N-Glucosyl Conjugates of Chlorinated Anilines: Spontaneous Formation and Cleavage

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N-Glucosides are known plant metabolites of chlorinated anilines, which are formed by *N*-glucosyltransferases utilizing UDP-glucose. Free D-glucose and free 3,4-dichloroaniline have now been found to also conjugate spontaneously under physiological conditions of pH and temperature. Rate constants for spontaneous formation and cleavage of the *N*-glucosyl conjugate of 3,4-dichloroaniline were determined. The dissociation equilibrium constant calculated from the two rate constants ($K_{diss} \approx 0.1$ M) was confirmed by direct measurement. Formation as well as cleavage of the *N*-glucoside showed general acid catalysis in the pH range of about 3–8. A previously proposed reaction mechanism is applied to interpret these results and to explain the known broad range of acid sensitivity of pesticidal *N*-glucosides. It should thereby become possible to predict the degree of cleavage of *N*-glucosyl conjugates under stomach conditions (pH 1–2, 37 °C), thus providing a first estimate of animal bioavailability of the pesticidal aglycon.

INTRODUCTION

Pesticides and other xenobiotics are usually metabolized in plants by transformation and conjugation reactions, followed by deposition in the plant tissue over considerable time intervals (Frear, 1976; Sandermann, 1987). This general pattern also applies to herbicides containing NH_2 groups such as metribuzin, pyrazon, amitrole, picloram, and chloramben which all form *N*-glucoside conjugates (Frear, 1976; Lamoureux and Frear, 1979; Lamourex and Rusness, 1986; Hatzios and Penner, 1982). In addition, many natural plant constituents, such as nucleic acid bases, cytokinins, or nicotinic acid, are known to form *N*-glycoside conjugates. The herbicide propanil is a well-studied example for sequential transformation by a plant amidase and conjugation of the hydrolysis product 3,4-dichloroaniline with D-glucosyl, malonyl, or lignin residues. These conjugation processes have been studied by feeding the direct precursor 3,4-dichloroaniline to intact plants (Still et al., 1968) or plant cell cultures (Winkler and Sandermann, 1989). A soybean *N*-glucosyltransferase specific for chlorinated anilines has been purified and shown to work with the energy-rich nucleotide sugar, UDP-glucose (Frear, 1968; Sandermann et al., 1991). In comparison to other xenobiotic plant conjugates, the *N*-glucosides of chlorinated anilines were unusually sensitive to acids. Complete bioavailability of the xenobiotic aglycons of these *N*-glucosides under the pH conditions of the human stomach (pH 1–2; 37 °C) is therefore expected (Sandermann, 1987; Winkler and Sandermann, 1989). In view of the importance of predictive methods in ecotoxicology, the acid sensitivity of the *N*-glucoside of 3,4-dichloroaniline has now been studied in a more comprehensive fashion. The kinetics for hydrolysis and formation of the *N*-glucoside of 3,4-dichloroaniline are described in order to characterize the equilibrium:

D-glucose + chloroaniline ⇌ chloroaniline *N*-glucoside

MATERIALS AND METHODS

Materials. 3,4-Dichloroaniline was purchased from Riedel-de-Haen, Seelze-Hannover, FRG. The *N*-glucoside of 3,4-dichloroaniline (F, 148–151 °C) was synthesized as previously described (Winkler and Sandermann, 1989).

HPLC Procedure. Two Model 114M solvent delivery pumps, a Model 420 control unit, a system organizer gradient mixer, and a Model 160 UV-absorbance detector (all from Beckman Co., Munich) were used. The aqueous sample (10 μL) was applied to a Lichrosphere RP-8 (12.5 × 0.4 cm) column, followed by elution with a binary solvent system: 0.085% (w/v) aqueous phosphoric acid (component I) and 0.085% (w/v) phosphoric acid in 90% (v/v) aqueous acetonitrile (component II). A gradient increasing from 25 to 85% component II was formed with 20% difference/min, at a flow of 1 mL/min. Retention volumes of 3.5 and 5.2 mL were obtained for 3,4-dichloroaniline *N*-glucoside and free 3,4-dichloroaniline, respectively. Substrate amounts were calculated from integrated peak areas, taking into account that the molar absorption of free 3,4-dichloroaniline was under the present conditions lower by a factor of 0.6 than that of the *N*-glucoside.

