quinolines. The yields were generally good to excellent in the reactions of ethyl cyanoacetate, 1,3-indandione, diethyl malonate, diethyl nitromalonate, ethyl nitroacetate (e.g. ethyl α-cyano-2-quinolineacetate in 88% yield), but acetone, acetophenone or phenylacetonitrile could not enter into the reaction. The reaction of quinoline 1-oxide using benzoyl chloride instead of acetic anhydride and those of pyridine 1-oxides in the presence of acetic anhydride proceeded similarly but in much lower yields.

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75. Hisao Tsukamoto, Hidetoshi Yoshimura, and Kiyoshi Tatsumi: Metabolism of Drugs. XXXV.*1 Metabolic Fate of Meprobamate. (3).*2 A New Metabolic Pathway of Carbamate Group——The Formation of Meprobamate N-Glucuronide in Animal Body.

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Glucuronic acid conjugation appears to be a common metabolic pathway of drugs which possess hydroxyl, carboxyl, and amino groups. The amino group conjugate, called N-glucuronide, has been extensively investigated in recent years,[1] however no other group has yet been found to form N-glucuronide.

Early in 1961, B. J. Ludwig, *et al.*[2] identified the hydroxylated metabolite of meprobamate (2-methyl-2-propyl-1,3-propanediol dicarbamate) in human urine and also commented on the formation of glucuronide of meprobamate, although they failed to obtain it in pure form. Therefore, its chemical structure was not delineated. Later, the authors*2 independently isolated meprobamate N-glucuronide with five other metabolites from urine of the rabbit which was administered meprobamate, however the purification and structure elucidation of this particular glucuronide have not been accomplished sufficiently.

We now wish to report that this metabolite has been further purified and that its structure was established to be meprobamate-N-mono-β-D-glucopyranosiduronic acid (I) by means of chemical synthesis.

Methods and Results

Paper Chromatography——In all cases, the development was performed by ascending method using Toyo Roshi No. 50 and the solvent systems of I : BuOH-AcOH-H_2O (4:1:5) and II : BuOH saturated with 3% NH_4OH. The spots on the paper chromatogram were revealed with (a) Ehrlichs' reagent (5% *p*-dimethylaminobenzaldehyde solution in MeOH and 1/3 volume of conc. HCl) followed by hot air or (b) $NaIO_4$ reagent (6.42 g. of $NaIO_4$ in 750 cc. of H_2O and 250 cc. of *t*-BuOH) followed by benzidine reagent (a mixture of 5.52 g. of benzidine in 500 cc. of *t*-BuOH and 48 g. of NH_4NO_3 in 500 cc. of H_2O). The results are shown in Table I.

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*1 Part XXXIV. K. Kato, K. Yoshida, H. Tsukamoto : This Bulletin, **10**, 1242 (1962).

*2 Part (2). A. Yamamoto, H. Yoshimura, H. Tsukamoto : *Ibid.*, **10**, 540 (1962); Part (I). *Idem* : *Ibid.*, **10**, 522 (1962).

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1) J. N. Smith, R. T. Williams : Biochem. J., **44**, 242 (1949); **44**, 250 (1949); E. Boyland, D. Manson, S. F. D. Orr. : *Ibid.*, **60**, 11 (1955); **65**, 417 (1957); S. R. M. Bushby, A. J. Woiwod : *Ibid.*, **63**, 406 (1956); S. Ôgiya, H. Kataoka : Yakugaku Zassi, **79**, 947 (1959); Ôgiya : *Ibid.*, **79**, 953 (1959).

2) B. J. Ludwig, *et al.* : J. Med. Pharm. Chem., **3**, 53 (1961).

