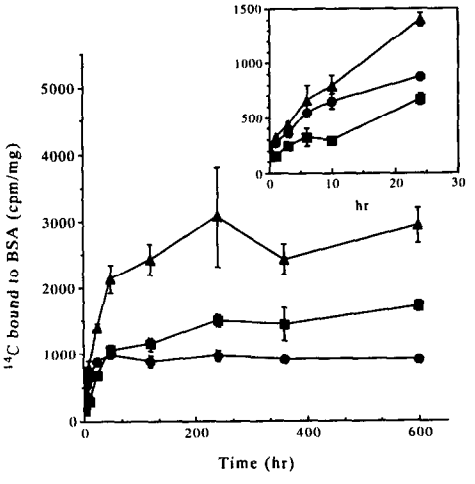

Fig. 4. Time course of covalent binding of radiolabeled sugars to BSA. BSA (0.1 mM) was incubated with 500 mM glucose (■), fructose (▲) or glucuronic acid (●) under the conditions described in the text. The inset expands the first 24-hr period of the incubation. The cpm values were normalized with respect to protein concentrations. Values are means ± SD, N = 3.

that the binding of the sugars to the proteins was determined by an equilibrium reaction as previously reported [32] and that there was more than one binding site per albumin molecule.

Incubations of the acyl glucuronides (2.5 mM) with BSA (0.1 mM) produced time-dependent covalent binding, as measured by drug liberated from the protein by hydrolysis with base, which was

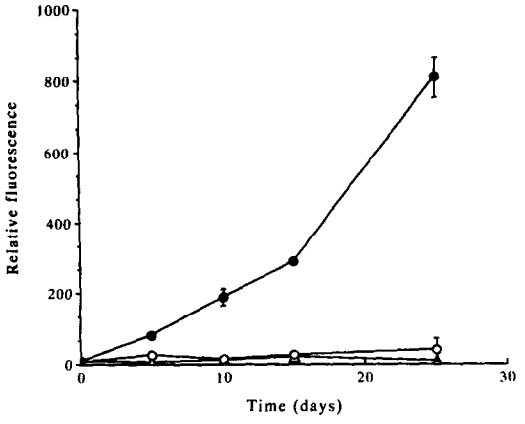

Fig. 5. Time course of fluorescence generated by methyl glucuronate and BSA. BSA (0.1 mM) was incubated with 500 mM glucuronic acid (●) or methyl glucuronate (○) under the conditions described in the text. Fluorescence values were normalized with respect to protein concentrations. A control solution of BSA (△) was incubated in the absence of sugars. Values are means ± SD, N = 3.

qualitatively similar to previously published data for zomepirac [15]. At 72 hr the amount of suprofen or zomepirac covalently bound to BSA indicated that there were 0.17 and 0.18 equivalents of drug bound per albumin molecule following incubations with each respective acyl glucuronide. Acyl glucuronides with radiolabel present in the glucuronic acid moiety were not available; however, previous data indicate that a significant fraction of the drug which becomes covalently bound is bound via the glucuronic acid moiety by the imine mechanism mentioned above [14].

To examine whether reactions occurring after covalent binding may be enhanced by contributions of the carboxylic acid group of glucuronic acid, glucuronic acid was converted to its methyl ester. After incubation of methyl glucuronate with albumin for 24 hr, a precipitate started to form. This precipitate may be due to covalent adduct which is less ionized and perhaps of lower solubility than native BSA. Although the cause of the precipitate is not clear and studies to address this are in progress, methyl glucuronate was almost totally devoid of ability to generate soluble fluorescent products with BSA. As shown in Fig. 5, methyl glucuronate produced very little fluorescence relative to incubations of glucuronic acid with albumin.

Reversible binding studies using radiolabeled sugars with 0.5 mM HSA consistently yielded a fraction unbound equal to 1.0 across a range of sugar concentrations from 0.05 to 500 mM. The very low fraction bound could not be measured accurately though it was estimated to be less than 1%. Using HPLC analysis, acyl glucuronides of suprofen and zomepirac had a fraction bound to BSA equal to 79.5 ± 4.9 and 88.6 ± 2.7% (N = 4), respectively, at a glucuronide concentration of 0.16 mM. There was a concentration dependency in the binding, with lower binding of the acyl glucuronide when their concentrations approached that of albumin. However, with equimolar concentrations of acyl glucuronide relative to albumin, the fractions bound reversibly to protein still exceeded 60%.

