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THE METABOLISM AND EXCRETION OF INDOLYLACRYLIC ACID IN THE RAT

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Abstract—A urinary excretion product following oral administration of tryptophan or indolylacrylic acid to rats, has been identified as indolylacryloylglucosiduronic acid, which accompanies the previously described indolylacryloyl-glycine. Under defined conditions *in vitro*, rat liver slices or whole homogenates form both these conjugates from the acid. Liver mitochondrial preparations synthesise solely the glycine derivative. Microsomal preparations synthesise solely the acyl-glucuronide, and only when fortified with UDP-glucuronic acid; stimulation of glucuronide formation by saccharolactone, UDP-*N*-acetyl-glucosamine or various nucleoside- or sugar-phosphates is observed, and is discussed. Glucuronic acid conjugation has not previously been reported with acids of the acrylic or cinnamic type.

THE URINARY excretion of indolylacrylic acid has been reported in connection with a number of pathological or dietary conditions.[1] It was identified in urine both as the free acid and conjugated with glycine. In our studies on the metabolism of L-tryptophan and other indoles under conditions simulating Hartnup Disorder, another conjugate of indolylacrylic acid is present in rat urine.[1] This was designated compound "G" and it was tentatively suggested that it might be a glucuronide. The evidence identifying the compound as indolyl-acryloyl glucosiduronic acid is now given, and the cell fractions responsible for the conjugation of indolylacrylic acid with both glycine and glucuronic acid have been identified.

MATERIALS AND METHODS

Compound "G"

Indolylacrylic acid (10 mg) was administered orally to rats using the methods already reported. The urine was collected over a period of 3 hr, reduced in volume using a rotary evaporator, and applied to thin-layer plates in concentrated bands. One way chromatography was carried out using *n*-butanol–acetic acid–water (12:3:5 by vol.). A small section of the plate was sprayed with Ehrlich's reagent to locate the position of the band containing compound G, the remainder of the plate being covered with a glass plate for protection. The located, but untreated, band was scraped off and the silica gel placed in about 50 ml acetone–water (50:50 by vol.). The solution was then filtered, evaporated to dryness, and the residue taken up in acetate buffer (5 ml, pH 6·2, 0·01 M). Two way thin-layer chromatography demonstrated that only one Ehrlich positive component was present.

The above solution containing compound G was incubated overnight at 37° with β-glucuronidase (5 mg) from *E. coli* (Sigma) or mollusc (Koch–Light). The incubation mixture was then treated with acetone, filtered and the filtrate evaporated to small volume for chromatography. Similar incubations were also carried out in the presence of saccharolactone (10 mg), and control incubations without enzyme.

Thin layer chromatography

Silica gel G (Merck) was used as adsorbant. One way chromatograms using a solvent system of ether–light petroleum (b.p. 100–120°)–formic acid (75:25:2 by vol.) were suitable for a quick preliminary detection of glycine conjugate but unsuitable for the glucuronide. For the study of incubation products two way thin-layer chromatography was carried out using 10×10 cm plates with solvent pair:—

isopropanol–ammonia–water (20:1:2 by vol.) followed by—

n-butanol–acetic acid–water (12:3:5 by vol.).

For indolic substances, the plates were dried and sprayed with Ehrlich's reagent. (10% w/v *p*-dimethylamino-benzaldehyde in conc. HCl, diluted 1:3 with ethanol before use.)

For locating uronic acids, the following reagent was mixed just before use: naphthoresorcinol 0·2% in acetone (5 volumes) with phosphoric acid9% in water (1 volume). The plates were sprayed and placed in an oven at 95° in which a beaker of hydrochloric acid was present. After a few minutes the colour developed.

Incubations with rat liver slices

Indolylacrylic acid (1 mg in 1 ml 0·15 M KCl at pH 7·4) was added to 1 g of rat liver slices (blade and tissue block) suspended in 15 ml of KCl. For glycine conjugation, the mixture was shaken at 37° for 1 hr while oxygen–carbon dioxide mixture (95:5; v/v) was bubbled through, or for 0·5 hr under nitrogen for glucuronide formation (see results). After incubation, the solutions were treated with acetone (10 ml) and filtered to remove protein. The filtrates were taken to dryness using a rotary evaporator and the residues dissolved in 1 ml of acetone–water (50:50 by vol.) for chromatographic examination.