Table I. Paper Chromatography of Meprobamate N-glucuronide (I) and Other Compounds

Compound	Solvent systems (Rf) I	Solvent systems (Rf) II	Color reagents
Meprobamate-N-mono-β-D-glucopyranosiduronic acid (I)	0.57	0.17	a
Meprobamate-N-mono-glucurone (II)	0.67	0.29	a
Meprobamate	0.84	0.83	a
Glucuronic acid	0.12	0	b
Glucuronolactone	0.32	0	b

Chemical Synthesis of Meprobamate-N-mono-β-D-glucopyranosiduronic Acid (I)——A mixture of glucuronic acid (5 g.) and meprobamate (5.65 g.) in 5% H_2SO_4 (5 cc.) was heated in water bath at 70° for 45 hr. with intermittent stirring. After an addition of 30 cc. of H_2O to this reaction mixture, it was made alkaline to pH 9 with 10% NaOH and the unreacted meprobamate was extracted with AcOEt. The alkaline aq. layer was then acidified to pH 3 with 10% HCl. This solution was shown to be a mixture of compound I and meprobamate-N-mono-D-glucofururonolactone (II) (approx. 3:1) by paper chromatographic examination. Isolation of these compounds was effected by a procedure diagramed in Chart 1. The procedure was rather tedious and the final sample of I was not obtained in crystalline form but as colorless amorphism, even though it was sufficiently pure for elemental analysis and paper chromatographic examination. *Anal.* Calcd. for $C_{15}H_{26}O_{10}N_2$: C, 45.68; H, 6.65; N, 7.10. Found : C, 45.43; H, 6.75; N, 6.61.

