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2. Masuo Akagi, Isamu Aoki, Takayoshi Uematsu, and Takashi Iyanagi : Studies on Food Additives. XI.[*1] N-Glucosiduronate Formation of Arylurea *in vivo* and *in vitro*.

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In the previous paper,[*1] it was reported that a new type of N-glucosiduronate was isolated from the urine of rabbits dosed with *p*-ethoxyphenylurea and assumed to be 1-[3-(*p*-ethoxyphenyl)ureido]-1-deoxy-D-glucosiduronate. It seems desirable to examine whether arylureas other than *p*-ethoxyphenylurea could form N-glucosiduronate in animals. This paper deals with N-glucosiduronate formations of some arylureas in the rabbits and the chemical condensation of free glucuronic acid with the arylurea in pyridine.

I. Isolation Procedure of Arylurea N-Glucosiduronate from the Urine of Rabbits administered with the Arylurea

It is well known that N-glucuronide is generally unstable, and its isolation from urine is difficult. After various attempts of separation, the following procedure was found to be suitable for this purpose.

The lead salt of glucuronide fraction was prepared according to the method of Williams.[1] It was suspended in water containing ammonium hydroxide and treated with hydrogen sulfide to remove lead. The resulting lead sulfide was filtered off and the filtrate was evaporated to dryness *in vacuo*. The obtained glucuronide gum was triturated with hot methanol. In the case of *p*-ethoxyphenylurea and *p*-chlorophenylurea, the methanol-insoluble part was dissolved in a small amount of water and could be solidified by addition of methanol. The methanol-soluble part was submitted to a column chromatography over a silica gel and eluted with the solvent system which was composed of chloroform, methanol and aqueous ammonia. The fractions which gave a rapid naphthoresorcinol test at 100° and slowly a yellow color with Ehrlich's reagent at room temperature by thin-layer chromatography were collected and crystallized. All glucuronides obtained by this manner are in the form of ammonium salt. Treatment with hydrogen sulfide of lead salt in the presence of ammonia was considered to be advantageous. By this isolation procedure, the urine of rabbits receiving *p*-chlorophenylurea, *o*-tolylurea and phenylurea, afforded crystals, m.p.

Table I. Urinary Excretion of *p*-Ethoxyphenylurea N-Glucuronide in Rabbit given *p*-Ethoxyphenylurea

Rabbit No.	Urinary *p*-ethoxyphenylurea N-glucuronide (%)	
	24 (hr.)	48 (hr.)
1	29	1
2	12	1
3	35	4

Rabbits each received 1.3 g. of *p*-ethoxyphenylurea orally and an aliquot of urine was used for estimation procedure.

*1 Part X : This Bulletin, **14**, 1 (1966).

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(decomp.) (IIa) 198°, 1.5 g., (IIIa) 153°, 92 mg., and (IVa) 165~166.5°, 90 mg., in yields respectively.

Further, in order to know the extent of N-glucosiduronate formation in metabolism, *p*-ethoxyphenylurea N-glucosiduronate in the urine was estimated by a spectrophotometry described by Akagi, *et al.*[2]

As shown in Table I, the urinary excretion of *p*-ethoxyphenylurea N-glucosiduronate was ca. 30% of the dose and roughly in accordance with unaccounted increased excretion of glucuronic acid estimated by naphthoresorcinol method. Although this excretion was not checked with various doses, this type of conjugation appeared to be a general pathway in regard to arylurea metabolism.

II. Chemical Condensation of Glucuronic Acid with Arylurea

Although glycosylureides have been prepared from urea homologs and sugars in the presence of acid as catalyst by Schoorl's method or its modification,[3~5] glucuronylureides have not been synthesized, except for 1-ureido-D-glucuronic acid,[6] which employed glucuronolactone as the starting material. Also it was reported by many investigators[7~14] that aromatic and aliphatic amines easily combined with glucuronic acid or glucuronate to give the amino N-glucosiduronate with or without acid in a good yield. However, the attempt to obtain the N-glucosiduronates of arylureas by above methods had not been successful because of a low basicity of ureido group.