Hydrolysis Assay. 3,4-Dichloroaniline *N*-glucoside (20 μL; 1.5 mM) in 50% (v/v) methanol was added to 480 μL of 1 M phosphate buffer adjusted to various pH values. The incubation was terminated by injection of a 10-μL aliquot onto the HPLC column.

Reversal of Hydrolysis. A hydrolysis assay mixture at pH 3 was incubated for 8 days at 25 °C so that the *N*-glucoside was completely hydrolyzed. A 10-μL aliquot of 555 mM D-glucose in water was added, and HPLC analysis was performed after various time intervals.

pH Dependence of N-Glucosidation. A 10-μL aliquot of 1.5 mM 3,4-dichloroaniline in methanol was added to a mixture of 480 μL of 1 M phosphate buffer of the desired pH value and 10 μL of 555 mM D-glucose in water. Aliquots (10 μL) of the incubation mixture were examined by HPLC after various time intervals.

Dependence of N-Glucosidation on D-Glucose Concentration. A 10-μL aliquot of 1.5 mM 3,4-dichloroaniline in methanol was added to 490 μL of 0.4 M phosphate buffer, pH 4.0, containing between 1.1 and 322 mM D-glucose. Aliquots (10 μL) were analyzed by HPLC after various time intervals.

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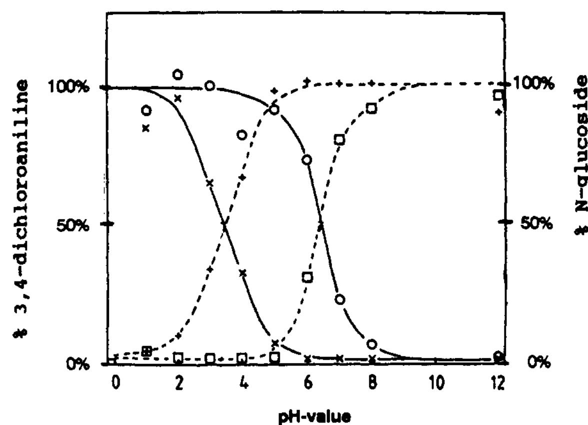

Figure 1. Hydrolysis of 3,4-dichloroaniline *N*-glucoside (31 μM) in 1 M sodium phosphate buffer of the indicated pH value at 25 °C. The concentrations of 3,4-dichloroaniline (× and ○) and of the *N*-glucoside (+ and □) were determined by HPLC after 10 min (×, +) and 23 h (○, □) incubation time, respectively. Experimental points were approximated by curves (—) for free 3,4-dichloroaniline and (- - -) for 3,4-dichloroaniline *N*-glucoside, respectively. The midranges of the curves intersect at pH 3.3 for the 10-min values and at pH 6.3 for the 23-h values, respectively.

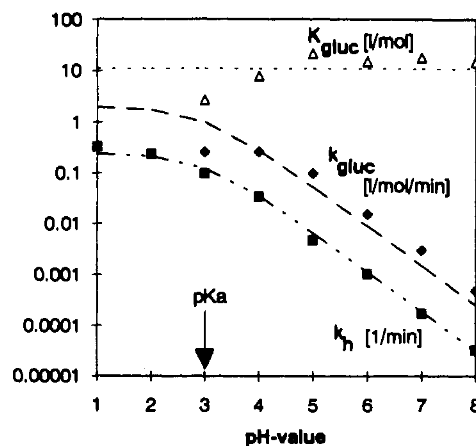

Figure 2. Dependence of kinetic constants on pH value. The experimental points (■) for the hydrolysis rate constant, k_h, are approximated by a curve calculated with the following empirical relationship: $k_h = 0.26/(1 + 10^{pH-pK_a})^{0.8}$. Here, pH is the experimental pH value. The pK_a of 3,4-dichloroaniline is 3.0. The experimental values (◆) of the rate constant for conjugate formation, k_{gluc}, are approximated by a curve calculated from the following empirical relationship: $k_{gluc} = 4.7/(1 + 10^{pH-pK_a})^{0.8}$. Division of k_{gluc} and k_h at each experimental pH value led to the data points (△) for the equiliibrium association constant, K_{gluc} (see eq 3 of text). K_{gluc} was about 11.0, as shown by the regression line (- - -).