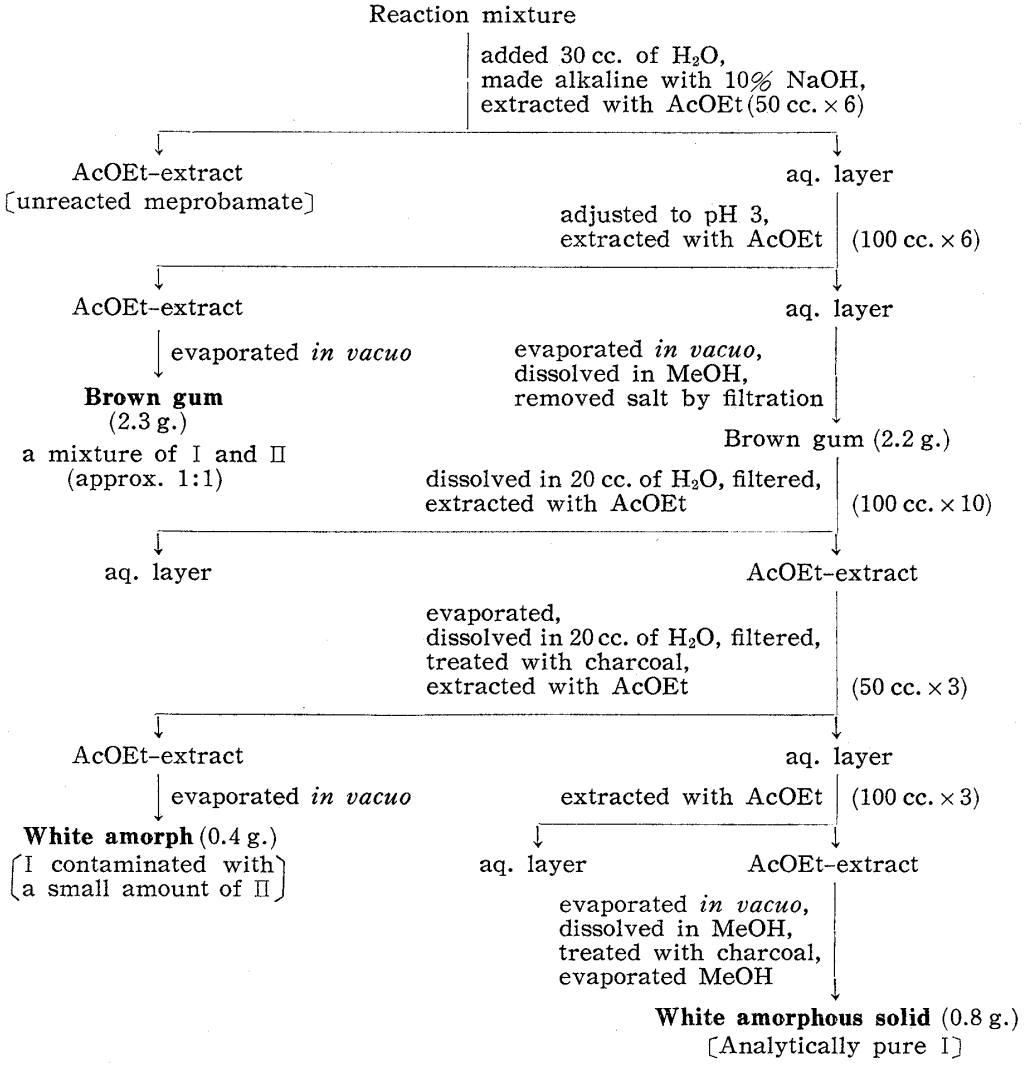

Chart 1. Isolation Procedure for I*4

*4 Amount of I and II was roughly estimated by paper chromatography.