Accordingly, when pyridine was employed as reaction solvent, the corresponding N-glucosiduronates were obtained in crystals, although the yields were poor.

An excess arylurea and glucuronic acid were dissolved in anhyd. pyridine. After standing at 37° for several days, the reaction mixture was poured into aqueous ammonia, unreacted arylurea that separated out was filtered off, and the filtrate was washed with ethylacetate and ether to remove excess arylurea and pyridine. The washed aqueous layer was evaporated to dryness *in vacuo* and the syrup was triturated with hot methanol to eliminate methanol-insoluble materials which were mainly excess glucuronic acid. The methanol-layer was concentrated *in vacuo* and this operation was again repeated to the obtained syrup. In order to isolate the pure main product, a solution of sirupy residure in methanol was passed through a column of silica gel and eluted with solvent as described above. Each elute was checked by thin-layer chromatography over a silica gel, and the fractions giving a spot of Rf corresponding to the substance isolated from the rabbit urine, were collected and crystallized. By the above mentioned procedure, conjugates of glucuronic acid with *p*-ethoxyphenylurea, *p*-chlorophenylurea, *o*-tolylurea and phenylurea afforded m.p. (decomp.) (I) 135°, in 3%, (IIb) 198°, in 2.8%, (IIIb) 153°, in 4.6%, and (IVb) 165~166°, in 1% yields respectively. They were identified by mixed fusion and infrared with the metabolites from the rabbit urine. Although other reaction products which would

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exist in the reaction mixture were detected by thin-layer chromatography, they were not able to obtain as crystals.

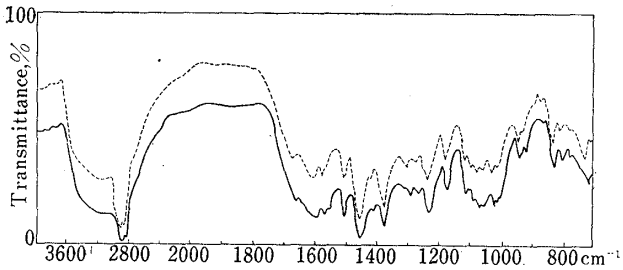

Fig. 1. Infrared Spectra of *p*-Ethoxyphenylurea N-Glucuronide (in Nujol)
——— *p*-Ethoxyphenylurea N-glucuronide isolated from the urine.*1
..... *p*-Ethoxyphenylurea N-glucuronide prepared from *p*-ethoxyphenylurea and glucuronic acid.

As discussed in the previous paper,*1 *p*-ethoxyphenylurea N-glucosiduronate was not hydrolyzed by β-glucuronidase and absorption intensity of ultraviolet spectra of it were not decreased but increased. Accordingly, the position of the bonding of sugar moiety was considered to be in 3-N of ureido group. Infrared absorption spectra of these compounds exhibited the band due to pyranose ring at 1030 cm^{-1}. All of their specific rotation were negative. It is known that the natural glucuronide has β-form.

Thereby, configurations of anomeric center of these glucuronides might be β-forms. From these facts, their structure was suggested to be ammonium 1-[3-(aryl)-ureido]-1-deoxy-β-D-glucopyranosiduronate.

Experimental*3

Synthesis of Ammonium 1-[3-(*p*-Ethoxyphenyl)ureido]-1-deoxy-D-glucopyranosiduronate (I)——D-glucuronic acid (10 g.) and *p*-ethoxyphenylurea (20 g.) were dissolved in 80 ml. of pyridine and maintained at 37° for 72 hr. The reaction mixture was poured into 250 ml. of H_2O and 50 ml. of conc. NH_4OH. The precipitates that separated out were discarded, and the supernatant was washed successively with 3×200 ml. of EtOAc and 2×200 ml. of ether to remove *p*-ethoxyphenylurea and pyridine. The aqueous layer was evaporated *in vacuo* at 45°, a syrup was extracted with 100 ml. of hot MeOH and the solvent was evaporated *in vacuo* from the extract. A small amount of MeOH was added to the sirupy residue and the mixture was allowed to stand, by which crystals were separated out. Recrystallization from MeOH afforded 0.6 g. (3%) of colorless needles, m.p. 135° (decomp.), $[\alpha]_D^{20}$ −46.2° (c=1.00, H_2O), (*Anal.* Calcd. for $C_{15}H_{23}O_8N_3$: C, 48.26: H, 6.00; N, 11.26. Found: C, 48.41; H, 6.12; N, 10.78). This compound showed no depression of melting point on admixture with the glucuronide isolated from the rabbit urine.*1

