UDC 615.78-092.21

83. Akira Yamamoto, Hidetoshi Yoshimura, and Hisao Tsukamoto : Metabolism of Drugs. XXVIII.*1 Metabolic Fate of Meprobamate. (1). Isolation and Characterization of Metabolites.

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Meprobamate, (2-methyl-2-propyl-1,3-propanediol dicarbamate), is widely used as one of the tranquilizing agents and abundant pharmacological reseaches have been done, but their metabolic fate has not been sufficiently elucidated.[1~4]

Agranoff, *et al.*[2] found that about 12~20% of the unchanged drug was excreted in the urine of human subject and that additional products were formed, including several conjugates at least, one of which was a glucuronide.

With the aid of ^{14}C-carbamate labeled meprobamate, Walkenstein, *et al.*[3] concluded that the major metabolite, accounting for approximately 60% of the dose in dogs, was excreted as hydroxy-meprobamate (2-hydroxymethyl-2-propyl-1,3-propanediol dicarbamate) and that two unknown glucuronides were found. They reported also that the carbamate groups were resistant to hydrolysis because of the failure to locate more than 1~2% of the labeled carbamate carbon in the respired air.

Since the series of investigation about biological oxidation of drugs acting on the central nervous system were being carried out in our laboratory, the authors wished to obtain more definite chemical information concernig the metabolism of meprobamate.

It is shown in this report that the six metabolites except unchanged meprobamate were found and characterized in the urine of dogs and rabbits. They were hydroxy-, keto-, and carboxy-meprobamate and three glucuronides. This result is quite different from those of Walkenstein, *et al.*[3]

Methods and Results

Isolation of Metabolites from the Urine of Rabbits——The animals used were male rabbits weighing 2.9~3.4 kg. They were housed in metabolism cages and 'Okara' (soybean curd residue). Meprobamate (0.3 g./kg. body weight) was administered as a suspension in 10% gum arabic by stomach tube, and the decomposition of metabolites in urine was prevented by the addition of toluene. A total dose of 10 g. of meprobamate was administered to 11 rabbits and the urine collected after 24 hr. was filtered through Hyflo-Supercel. The filtrate was brought to pH 2.5 with 20% H_2SO_4 and extracted continuously with AcOEt for 20 hours. The AcOEt extracts were combined, evaporated to dryness *in vacuo*, and dissolved in 50 cc. of H_2O.

The aqueous solution was made alkaline with $NaHCO_3$ and extracted 4 times with 150 cc. portions of AcOEt. The AcOEt extracts were combined, washed with a small volume of H_2O, dried over anhyd. Na_2SO_4, and evaporated *in vacuo*. The residual 3.65 g. of faint yellow syrup was dissolved in benzene-AcOEt (1:1) and chromatographed through 100 g. of Al_2O_3, using a developing solvent system as shown in Fig. 1. Each fraction was consisted of 50 cc. of volume and components of several fractions were examined by paper chromatography as mentioned in later section.

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*1 Part XXVII. This Bulletin, **10**, 91 (1962).

*2 Katakasu, Fukuoka (山本 陽, 吉村英敏, 塚元久雄).

1) F. M. Berger : J. Pharmacol. Exptl. Therap., **112**, 413 (1954).
2) B. W. Agranoff, R. M. Bradley, J. Axelrod : Proc. Soc. Exptl. Biol. Med., **96**, 261 (1957).
3) S. S. Walkenstein, C. M. Knebel, *et al.* : J. Pharmacol. Exptl. Therap., **123**, 254 (1958).
4) J. L. Emmerson, T. S. Miya, G. K. W. Yim : *Ibid.*, **129**, 89 (1960).

From the fractions of Nos. 17～47, 0.35 g. of colorless crystals, m.p. 104～105°, were obtained and confirmed to be identical with authentic meprobamate by the melting point on admixture and infrared spectrum.

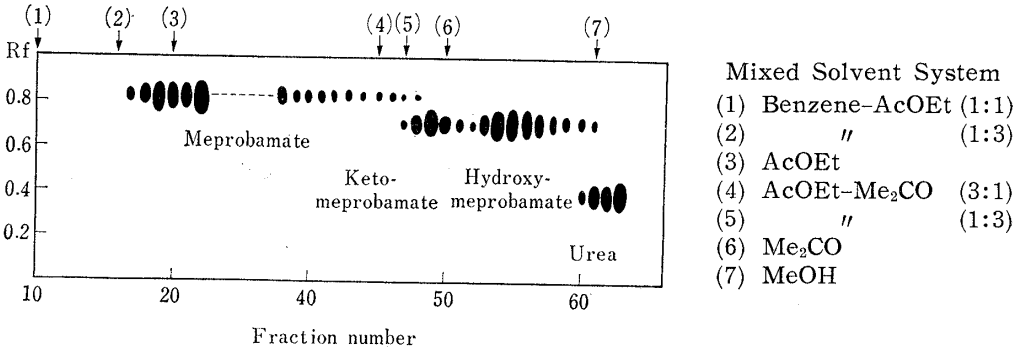

Fig. 1. Paper Chromatogram ($BuOH-AcOH-H_2O=4:1:5$) of Fractions eluted from Alumina Chromatography