Circular dichroism measurements provide an estimate of the acyclic free aldehyde fraction of the sugars in solution [33]. From the spectra obtained relative to camphor standard, fructose had approximately 2%, glucuronic acid 0.020% and glucose 0.0026% free aldehyde present in phosphate buffer at pH 7.4. These values for fructose and glucose agree with previous literature values [33]. Attempts to measure the circular dichroism spectra of acyl glucuronides were unsuccessful due to the limited amount of material available. If sufficient acyl glucuronide material were available, it is questionable whether the acyl glucuronide could provide adequate spectra, as suprofen glucuronide is a diastereomeric mixture due to suprofen being racemic, and both suprofen and zomepirac have ketone functional groups that may obscure the signal of any free aldehyde present in glucuronic acid.

DISCUSSION

These data show that glucuronic acid, through labile and reactive acyl glucuronide metabolites, can bind to albumin, leading to the formation of fluorescent AGEs. The reactivity of glucuronic acid was expected based upon much previous data on nonenzymic glycation via reducing sugars; however, the fact that the formation of AGE was facilitated by acyl glucuronides has not been reported previously.

The comparison of the abilities of glucose and fructose to form fluorescent products with albumin, shown in Fig. 2, agrees qualitatively with a previous report where fructose produced about 10 times more fluorescence than glucose [5]. This relative fluorescence correlates with the rates of imine formation with hemoglobin for fructose and glucose [7]. As shown in Fig. 1, the fluorescence spectra were similar for all three sugars with maximum excitation/emission at 350/410 nm as previously reported for glucose and fructose [5]. Although the formation of fluorescence products with albumin was much greater for glucuronic acid relative to glucose and fructose, from the studies using radiolabeled material presented in Fig. 4, it is apparent that covalent binding to albumin does not rank correlate with the ability to subsequently form fluorescent products. If a comparison were made between only glucose and fructose, the fluorescence (Fig. 2) and radiolabel incorporation (Fig. 4) do rank correlate, though the relative fluorescence was generated at about twice the value of the ratio for binding of radiolabel at day 25. The relative ratio of fluorescence/radiolabel incorporation was 1.0, 2.3 and 13.1, respectively, for glucose, fructose and glucuronic acid. This suggests that reactions which lead to fluorescent products and occur after covalent binding are more efficient for fructose than glucose and underscores the potential role that fructose may have in forming AGEs in some tissues of the body.

When fluorescent product is compared relative to binding of radiolabeled material, it is evident that

reactions that occur after covalent binding are facilitated for glucuronic acid compared to reactions for fructose or glucose. Previous studies on the Maillard reaction of lysine and glucuronic acid indicates that imines formed with glucuronic acid undergo the Amadori rearrangement almost instantly [34]. This suggested that if glucuronic acid did form an imine with albumin in a manner similar to hexoses, then its rate of irreversible binding to protein might be much higher and the subsequent formation of fluorescent products could be enhanced. This was not observed, as the incorporation of radiolabeled glucuronic acid with albumin reached an apparent equilibrium after 5 days that was substantially below that of either glucose or fructose (Fig. 4). Therefore, the disparity in the propensity of the reducing sugars to form fluorescent products with albumin may be due to other factors beyond the initial formation of the covalently bound imine or Amadori intermediates. These factors may include binding to different sites on albumin or the inherent ability of the bound sugar to undergo further reactions to generate the presently unidentified fluorescent products. The later reaction, that of forming fluorescent products, appears to be faster for glucuronic acid since its fluorescence showed no apparent plateau and increased substantially between days 15 and 25 (Fig. 2).

Since glucuronic acid bound to albumin was many fold more efficient in forming fluorescent products than glucose or fructose, the major distinction between these sugars, namely the carboxylate at C-6 was investigated for its potential role in facilitating the formation of the fluorescent products. Methyl glucuronate was devoid of ability to form soluble fluorescent products with albumin (Fig. 5), though it also reduced the solubility of most of the albumin within 24 hr of incubation. Whether this altered solubility is due to possible protein cross-linking or an alteration in the nature of lysine residues on albumin has not yet been evaluated. Though the covalent binding of radiolabeled methyl glucuronate to albumin has not been examined, the results do suggest that the free carboxylate of glucuronic acid may be critical for its enhanced formation of fluorescent product with albumin. The influence of charge of the reducing sugar on the effects of covalent binding to protein has been discussed [7]; however, this effect does not explain the differences in the extent of fluorescent products formed by glucuronic acid compared to glucose and fructose, since radiolabeled glucuronic acid had lower covalent binding to BSA than either glucose or fructose (Fig. 4).