Isolation of *p*-Chlorophenylurea N-Glucosiduronate (IIa) from the Urine of Rabbits——The rabbits used weighed 2~3 kg. They were fed with a standard diet (oats 50 g., carrot 100 g., and cabbage 200 g.) and were kept separately in metabolism cage designed to permit separate collection of urine and feces. *p*-Chlorophenylurea (1.5 g.) was administered orally to each of four rabbits. The collected 48 hr. urine was brought to pH 4 with glacial acetic acid, and treated with saturated normal lead acetate solution until no further precipitation occured. The precipitates were removed by filtration. The filtrate was brought to pH 7.5~8.0 with NH_4OH and saturated basic lead acetate solution was added in excess. The precipitates were collected and washed with water, successively EtOH and ether. The lead salt was suspended in 100 ml. of water containing 10 ml. of conc. NH_4OH and decomposed by treatment with H_2S. After removal of PbS by filtration, the filtrate was evaporated to dryness at 45° *in vacuo*. The sirupy residue was triturated with 25 ml. of hot MeOH. After standing for 2 days at room temperature, the mixture was divided into the solid (A) and mother liquor (B). To the solid (A) was added 3 ml. of water, the insoluble materials were discarded by filtration and MeOH was added to the filtrate until a slight turbidity developed. After standing overnight in a refrigerator, 1.4 g. of white crystals was obtained. The mother liquor (B) was concentrated nearly to 2 ml. *in vacuo* which was chromatographed over a silica gel column (40 g. × 2.5 cm.). Elution with $CHCl_3$–EtOH–H_2O (containing conc. NH_4OH, 1 ml. in 2 ml.) (5:4:1) gave 130 mg. of crystals. They were recrystallized from MeOH to colorless needles, m.p. 198° (decomp.), $[\alpha]_D^{19}$ −49° (c=2.00, H_2O), (*Anal.* Calcd. for $C_{13}H_{18}O_7N_3Cl$: C, 42.90; H, 4.99; N, 11.20. Found: C, 43.00; H, 5.29; N, 11.43).

*3 All melting points were uncorrected. Silica gel used was Kanto Chemical Co. Ltd. (100~200 mesh for chromatography).

Synthesis of Ammonium 1-[3-(*p*-Chlorophenyl)ureido]-1-deoxy-D-glucopyranosiduronate (IIb)——A mixture of 10 g. of *p*-chlorophenylurea, 5 g. of glucuronic acid and 0.5 ml. of conc. H_2SO_4 in 50 ml. of pyridine was allowed to stand for 4 days at 37°. The reaction mixture was poured into 50 ml. of water and 50 ml. of conc. NH_4OH. After standing for 1 hr. at 0°, the unreacted *p*-chlorophenylurea that separated out was removed by filtration, and the filtrate was washed with 5×100 ml. of ether and concentrated to dryness *in vacuo*. The concentrate was introduced into 100 ml. of MeOH, and refluxed for several min., the precipitates that yielded were filtered off, and the supernatant was evaporated to dryness *in vacuo*. The residue was again treated with 100 ml. of MeOH and the methanolic solution was concentrated *in vacuo* to dryness. The sirupy residue was dissolved in 2 ml. of MeOH, adsorbed on a column of silica gel (30 g., ×2.5 cm.) and eluted with $CHCl_3$–MeOH–half saturated NH_4OH (5:4:1) by gradient elution. The effluent was collected in 10 ml. fractions, and an aliquot of them was submitted to thin-layer chromatogram with the solvent as mentioned above and compared with the glucuronide obtained from the urine. The fractions which gave a spot of Rf corresponding to that of the metabolite were collected, evaporated *in vacuo*, and the syrup was crystallized from MeOH to give 284 mg. (2.8%) of needles. Recrystallization from MeOH containing NH_4OH (1 ml. in 100 ml.) afforded an analytical sample, m.p. 198° (decomp.), $[\alpha]_D^{18}$ −55° (c=0.20, H_2O), (Found : C, 42.67; H, 5.12; N, 11.32). A mixture of this compound and IIa showed no melting point depression.