Moreover, two crystalline compounds, metabolite (I) and (II) were obtained from the fractions of Nos. 48～49, and Nos. 51～60, respectively. Metabolite (I) was recrystallized from AcOEt to colorless prisms, m.p. 122～123° (yield, 0.023 g.), and (II) was recrystallized from H_2O to colorless plates, m.p. 39～40° (yield, 0.91 g.).

The $NaHCO_3$-alkaline aqueous solution separated from AcOEt extract described above, was adjusted to pH 2.5 with 20% H_2SO_4, extracted 4 times with 150 cc. portions of AcOEt. The AcOEt extracts were combined, washed, and evaporated *in vacuo*. The brown oily substance was dissolved in small amounts of AcOEt and allowed to stand overnight in a refrigerator. The white crystalline compound (metabolite III) separated was collected and recrystallized from H_2O and AcOEt to colorless needles, m.p. 131° (yield, 0.65 g.).

Furthermore, the isolation of glucuronides was carried out according to the general method described by Williams.[5] The urine remained after continuous extraction with AcOEt was treated with normal and basic lead acetate.

The resulting Pb-salts of glucuronides were finely suspended in MeOH and converted to free glucuronides by H_2S. The red gum obtained after evaporation of MeOH *in vacuo* at 25°, was dissolved in 10 cc. of H_2O, filtered and extracted 5 times with 100 cc. portions of AcOEt. After evaporation of AcOEt, it gave 0.2 g. of faint yellow gum (metabolite IV).

The aqueous layer remained after extraction with AcOEt was evaporated *in vacuo* at 35°, dried over anhyd. $CaCl_2$ for 24 hours *in vacuo*. The resulting residue was dissolved in a small volume of MeOH, and stirred into a large excess of dehyd. Et_2O and immediately grayish-white precipitate was separated to give 0.1 g. of hygroscopic powder as a mixture of metabolite (V) and (VI).

Isolation of Metabolites from the Urine of Dogs——Meprobamate wrapped in a piece of meat was administered in doses of 0.3 g./kg. body weight to dogs weighing about 8 kg. and a total dose of 10 g. was given. The collected urine were treated same as in the rabbits and 1.1 g. of unchanged meprobamate was obtained in this case. Although metabolite (I) and (III) could not be isolated, other metabolites, (II), (IV), (V), and (VI) were also obtained in dogs. Their identities with those from rabbits urine were confirmed by mixed melting point determination, infrared spectra, paper chromatography, and paper electrophoresis.

The yields from rabbits and dogs urine were summarized in Table I.

5) R. T. Williams: Biochem. J., **50**, 235 (1951).

Table I. Yield of Isolated Metabolites from the Urine of Rabbits and Dogs

Dose 0.3 g./kg.	Total dose g.	Unchanged meprobamate		Keto-meprobamate		Hydroxy-meprobamate		Carboxy-meprobamate	
		g.	%[a]	g.	%[a]	g.	%[a]	g.	%[a]
Dog	10	1.10	11.0			0.80	7.4		
〃	10	1.82	18.2			0.65	6.1		
〃	10	1.66	16.6			0.83	7.7		
Rabbit	10	0.35	3.5	0.023	0.21	0.91	8.4	0.65	5.6
〃	10	0.39	3.9	—		0.52	4.8	0.73	6.3
〃	10	0.32	3.2	—		0.60	5.5	0.70	6.0
〃	10	0.35	3.5	0.03	0.27	0.92	8.5	0.25	2.1

a) % of dose

Paper Chromatography and Paper Electrophoresis of Metabolites——The development was made by ascending method using Toyo Roshi No. 50. Solvent system used were (1) BuOH-AcOH-H_2O (4:1:5), (2) BuOH saturated with 3% NH_4OH, and (3) BuOH-H_2O (100:15).[3] For the electrophoresis, the horizontal open-strip type apparatus (plastic frame support) was used with constant voltage. Electrolyte used was 1% borax. The current of 400 V for the electrolyte was applied for a filter paper strip (24×12 cm.) and charged for 1 hour.