RESULTS

Starting Observation: Spontaneous *N*-Glucoside Formation. We have previously reported on the mineralization of lignin-bound and free chloroanilines by the white-rot fungus, *Phanerochaete chrysosporium* (Arjmand and Sandermann, 1985). HPLC analysis of fungal cultures containing 32 μM 3,4-dichloroaniline and 11–55 mM added D-glucose showed rapid, but transient formation of a conjugate that was identical with 3,4-dichloroaniline *N*-glucoside under gradient as well as isocratic HPLC conditions (see Materials and Methods; Winkler and Sandermann, 1989). The *N*-glucoside also formed in growth medium without fungal inoculum and was under these conditions (pH 4.5, 37 °C) stable for several days. Addition of an equal volume of 1 N HCl at room temperature led to complete disappearance of the conjugate. Acid sensitivity is a characteristic property of chloroaniline *N*-glucosides (Winkler and Sandermann, 1989). *N*-(Chlorophenyl)glucosides are formed in plants by a specific *N*-glucosyltransferase that utilizes UDP-glucose (Frear, 1968; Sandermann et al., 1991). In contrast, 3,4-dichloroaniline *N*-glucoside appeared to be formed in fungal growth medium by a spontaneous chemical process from free 3,4-dichloroaniline and free D-glucose. The spontaneous formation of pesticidal conjugates is a rare process because usually energy-rich donor molecules and rather specific enzymes are involved (Sandermann, 1992). Hydrolysis and formation of 3,4-dichloroaniline *N*-glucoside have therefore been studied in detail.

***N*-Glucoside Hydrolysis.** As an improvement of previous gas-chromatographic measurements (Winkler and Sandermann, 1989) the hydrolysis of the *N*-glucoside of 3,4-dichloroaniline has now been determined by a newly developed, about 10-fold more sensitive, HPLC method (Figure 1). The degree of hydrolysis was determined after 10 min or 23 h incubation at 25 °C. Complete cleavage was reached at both incubation times. However, a much more acidic pH value (pH 3.3) was required for 50% hydrolysis after 10 min than after 23 h incubation time (pH 6.3). When log (percent cleavage) of these and additional experiments was plotted against incubation time, straight lines were obtained at the studied constant pH values of between 1 and 8. Greatly differing half-lives of between 2.1 min at pH 1 and 2800 min at pH 8 could be calculated.

These results pointed to a first-order chemical reaction where the following equation applies (v_h, rate of hydrolysis; k_h, hydrolysis rate constant; [*N*-glucoside], concentration of 3,4-dichloroaniline *N*-glucoside):

$$v_h = k_h[N\text{-glucoside}] \qquad (1)$$

A strong pH dependence was visible when the rate constants (k_h) were plotted against pH value (Figure 2).

Reaction velocity became very small at alkaline pH. At acidic values of pH ≤ 3 cleavage rates did not further increase. This was probably due to protonation of the anomeric nitrogen rather than the ring oxygen (see Discussion). General acid catalysis was thereby limited to the pH range between 3 and 8. The data points of Figure 2 could be fitted by an empirical equation (see caption for Figure 2).

***N*-Glucoside Formation.** *N*-Glucoside formation was tested after addition of 35 mM free D-glucose to 32 μM 3,4-dichloroaniline in 1 M phosphate buffer, pH 3.0, in order to examine for a mass–action equilibrium that could be shifted from hydrolysis to conjugate formation. *N*-Glucoside formation was confirmed by HPLC analysis. There was rapid formation of *N*-glucoside, with 3% conversion after 3 min, 6% conversion after 10 min, and up to 10% conversion after 50 min. Further incubations at different pH values used substrate concentrations of 62 μM 3,4-dichloroaniline and 11.1 mM D-glucose. The amounts of conjugate were measured by HPLC after 10 min, 23 h, 6, 19, and 28 days. After 10 min, the product was already clearly detectable with a maximum at pH 3–4. *N*-Glucoside formation after ≥23 h was much higher and had a maximum at pH 5–6 (Figure 3).