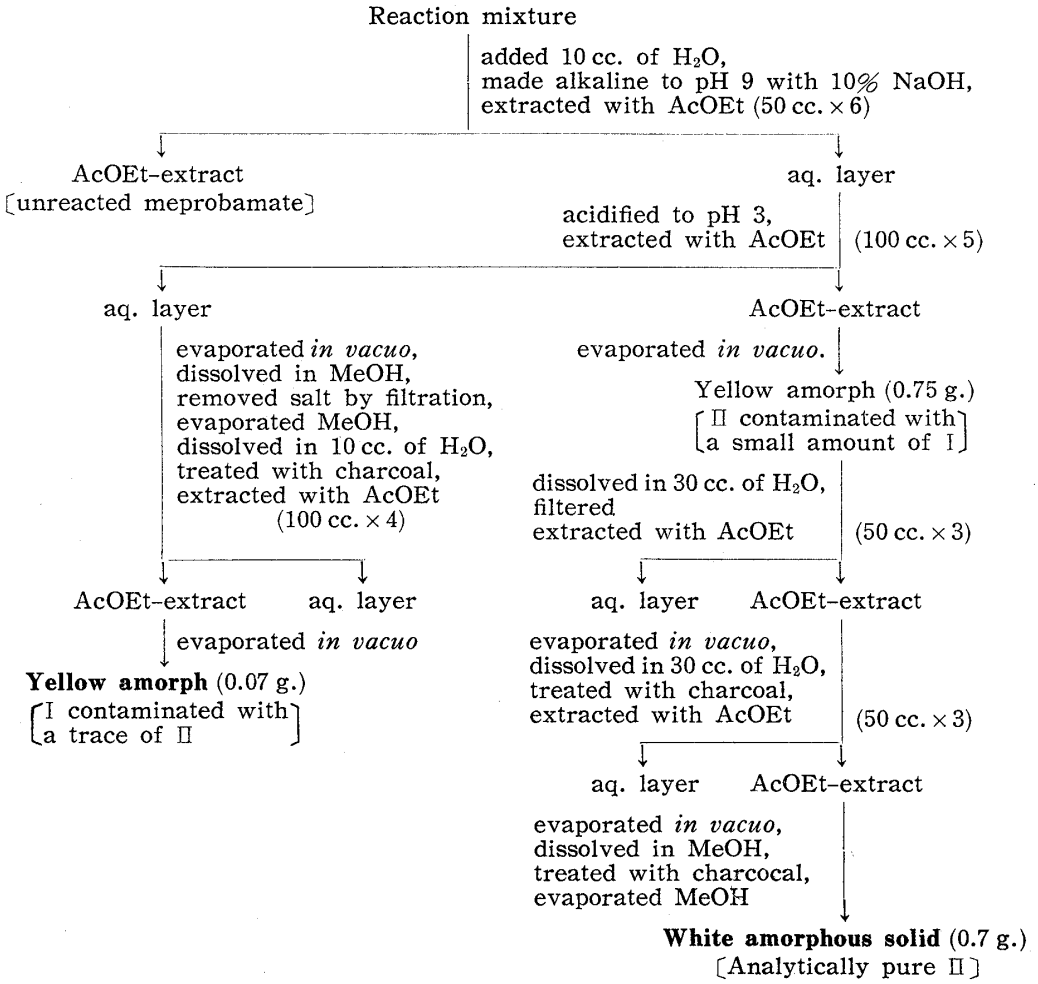

Chart 2. Isolation Procedure for II*4

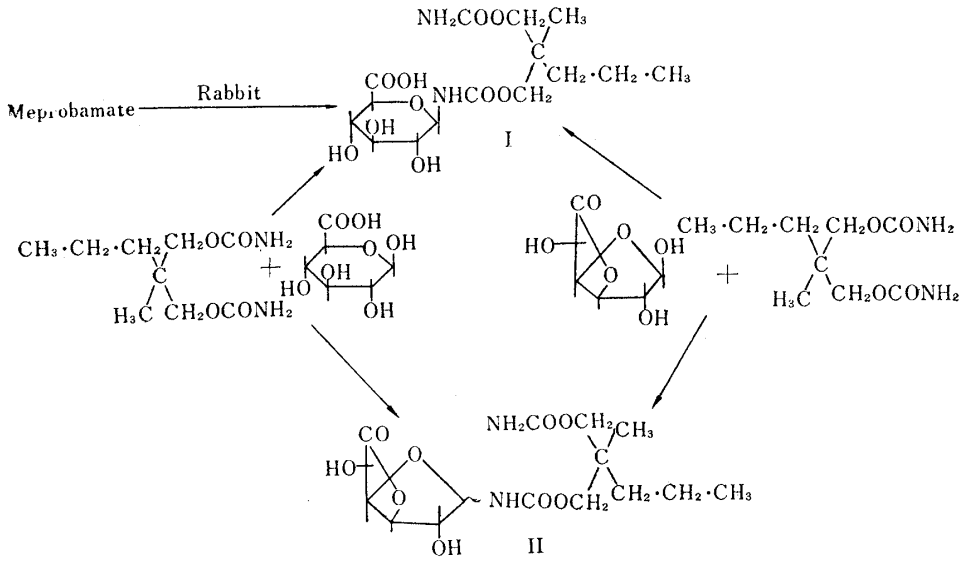

Chart 3.

The IR spectrum indicated to be mono-glucuronide by absorption characteristics of its amide II bands of prim. and sec. carbamate groups. IR λ_{max}^{KBr} μ : 2.90 ($\nu_{NH_2, OH}$), 5.78~5.80 ($\nu_{C=O}$), 6.19 (δ_{NH_2}), 6.50 (δ_{NH}). The glucuronide structure was also suggested by a positive naphthoresorcinol test and negative Fehling's test. In addition, meprobamate, glucuronic acid, and glucuronolactone*5 after acid hydrolysis were detected by paper chromatography.

A methyl acetyl derivative III, methyl meprobamate-N-mono-2,3,4-tri-O-acetyl-β-D-glucopyranosiduronate, was prepared from I as follows: To a solution of 0.1 g. of I, Et_2O solution of CH_2N_2, which was freshly prepared from 5 g. of nitrosomethylurea, was added. The mixture was allowed to stand overnight in a refrigerator, filtered, and the filtrate was evaporated under reduced pressure. This residue was dissolved, in 2 cc. of pyridine, and then 1.4 cc. of Ac_2O was added and the mixture was allowed to stand overnight at room temperature. The reaction mixture was poured into 20 cc. of ice water while stirring and extracted with Et_2O. Et_2O extract was successively washed with dil. HCl and H_2O, dried over anhyd. Na_2SO_4, and evaporated to dryness under reduced pressure. The residue was submitted to silica gel (5 g.) chromatography using benzene, benzene-$CHCl_3$ (1:1), and $CHCl_3$ as effluent solvents. From the fractions eluted with $CHCl_3$, III was obtained as colorless amorphism. The IR spectrum showed clearly the β-glucopyranoside structure[3] by its strong bands at 9.34 μ and a peak at 9.63 μ. IR λ_{max}^{KBr} μ : 2.79, 2.88 (ν_{NH_2}), 5.69 ($\nu_{C=O}$), 6.22 (δ_{NH_2}), 6.51 (δ_{NH}), 9.34 ($\nu_{\beta\text{-pyranose C-O-C}}$). Analytical data also supported the above structure. *Anal.* Calcd. for $C_{22}H_{34}O_{13}N_2$: C, 49.44; H, 6.37; N, 5.24. Found : C, 49.18; H, 6.31; N, 5.02.