Incubations with liver homogenates and fractions

Rat liver homogenates (20% w/v) were prepared in cold 0·15 M KCl-phosphate at pH 7·4, using a hand homogeniser with Teflon pestle.

For cell fractions, KCl homogenates were also used. After removal of debris and nuclei, a mitochondrial fraction was obtained by centrifuging at 24,000 *g*/10 min/2°, and a microsomal fraction obtained at 105,000 *g*/1 hr. (Beckman Model L2). The supernatant from this is referred to as "cell sap". Both fractions were suspended in KCl, recentrifuged, and resuspended to be equivalent to the original homogenate in concentration. Incubations were carried out using 1 mg of indolylacrylic acid per 10 ml of homogenate or suspension, equivalent to 2 g fresh liver. The following factors were added as appropriate per 10 ml of incubation mixture:

UDP-glucuronic acid (Sigma), (6 mg); UDP-*N*-acetyl glucosamine (Sigma), (9 mg); saccharolactone (Calbiochem.), (2 mg); UDP-glucose (Sigma), (6·1 mg); glucose-1-phosphate (Sigma), (4 mg); glucuronic acid-1-phosphate (Sigma), (4 mg); ATP (Boehringer), (11 mg); NAD (Boehringer), (10 mg).

RESULTS

Identification of glucuronide conjugate

After the oral administration to rats of indolylacrylic acid, chromatography revealed three components reacting with Ehrlich's reagent to give the colour and fluorescence characteristic of indolylacryloyl derivatives[1] [pink with yellow fluorescence in u.v. (Woods) 366 mμ light]. Two of these components were chromatographically identical with the free acid and the glycine conjugate; the third, present in smaller amount, was designated compound G and was studied as described under Methods. Occasionally, traces of indolylacryloyl glycine were detectable in control urines, but never the other two components. Chromatography of our preparation of G gave only one component, which reacted with naphthoresorcinol reagent to give the blue colour expected with glucuronides, in the position corresponding exactly to the position of the Ehrlich-reacting substance on a duplicate chromatogram. No reaction with naphthoresorcinol occurred on a duplicate control chromatogram at this position.

After incubation of G with β-glucuronidase, chromatography revealed that G had disappeared, to be replaced by indolylacrylic acid. Saccharolactone completely inhibited the effect of β-glucuronidase in liberating free acid, and no liberation occurred in the absence of enzyme.

Conjugation in vitro *with glycine and with glucuronic acid*

After indolylacrylic acid had been incubated with rat liver slices, its glycine conjugate was readily detected chromatographically, with the glucuronide present in smaller amount.

Glycine conjugation also occurred readily using whole liver homogenates. However, no glucuronide could be detected unless the whole homogenate was fortified by the addition of UDP-glucuronic acid and the incubation carried out under nitrogen. Addition of saccharolactone further increased the yield of glucuronide, presumably due to β-glucuronidase inhibition. The level of glycine conjugation was markedly raised by the addition of free glycine to the incubation mixture, an effect also seen with liver slices.

Incubation of indolylacrylic acid with liver mitochondrial fractions resulted in considerable conjugation with glycine, further raised by the addition of glycine. No conditions or additions were found which would cause detectable glucuronide formation with this fraction.

The microsomal fraction of liver was found to catalyse the conjugation of indolylacrylic acid with glucuronic acid, but only after the addition of UDP-glucuronic acid to the incubation mixture. The amount of accumulated glucuronide was further increased by the addition of saccharolactone to this mixture.

Although strictly quantitative measurements were not made, some indication of the degree of glucuronidation under different conditions was obtained by comparison of the spot sizes on the chromatograms. Stimulation of glucuronidation by added UDP-*N*-acetylglucosamine and/or ATP, and by UDP-glucose and glucuronic acid-1-phosphate together, is in accord with the general suggestions of Pogell and Leloir.[2]

The microsomal fraction did not cause detectable conjugation with added glycine.