The initial rate of formation of the *N*-glucoside (v_{gluc}) should in a mass–action situation show the following dependence:

$$v_{gluc} = k_{gluc}[\text{D-glucose}][\text{3,4-dichloroaniline}] \qquad (2)$$

Here, k_{gluc} is the rate constant for formation, [D-glucose] is the free D-glucose concentration, and [3,4-dichloroaniline] is the concentration of 3,4-dichloroaniline. Values of k_{gluc} are plotted against pH value in Figure 2. The

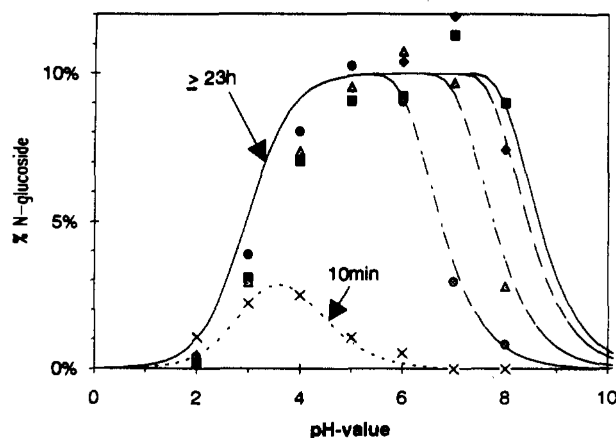

Figure 3. N-Glucosylation of 3,4-dichloroaniline (60 μM) in 1 M sodium phosphate buffers of the indicated pH value. The amount of *N*-glucoside (%) formed after addition of 11 mM D-glucose was determined by HPLC after the following incubation times: 10 min (×), 23 h (○), 6 days (△), 19 days (◆), and 28 days (■). The incubation temperature was 25 °C.

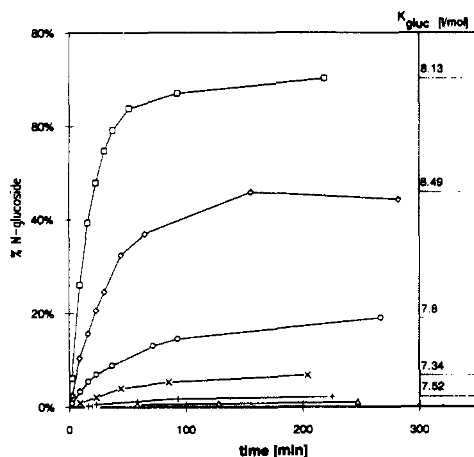

Figure 4. N-Glucosylation of 3,4-dichloroaniline (62 μM) in 0.4 M sodium phosphate buffer, pH 4.0, upon addition of the following concentration of D-glucose (mM): 1.1 (△), 3.3 (+), 11.1 (×), 33.3 (○), 111.1 (◇), and 333.3 (□). The amount of *N*-glucoside (%) was determined by HPLC after various incubation times (min). The approximate plateau levels of *N*-glucoside yielded the association equilibrium constants, K_{gluc}, shown in the right-hand panel (use of eq 4 of text).

curve obtained shows that in the pH range of pH 5–8 there is a high degree of pH dependence. As in the case of hydrolysis, general-acid catalysis appeared to exist. A possible molecular explanation is given in the Discussion.

Equilibrium Constant. The quotient of the rate constants k_{gluc} and k_h was formed for each pH value used. This treatment gives the equilibrium association constant, K_{gluc}, according to

$$K_{gluc} = k_{gluc}/k_h \quad (3)$$

From the individual k_{gluc} and k_h values shown in Figure 2 for each pH value, an approximate overall value of K_{gluc} of 11.0 M^{-1} was obtained, corresponding to an approximate dissociation equilibrium constant of $K_{diss} \approx 0.1$ M.