Chemical Synthesis of Meprobamate-N-mono-D-glucofururonolactone (II)——This was carried out by a method similar to I using 1 g. of glucuronolactone and 1.2 g. of meprobamate. Both compounds were dissolved in 5% H_2SO_4 (1 cc.) and heated in water bath at 70° for 14 hr. The isolation procedure is shown in Fig. 2. The sample of II thus obtained was also a white amorphous solid. Elementary analysis values and IR spectral data showed a good agreement with those of the structure of meprobamate-N-mono-D-glucofururonolactone. *Anal.* Calcd. for $C_{15}H_{24}O_9N_2$: C, 47.87; H, 6.43; N, 7.44. Found : C, 48.12; H, 6.67; N, 7.29.

IR λ_{max}^{Nujol} μ : 2.90 ($\nu_{NH_2, OH}$), 5.75~5.88 ($\nu_{C=O}$), 6.20 (δ_{NH_2}), 6.50 (δ_{NH}). It exhibited a positive naphthoresorcinol test and liberated meprobamate and glucuronolactone upon acid hydrolysis. A negative Fehling's reaction also suggested glucuronide structure indicating that the aldehyde group at C_1 of glucurone was blocked. II was acetylated by the usual method and the resulting acetyl derivative IV showed the following IR absorption characteristics : IR $\lambda_{max}^{CHCl_3}$ μ : 2.75, 2.84, 2.90 (ν_{NH_2}), 5.50, 5.65~5.84 ($\nu_{C=O}$), 6.29 (δ_{NH_2}), 6.66 (δ_{NH}).

Isolation of I from Rabbit Urine——A 24-hr's urine of 20 rabbits which were administered totally 10 g. of meprobamate (0.25 g./kg. body wt.), was continuously extracted for 20 hr. with AcOEt at pH 2.5. A crude meprobamate N-glucuronide gum was then obtained from the aq. layer by the procedure described in the previous paper.*2 The gum was then dissolved in a small volume of H_2O, adjusted to pH 9 with 10% NaOH and washed with AcOEt. The alkaline aq. layer was acidified to pH 3 with 10% HCl and extracted exhaustively with AcOEt. This extract was dried over anhyd. Na_2SO_4 and evaporated to dryness under reduced pressure, leaving a almost pure sample of I as a little colored gum. The yield was about 0.3 g.

The residue was, then, dissolved in AcOEt containing a small volume of MeOH and submitted to a silica gel (15 g.) chromatography. The column was eluted stepwise with AcOEt, AcOEt-MeOH (49:1), and AcOEt-MeOH (4:1). The fractions collected by elution with AcOEt-MeOH (4:1), which consisted only of I as shown by paper chromatography, were combined and evaporated under reduced pressure.

A white amorphous solid was obtained and the IR spectrum of this material was found to be superimposable with that of synthetic sample (I) chromatographed through silica gel. This metabolite exhibited also a positive naphthoresorcinol reaction and liberated meprobamate upon acid hydrolysis in the same manner as in the case of the synthetic compound.

The IR spectrum of the methyl acetyl derivative III of this metabolite was also identical with the spectrum of synthetic methyl acetyl derivative III. The former was prepared by the same procedure described in this paper for the synthesis of methyl acetyl derivatives.

Enzymatic Synthesis of I——Enzymatic synthesis of I was performed by employing the enzymatic system of rat liver suspension and crude active factor from rabbit liver according to the procedure descrived by Dutton and Storey.[4]

Four flasks were prepared having a complete system, each of which contained 1.5 cc. of 0.5*M* phosphate buffer (pH 7.6), 1 cc. of a solution of 45.8 mg. of meprobamate in 100 cc. of H_2O, 0.5 cc. of 0.3*M* $MgCl_2$ solution, 2.5 cc. of 10% liver suspension, 5 cc. of crude active factor, and 4.5 cc. of H_2O

*5 Glucuronolactone was produced from glucuronides as the result of its equilibrium with glucuronic acid in acidic medium.