Cis-trans-*isomerism*

Chromatography of a freshly prepared solution of indolylacrylic acid always revealed two Ehrlich-reacting spots, the major with R_f 0·50 and the minor with R_f

0·68. However, if the same solution was handled in subdued light and chromatographed in the dark, only the major component was detected. Irradiation of the solutions with ultra-violet light (λ 366 mμ) for several hours resulted in approximately equal amounts of the two materials being found. Elution from an untreated chromatography plate allowed isolation of each substance. Chromatography of each isolate in the dark showed it contained a single substance with characteristic R_f value. Chromatography under the usual conditions of lighting resulted in the same two substances from both (R_f 0·50 and R_f 0·68). This supports the suggestion of Hansen and Crawford[3] that indolylacrylic acid in solution under normal conditions exists in two isomeric forms, *cis* and *trans* about the side chain double bond. Likewise, pure indolylacryloyl-glycine, and indolylacryloyl-glycine formed *in vivo* or *in vitro*, usually showed two components (R_f 0·10 and 0·26) when chromatographed in the light, the faster running (*cis* compound) being present only in small amount. Both Ehrlich-reacting materials, considered to be isomers of indolylacryloyl-glycine, were shown by elution and hydrolysis to be glycine conjugates. The same mixture of conjugate isomers was obtained after chromatography of urine from rats administered either "dark" or "irradiated" indolylacrylic acid.

DISCUSSION

The results show that indolylacrylic acid administered to rats is excreted mainly as the glycine conjugate, although some may appear as a glucuronic acid conjugate or as free acid. The glucuronide has not previously been reported.

The conjugation of the indolic acid must be through either the 1 N position (*N*-glucuronide) or the carboxy group (acyl-glucuronide). The former would be expected to be very stable to alkali, which the urine conjugate is not. On the other hand, the conjugate can be chromatographed in moderately basic solvents like isopropanol-ammonia, with only slight decomposition. This stability is somewhat greater than might be expected for an acyl glucuronide, e.g. indolylacetyl glucosiduronic acid is completely converted to indolylacetamide under these conditions.[4] However, indolylacrylic acid is the vinylogue of 3-carboxy-indole, which forms acyl derivatives of exceptional stability because of the relationship of the carbonyl attached to C-3 with the NH, through the ring —CH = CH—.

The evidence thus favours the ester (acyl) type glucuronide, indolylacryloyl glucosiduronic acid. Formed *in vitro*, or as excreted in urine, the glucuronide was not detectable if its solution were kept more than a day even in the cold. Tissue or urinary glucuronidase may contribute to this instability.

The studies with liver slices, homogenates and sub-cell fractions confirm that glycine conjugation occurs in liver mitochondria whilst glucuronide formation takes place in the liver microsomal fraction. When glycine was added to the incubation mixtures, the level of glycine conjugation increased considerably. This infers that the rate limiting step may be the availability of glycine rather than the enzymatic activity, which could have important consequences in relating drug metabolism to diet. Although the two isomers of indolylacrylic acid are found as glycine conjugates, it was not possible to show if the *cis* and *trans* isomers of the free acid conjugate with glycine individually, since isomerism could take place after conjugation.

The conjugation with glucuronic acid was difficult to simulate *in vitro*. The levels of conjugate rose when saccharolactone was added to the incubation flasks. Since this

substance is a known inhibitor of β-glucuronidase,[5] the levels of glucuronidase must be high in liver tissue, thus breaking down any glucuronide formed. UDP-glucuronic acid was required with all homogenates, although liver slices had a sufficient level of available UDP-glucuronic acid to enable the glucuronide synthesis to take place without further additions. The only evidence at present to show that this glucuronide is excreted in humans was obtained by us recently from examinations of urine from a patient with Hartnup Disorder. The patient had been administered a tryptophan load 24 hr prior to collection of the urine.

Glucuronic acid conjugation has not been reported previously with compounds of this type. Cinnamic acid forms a glycine conjugate and undergoes β-oxidation of the side chain but no glucuronide formation has been reported.[6] The mode of formation and the various additions required for stimulating its formation *in vitro* appear to be similar to those reported for phenolic substances such as *p*-nitrophenol or *o*-aminophenol[2, 7] and suggests that the same glucuronyl transferase is involved in both conjugations.

There is increasing interest in the fate of minor dietary components of the acrylic and cinnamic acid types;[8, 9] acyl glucuronide formations must now be added to the list of their metabolic pathways.

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