To come to an independent estimate of K_{gluc}, a fixed concentration of 62 μM 3,4-dichloroaniline was reacted with varying D-glucose concentrations of between 1 and 333 mM at pH 4. Steady-state concentrations of the *N*-glucoside increased from an initial amount of ≤1% to ~60% conversion in a relatively slow reaction requiring >1 h (Figure 4). The equilibrium association constant K_{gluc} for formation of the *N*-glucoside could be calculated for the plateau region of each curve from

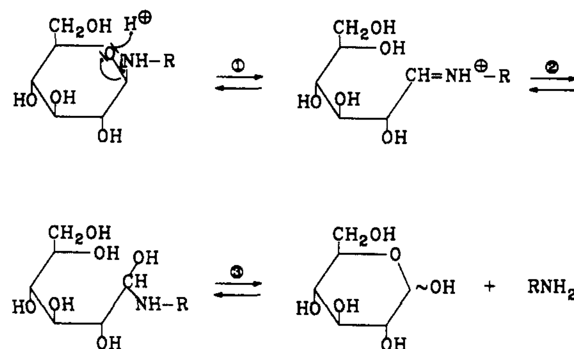

Figure 5. Chemical mechanism for cleavage and formation of *N*-glucosides. Reaction 1 is the reversible protonation of the ring oxygen and Schiff's base formation. Reaction 2 is the reversible isomerization of the Schiff's base to the aminol derivative. Reaction 3 is the reversible cleavage of the aminol derivative to free D-glucose and free amino compound.

$$K_{gluc} = [N\text{-glucoside}]/([3,4\text{-dichloroaniline}][\text{D-glucose}]) \quad (4)$$

The individual values are shown in Figure 4 (right panel). An average value of $K_{gluc} \approx 8\ M^{-1}$ could be estimated, corresponding to a dissociation equilibrium constant of $K_{diss} \approx 0.125$ M. This value is close to the above value of 0.1 M that was determined by a completely different method from the individual rate constants.

DISCUSSION

Mechanistic Interpretation of Results. The above results document that a pesticidal conjugate can under physiological conditions form and decompose by nonenzymatic spontaneous processes. General-acid catalysis was demonstrated over the pH range of 3–8 for cleavage as well as formation (Figure 2). This result is best interpreted by reference to reaction mechanisms suggested in the literature (Capon, 1969; Simon and Palm, 1965; Paulsen and Pflughaupt, 1980). As shown in Figure 5, an open-chain aminol derivative is the central intermediate in formation as well as cleavage of *N*-glucosides. It is also involved in mutarotation (not shown). The aminol derivative can be formed (a) by protonation of the ring oxygen of the *N*-glucoside, followed by Schiff's base formation (reactions 1 and 2) and (b) by attack of the nonprotonated amino group ($R-NH_2$) at C-1 of D-glucose (back-reaction 3). The protonation reactions involved in both directions of steps 1–3 may explain the observation of general-acid catalysis in formation as well as hydrolysis. Elucidation of the partial steps involved will, however, require further study. If in reaction 1 the N atom rather than the ring oxygen of the *N*-glucoside is protonated, the catalytic sequence of Figure 5 is interrupted. The pK value of the glycosidic *N*-atom which depends on mesomeric and inductive effects in the aglycon can therefore become rate-determining (Simon and Palm, 1965; Paulsen and Pflughaupt, 1980; Winkler and Sandermann, 1989). As a general rule, electron withdrawal from the glycosidic N atom will increase acid stability.

Acid Sensitivity of *N*-Glucosides. The catalytic sequence of Figure 5 has previously been used to explain the greatly different acid sensitivity of structurally different *N*-glucosides with different mesomeric or electronic structures (Simon and Palm, 1965; Paulsen and Pflughaupt, 1980; Winkler and Sandermann, 1989). For example, the stability of *N*-ribosyl purine and pyrimidine bases (Micheel and Heesing, 1960) and the greatly increased acid sensitivity of the reduced redox cofactor

NADH over its oxidized form, NAD^+, can be explained. The general rule derived from many individual studies is that acid stability of *N*-glucosides increases in the following sequence of aglycons: aliphatic amine ≈ amino acids and their esters < aromatic amines < acid amides ≈ urea, and thiourea derivatives (Micheel and Heesing, 1960; Paulsen and Pflughaupt, 1980). Similar rules apply to pesticidal *N*-glucosides.

In plant physiology, the *N*-glucosides of the cytokinin phytohormones are of special importance. These *N*-glucosides should be rather stable due to resonance delocalization of the free electrons of the anomeric nitrogen atom. Cytokinin *N*-glucosides are in fact considered to be terminal metabolites in plants. No plant hydrolases for cytokinin *N*-glucosides have been reported, although more than 30 *N*-glycosyl hydrolases (EC 3.2.2) are known from other sources (IUB, 1984). A hydrolase acting on cytokinin *N*-glycosides has recently been described as the *rolC* gene product of *Agrobacterium rhiizogenes*. Cytokinin *O*-glucosides were also reported as substrates (Estruch et al., 1991).