3) Y. Nitta, *et al.* : Yakugaku Zassi, **81**, 1160 (1961).

4) G. J. Dutton, I. D. E. Storey : Biochem. J., **57**, 275 (1954).

(final substrate concentration : $1.4\times10^{-4}M$). These were incubated at 37° for 1 hr. Two additional flasks having an incomplete system were incubated also as controls. One contained a boiled 10% liver suspension instead of 10% liver suspension and the other, H_2O instead of meprobamate solution. After incubation, these mixture were boiled for 3 min., centrifuged, and the supernatant was evaporated to dryness under reduced pressure. The residue was again dissolved in a small volume of H_2O and filtered. The filtrate was adjusted to pH 9 with 10% NaOH and extracted with AcOEt for unreacted meprobamate. The alkaline aq. layer was then acidified to pH 3 with 10% HCl, and extracted exhaustively with AcOEt. After drying over anhyd. Na_2SO_4, it was evaporated to dryness under reduced pressure. The residue was dissolved in a small volume of MeOH and examined for the formation of I by paper chromatography.

The sample having a complete system showed a distinct appearance of I on the paper chromatogram while the two samples having an incomplete system did not result in any formation of I. The formation of I in the former was further proved by a positive naphthoresorcinol test of the area corresponding to I on the paper chromatogram and by the appearance of meprobamate spot after re-chromatography of the hydrolysate from the same area.

Hydrolysis of I with β-Glucuronidase——β-Glucuronidase was prepared according to the method of Fishman.[5] A solution of 7 mg. of I in 4.5 cc. of 0.1*M* acetate buffer (pH 4.5) was incubated in 0.5 cc. of β-glucuronidase solution (6750 units/cc.) at 38° for 1.5 hr. The incubated mixture was then boiled in a water bath for 3 min. and the precipitate was removed by centrifugation. The supernatant was extracted three times with Et_2O, dried over Na_2SO_4, and evaporated to dryness. The residue was dissolved in a few drops of EtOH and submitted to paper chromatography. No spot corresponding to meprobamate was observed. However, 2-naphthol was liberated*6 after incubation of 2-naphthyl-β-D-glucopyranosiduronic acid with the same β-glucuronidase preparation under the same conditions used in the above experiment.

Experiment on Possible Non-enzymatic Formation of I during the Extraction——A solution of 0.1 g. of meprobamate and 0.5 g. of glucuronic acid in 200 cc. of normal rabbit urine was adjusted to pH 2.5 with 20% H_2SO_4 and allowed to stand at room temperature for 10 days. The isolation of glucuronide was conducted using normal and basic $(AcO)_2Pb$ according to a general method described by R. T. Williams.[6] The resulting Pb-salt of glucuronide fraction was suspended in MeOH and converted to a free form by H_2S. MeOH suspension was filtered and the residue remained after the evaporation of filtrate was subjected to paper chromatographic examination. The presence of I in this residue was not revealed by chromatography.

Discussion

It is assumed that amino, carbamoyl, and amide compounds could form N-glucuronides as their metabolic end products, but only amino compounds have been actually reported so far to form N-glucuronides in animal body.[1] The present study, showed that even NH_2 of carbamate group could be conjugated with glucuronic acid as a N-glucuronide and that I was excreted as one of the principal metabolites.*2 This is significant because this finding may be applied not only to all drugs possessing a carbamate group, but also to ureido or amide compounds which are used widely in medicine. M. Akagi, *et al.*[7] recently furnished an evidence to this proposal in that N-glucuronide could be isolated from urine of rabbits administered dulcin, (*p*-ethoxyphenylurea). Further research is being continued in this laboratory to decide if this consideration is generally acceptable.

To exclude any doubt that meprobamate might be linked nonenzymatically with glucuronic acid during the course of a long extraction procedure in acidic medium, additional experiments were performed to prove that its formation could only occur by enzymatic action in animal body. Such an action had been well established for O-glucuronides[4,8] and for aniline-N-glucuronide[9] formations.