The importance of the open chain, acyclic form of the sugar and its aldehyde as a reason for different initial rates of glycation for the sugars has been discussed previously [7]. Using circular dichroism measurements, the fraction of glucuronic acid present in the open chain form is estimated to be 0.02% in contrast to 2% for fructose and 0.0026% for glucose. This may explain the initial rates of binding observed for the radiolabeled material (inset of Fig. 4), but does not provide support for either the relative covalent binding of glucuronic acid to albumin at equilibrium compared to the other reducing sugars (Fig. 4) or the enhanced formation of fluorescent products (Fig. 2).

The reactions of glucuronic acid with proteins may be of little consequence if its concentrations are very low *in vivo*. However, since there are measurable levels of unstable acyl glucuronides *in vivo* which covalently bind to plasma proteins [15, 18, 19, 21] and evidence exists for the formation of an imine-mediated adduct between the isomeric conjugates and albumin [14], the potential for these metabolites to generate fluorescent products with albumin was examined. On a molar basis, the acyl glucuronides of suprofen and zomepirac were about 280 and 27 times more efficient than glucose or free glucuronic acid, respectively, in forming fluorescent products with albumin *in vitro*. Thus, although levels of acyl glucuronides may be much lower than that of glucose (2.5 to 5.0 mM) *in vivo* (e.g. zomepirac glucuronide has an average maximum plasma concentration of 5.8 μM in humans [20]), there is the potential for the formation of fluorescent AGE *in vivo* due to what could be described as "acyl glucuronide induced glycation". As described for the glycation of proteins by reducing sugars [1–4], acyl glucuronide induced glycation with glucuronic acid would be expected to accumulate over long periods of chronic dosing of such acidic drugs, especially for long-lived proteins. It is also possible that certain organs, such as the liver and kidney, may be exposed to much higher concentrations of reactive acyl glucuronides due to their respective roles in forming and eliminating acyl glucuronides.

The enhanced efficiency in forming fluorescent products with albumin for the acyl glucuronides compared to free reducing sugars may be explained if reversible binding to the protein is a requisite for covalent binding of the acyl glucuronides. Lipophilic acids, such as the nonsteroidal antiinflammatory drugs and bilirubin, are generally reversibly bound to albumin to a high extent. Though conjugation with glucuronic acid substantially increases the polarity of the glucuronide relative to the parent, the fractions of zomepirac glucuronide and suprofen glucuronide reversibly bound to albumin were still high, ranging from 60 to 90%. The fraction of the free reducing sugars reversibly bound to albumin were not measurable, indicating that almost all sugar is free, not reversibly bound, in solution. Thus, the high reversible binding of acyl glucuronides relative to free sugars is a probable explanation for their enhanced formation of fluorescent products when incubated with albumin. The stoichiometry of the covalent binding of the acyl glucuronides to BSA after 72 hr, as measured by drug covalently bound, was about 0.18:1 when 25-fold excess acyl glucuronide to BSA was employed. In contrast, the 5000-fold excess (500 mM sugar:0.1 mM BSA) of glucuronic acid provided a stoichiometry for covalent binding of 1.2:1. The observed stoichiometries did correspond to relative fluorescence produced as shown by a comparison of glucuronic acid in Fig. 2 to acyl glucuronides in Fig. 3; however, it is evident that the acyl glucuronides were much more efficient on a molar basis than the free sugars for the initial step of covalent binding to BSA. In summary, the data indicate that, on a molar basis, the acyl

glucuronides undergo much greater covalent binding to albumin and, once covalently bound, glucuronic acid is more efficient than the other reducing sugars studied in progressing to fluorescent products.

When considered together, these data indicate that glucuronic acid is a reducing sugar that may have significant potential for the glycation of some proteins *in vivo*. Though levels of glucuronic acid are not significant relative to other sugars *in vivo*, acyl glucuronides, a common metabolite of many acidic xenobiotics and endogenous compounds, may provide an efficient means for glycation of some proteins with glucuronic acid and such binding might be expected to be cumulative during chronic dosing of drugs metabolized to these reactive acyl glucuronides. Once covalently bound to protein via the reactive acid glucuronides, the results reported here with albumin indicate that glucuronic acid undergoes subsequent reactions to fluorescent, Maillard products with greater facility than either glucose or fructose. The importance of these reactions of acyl glucuronides with proteins *in vivo* are currently being investigated for their potential relationships to the toxicity of acidic drugs and bilirubin.

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