Isolation of *o*-Tolylurea N-Glucosiduronate (IIIa) from the Urine of Rabbits——Five rabbits were each given 1 g. of *o*-tolylurea orally. In 48 hr. 1.1 L. of urine was collected, acidified with a few drops of glacial acetic acid and treated with saturated normal lead acetate until no further precipitate was formed. The filtrate was neutralized with NH_4OH and treated with excess saturated basic lead acetate solution. The lead salt was suspended in water containing NH_4OH and decomposed with H_2S. The filtrate from the PbS was concentrated *in vacuo* at 45~50°. The syrup was treated with 10 ml. of MeOH, the insoluble materials were filtered off, and the MeOH-layer was poured into 50 ml. of EtOH gradually. The precipitates that separated out were removed by filtration, the filtrate evaporated to dryness *in vacuo* and the residue dissolved in 3 ml. of MeOH containing water. The solution was poured onto a silica gel column, and eluted with the solvent as described above. The fractions that gave colors with Ehrlich's reagent and naphthoresorcinol on thin-layer chromatography, were evaporated *in vacuo* and solidified from MeOH. The crystals were recrystallized from $CHCl_3$–MeOH–NH_4OH to 92 mg. of colorless needles, m.p. 153° (decomp.) $[\alpha]_D^{20}$ −51° (c=0.13, H_2O), (*Anal.* Calcd. for $C_{14}H_{21}O_7N_3 \cdot 1/2H_2O$: C, 47.72; H, 6.22; N, 11.93. Found : C, 48.12; H, 6.09; N, 11.79).

Synthesis of Ammonium 1-[3-(*o*-Tolyl)ureido]-1-deoxy-D-glucopyranosiduronate (IIIb)——A solution of *o*-tolylurea (10 g.), glucuronic acid (5 g.) and 0.5 ml. of H_2SO_4 dissolved in 60 ml. of dehyd. pyridine was maintained at 37° for 48 hr. until rotation further unchanged. The reaction mixture was introduced into 50 ml. of water and 50 ml. of NH_4OH, the resultant solid filtered off, the filtrate washed with 5×100 ml. of ether and concentrated *in vacuo* to dryness. MeOH (100 ml.) was added to the syrup, refluxed for 10 min. and evaporated to dryness. This treatment was repeated to the residue again. The syrupy residue was dissolved in a small amount of MeOH, which was chromatographed on 50 g. of silica gel. Elution with $CHCl_3$–MeOH–NH_4OH and crystallization from $CHCl_3$–MeOH (containing a trace of NH_4OH) gave 450 mg. (4.6%) of colorless needles, m.p. 153° (decomp.), $[\alpha]_D^{20}$ −52° (c=0.25, H_2O), (Found : C, 47.41; H, 6.16; N, 11.79). This compound showed no depression of melting point with IIIa.

Isolation of Phenylurea N-Glucosiduronate (IVa) from the Urine of Rabbits——Phenylurea (1 g.) was fed orally to each of six rabbits. The collected 48 hr. urine was worked up as in the other two cases, the obtained gum was treated with 100 ml. of hot MeOH and the MeOH-layer concentrated nearly to 5 ml. *in vacuo*. The concentrated was chromatographed over 45 g. of silica gel (×2.5 cm.) with the system of $CHCl_3$–MeOH–NH_4OH. The effluent was collected in 10 ml. fractions. The fraction No. 12~30 which gave very rapid naphthoresorcinol reaction and a yellow color with Ehrlich's reagent on thin-layer chromatography, was evaporated *in vacuo* to dryness. The syrup was rechromatographed over 20 g. of silica gel with same system, and fraction No. 14~22 was concentrated *in vacuo* and crystallized from MeOH to 90 mg. of needles. Recrystallization from MeOH containing a trace of ammonia gave the analytical specimen, m.p. 165~166.5° (decomp., micro.), $[\alpha]_D^{20}$ −60° (c=0.20, H_2O), (*Anal.* Calcd. for $C_{13}H_{19}O_7N_3$: N, 12.76. Found : N, 13.25).