*6 Detected with diazotized benzene sulfonic acid on paper chromatogram using solvent system (I), (Rf 0.89).

5) W. H. Fishman, *et al.* : J. Biol. Chem., **166**, 757 (1946).
6) R. T. Williams : Biochem. J., **50**, 235 (1951).
7) M. Akagi, I. Aoki, T. Uematsu : Abst. of 15th Annual Meeting of Pharm. Soc. Japan, p. 161 (1962).
8) K. J. Isselbacher, J. Axelrod : J. Am. Chem. Soc., **77**, 1070 (1955); J. Strominger, *et al.* : J. Biol. Chem. **224**, 79 (1957); G. J. Dutton : Biochem. J., **64**, 693 (1956).
9) J. Axelrod, J. K. Inscoe, G. M. Tomkins : Nature, **179**, 538 (1957); J. Axelrod, *et al.* : J. Biol. Chem. **232**, 835 (1958).

One more remarkable characteristic of this glucuronide is its considerable stability in acid and alkali as it had been also indicated by J. Ludwig, *et al.*[2] while amine-N-glucuronide is very labile in acid.[9,10] β-Glucuronidase, as expected, did not act on this metabolite such as it did on aniline-N-glucuronide.[10]

It followed, then, that the final proof of chemical structure of this metabolite lays in the comparative examination with its synthetic counterpart.

The authors had at first undertaken a more unequivocal synthetic approach than that of the present method, however all attempts, to prepare I by condensation of methyl 1,2,3,4-tetra-O-acetyl-β-D-glucopyranuronate or methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl-α-D-glucopyranuronate with meprobamate under various conditions followed by hydrolysis, did not succeed.

Recently, F. Micheel and R. Habendorff,[11] and Y. Nitta, *et al.*[12] reported extensive studies on the synthesis of 1-deoxy-1-thioureido-β-D-glucuronic acid derivatives by utilization of methyl 1-deoxy-1-isothiocyanate (or 1-azido)-2,3,4-tri-O-acetyl-β-D-glucopyranuronate. An application of this method to the present study resulted only in a number of difficulties. Finally, the method of C. Neuberg and W. Neiman,[13] which favors a method for the condensation of urea directly with glucuronic acid, was adopted.

Therefore, one mole each of meprobamate and glucuronic acid were condensed in 5% H_2SO_4 solution and a product was isolated which was a glucuronide identical with that of the metabolite. The problems remained, however, to isolate and purify the glucuronide from the mixture contaminated with meprobamate N-mono-glucurone and to isolate it in acrystalline form. The mono-glucuronide structure, in which glucuronic acid was linked with one of two carbamate groups in meprobamate, was confirmed by elemental analysis and infrared spectrum examination. Whether or not it had a α- or β-configuration and whether or not it had a pyranoside or furanoside structure were indicated only by the infrared spectrum study of its methyl acetyl derivative. It is known that natural glucuronide possesses a β-pyranoside structure.

Synthesis of meprobamate-N-mono-glucurone (II) from glucronolactone and meprobamate was conducted just for confirming the compound which was produced as a by-product in the synthesis of meprobamate N-mono-glucuronic acid (I). Equilibration between glucuronic acid and glucuronolatone in acidic medium is a phenomenon which is well known and so it is quite reasonable to believe that both compounds (I) and (II) could be isolated in either method.

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Summary

The structure of meprobamate-N-glucuronide, one of the principal metabolites of meprobamate, was established as meprobamate-N-mono-β-D-glucopyranosiduronic acid by direct condensation of meprobamate and glucuronic acid.

It was also proved that this glucuronide was not formed spontaneously during the course of extraction but only by enzyme system in animal body.

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10) S. Takitani : This Bulletin, **7**, 845 (1959).
11) F. Micheel, R. Habendorff : Ber., **90**, 1590 (1957).
12) Y. Nitta, *et al.* : Yakugaku Zassi, **81**, 1166, 1171 (1961); M. Kuranari : *Ibid.*, **81**, 1179, 1185, 1189, 1195 (1961).
13) C. Neuberg, W. Neiman : Z. Physiol. Chem., Hoppe-seyler's, **44**, 97 (1905).