Pesticidal N-glucoside conjugates of metribuzin and chloramben were reported to represent stable terminal metabolites (Frear et al., 1983; Frear, 1976; Lamoureux and Rusness, 1986). More generally, the metabolic stability of pesticidal *N*-glucosides will depend on chemical structure. The existence of a great range from highly labile to highly stable pesticidal *N*-glucosides has previously been discussed (Winkler and Sandermann, 1989). There is a clear difference to *O*-glucopyranosides which do not have such a broad range of stability. Acid sensitivity of pesticidal *N*-glucosides should be related to hydrolysis rates under stomach conditions (pH 1–2; 37 °C). Chemical predictions of bioavailability have in fact been made for the *N*-glucosyl conjugates of chlorinated anilines (Sandermann, 1987; Winkler and Sandermann, 1989) and also for certain lignin conjugates of chlorinated anilines (Sandermann et al., 1992). Chemical lability also appears to be important in normal plant metabolism since a nonenzymatic hydrolysis of sucrose has been reported for *Citrus anrautifolia* (Echeverria, 1990). During the developmental acidification of vacuoles spontaneous sucrose hydrolysis was observed at pH values of ≤2.5.

Spontaneous Formation of *N*-Glucosides. While much literature is available on the acid hydrolysis of *N*-glucosides, there are few reports on the spontaneous formation of *N*-glucosides (or other conjugates) under physiological conditions. Acid-labile alkylamine *N*-glucuronides were formed spontaneously in aqueous solution (Takitani, 1959). The acid-labile *N*-glucuronide of 5-aminosalicylic acid was formed nonenzymatically in phosphate buffer of pH 7.4 (Tjornelund et al., 1989).

Available reviews dealing with pesticidal *N*-glucosides do not mention their spontaneous formation (Edwards et al., 1982; Hatzios and Penner, 1982; Lamoureux and Rusness, 1986; Lamoureux and Frear, 1979; Frear, 1976). In fact, plants metabolize chloroanilines with the aid of a highly active *N*-glucosyltransferase that works with the energy-rich nucleotide sugar, UDP-glucose (Frear, 1968; Sandermann et al., 1991). This enzyme reaction is much more efficient than the spontaneous reaction described here. *N*-Glucosyltransferases for metribuzin (Frear et al., 1983) and 3-isoxazolin-5-one (Murakoshi et al., 1975) have also been shown to use UDP-glucose rather than free D-glucose. In addition parsley contains a UDP-glucose-dependent *N*-glucosyltransferase for nicotinic acid (Upmeier et al., 1988). Due to the permanent positive charge on nitrogen the rate of hydrolysis of nicotinic acid *N*-glucoside is predicted to be extremely slow. This *N*-glucoside was in fact observed to be chemically stable at pH 1 and 37 °C (TLC assay; H. Sandermann, unpublished). Interestingly, the *N*-glucoside of nicotinic acid was reported to be energy rich, because in the enzyme reaction there was free equilibrium with UDP-glucose (Upmeier et al., 1988). A previous report has determined equilibrium constants for the reaction: D-glucose + substituted aniline ⇌ *N*-glucoside (Holton and Runquist, 1961). A complex polarometric method was used, and dissociation equilibrium constants near 0.09 M were obtained. In view of the different methods and assumptions used, these values are reasonably close to the value obtained here (0.1 M). The in vivo formation of protein adducts with D-glucose and subsequent rearrangement reactions are well known for animals and man. These processes contribute to ageing and disease reactions (Cerami, 1986). In plants, sucrose rather than D-glucose usually is the predominant carbohydrate, so that spontaneous glucose addition reactions and the risk of senescence processes are avoided. However, recently N-glucosidation and formation of Amadori and Maillard products were reported to occur in seed deterioration (Wettlaufer and Leopold, 1991).

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Registry No. 3,4-Dichloroaniline *N*-glucoside, 17708-78-0; 3,4-dichloroaniline, 95-76-1; D-glucose, 50-99-7.