Synthesis of Ammonium 1-[3-(Phenyl)ureido]-1-deoxy-D-glucopyranosiduronate (IVb)——A mixture of phenylurea (10 g.), glucuronic acid (5 g.) and pyridine (50 ml.) was allowed to stand at 37° for two days. The reaction mixture was treated in a similar manner. The resultant solid was recrystallized from MeOH–NH_4OH to give 100 mg. (1%) of colorless needles, m.p. 165~166° (decomp.), $[\alpha]_D^{21}$ −60° (c=1.00, H_2O), (*Anal.* Calcd. for $C_{13}H_{19}O_7N_3$: C, 47.41; H, 5.82; N, 12.76. Found : C, 47.62; H, 6.01; N, 13.34). This compound showed no mixed melting point depression with IVa.

Estimation of *p*-Ethoxyphenylurea N-Glucuronide in the Urine of Rabbits——Free *p*-ethoxyphenylurea : One to three ml. of dosed urine was extracted into benzene-EtOAc and measured according to the method of Akagi, *et al.*[2] Total *p*-ethoxyphenylurea : An equal volumes of 1*N* HCl was added to the urine and boiled on a water bath for 8 min. After cooling, an equal volume of 1*N* NaOH was

introduced into the treated urine and carried out as mentioned above. Recovery was 83.2(±0.5)%. Total *p*-ethoxyphenylurea minus free *p*-ethoxyphenylurea equals *p*-ethoxyphenylurea N-glucuronide.

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Summary

N-Glucosiduronates of arylureas excreted in the rabbit urine dosed with arylureas were isolated and confirmed to be identical with ammonium 1-[3-(aryl)ureido]-1-deoxy-D-glucopyranosiduronates which were synthesized from glucuronic acid and arylureas in pyridine.

It is suggested that N-glucosiduronate conjugation is a general pathway of arylurea metabolism in the rabbit.

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3. Masuo Akagi, Isamu Aoki, Masanobu Haga, Takayoshi Uematsu, and Masakatsu Sakata : Studies on Food Additives. XII.*1 Synthesis of *p*-Ethoxyphenylurea N-Glucuronide, a Metabolite in Rabbit.

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In the preceding papers,*1,1) it was reported that oral administration of *p*-ethoxyphenylurea, *o*-tolylurea, *p*-chlorophenylurea and phenylurea to rabbits resulted in the excretion of a new type N-glucuronide and these were identical with the compounds prepared by the condensation of glucuronic acid and arylureas in pyridine.

In the present paper, the synthesis of some arylurea N-glucuronides and the determination of their structure are described.

The condensation of arylamine with glycosyl-isocyanate and isothiocyanate had been favorably used to obtain glycosylurea and thiourea.[2,3] 2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosylisocyanate[4] was refluxed with *p*-phenetidine in chloroform-pyridine, and 1-[3-(*p*-ethoxyphenyl)ureido]-1-deoxy-2,3,4,6-tetra-O-acetyl-β-D-glucopyranose (Ⅶ) was obtained in a yield of 73%. The same compound was also prepared from 1-[3-(*p*-ethoxyphenyl)thioureido]-1-deoxy-2,3,4,6-tetra-O-acetyl-β-D-glucopyranose (Ⅸ) in a yield of 20% when the aqueous methanolic solution of Ⅸ was desulfurized with silver nitrate. Ⅸ was synthesized from 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosylisothiocyanate and *p*-phenetidine in the similar way as in the case of the isocyanate in a good yield. Ⅶ was converted to 1-[3-(*p*-ethoxyphenyl)ureido]-1-deoxy-β-D-glucopyranose

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