Metabolites were visualized on filter paper by spraying the following reagent: (1) Ehrlichs' reagent,[6] 2% *p*-dimethylaminobenzaldehyde in conc. HCl, followed by hot air, (2) 20% $SbCl_3$ in $CHCl_3$, followed by fresh furfural.[7] The results were listed in Table II.

Table II. Paper Chromatography and Paper Electrophoresis of Metabolites

Compounds	Rf Solvent System I	II	III	Electrolyte 1% Borax (MD)
Metabolite I	0.72	0,62	0.63	− 0.9
〃 II	0.72	0.62	0.63	− 0.9
〃 III	0.70	0.60	0.05	+32
〃 IV	0.57	0.47	0.15	+23
〃 V	0.31	0.08	0.03	+20
〃 VI	0.20	0.03	—	—
Meprobamate	0.85	0.81	0.80	− 0.9
Urea	0.40	0.25	0.23	− 0.9

Characterisation of Metabolites——All six metabolites isolated here showed an existance of carbamate groups by color reaction.

Metabolite (I): Analysis of this compound, m.p. 122~123°, suggested that it should be a ketone which had still meprobamate skeletone. *Anal.* Calcd. for $C_9H_{16}O_5N_2$: C, 46.55; H, 6.89, N, 12.06. Found: C, 46.61; H, 7.10; N, 12.00.

It formed easily 2,4-dinitrophenylhydrazone by usual procedure, and recrystallized from hydr. EtOH to orange-yellow plates, m.p. 171~173°. *Anal.* Calcd. for $C_{15}H_{20}O_8N_6$: C, 43.68, H, 4.85, N, 20.38. Found: C, 43.95, H, 5.08, N, 20.31.

Iodoform test for this metabolite was carried out with quite positive result and moreover, the crystalline iodoform, m.p. 119°, was isolated from the reaction mixture, which rigorously proved a location of CH_3CO-grouping in the structure. The possibility of aldehyde structure was ruled out by its negative Tollens' test. It was also found to be identical with the oxidation product of metabolite (II) as shown in the following experiment and the infrared spectrum was agreed with this keto-meproobamate structure.

Metabolite (II): This metabolite was oily as the beginning, but careful recrystallization

6) M. Shimizu, S. Ichimura: Yakugaku Zasshi, **78**, 1183 (1958).
7) B. J. Ludwig, A. J. Hoffman: Arch. Biochem. Biophys., **72**, 234 (1957).

from H_2O gave colorless plates, m.p. 39~40°. The analytical result showed a good agreement with a hydroxymeprobamate and the ceric nitrate test for a hydroxy group suggested also an existence of OH, although its infrared spectrum could not be distinguishable the stretching absorption peaks between newly formed OH, and NH in carbamate groups which appeared in the same region. *Anal.* Calcd. for $C_9H_{18}O_5N_2$: C, 46.16 H, 7.69, N, 11.97. Found : C, 46.31, H, 7.57, N, 11.88.

Iodofrom reaction for this metabolite gave also positive result and suggested an existence of CH_3CHOH-grouping.

It was then acetylated with Ac_2O and BF_3-Et_2O as followed : To a solution of 0.3 g. of metabolite (II) in 1.5 cc. of Ac_2O, 0.9 cc. of BF_3-Et_2O was dropped and allowed to stand over-night at room temperature. The reaction mixture was poured into ice-water and extracted 3 times with 20 cc. portions of AcOEt. The combined extracts were washed, dried and evaporated. The remained oily substance was purified by alumina chromatography, using benzene-AcOEt (3:2) as solvent. The product was recrystallized from hydr. EtOH as prisms, m.p. 127~129°. *Anal.* Calcd. for $C_{15}H_{24}O_8N_2$: C, 50.00 H, 6.66 N, 7.77. Found : C, 50.17 H, 6.67 N, 7.71.

Metabolite (II) was further oxidized with $KMnO_4$ in acidic solution as followed : To a solution of 1 g. of metabolite (II) in 13.8 cc. of 5% H_2SO_4, 0.7 g. of $KMnO_4$ was added in portions for 3 hours at room temperature with stirring. After dissolving the precipitates with $NaHSO_3$, the reaction mixture was extracted 3 times with 50 cc. portions of AcOEt. Evaporating the solvent from the extract, 0.4 g. of colorless crystals, m.p. 120~122°, were obtained. It was recrystallized from AcOEt to colorless prisms, m.p. 122~123°, which showed no depression of melting point by an admixture with metabolite (I) and also gave identical infrared spectrum with those of (I).

Metabolite (III) : This acidic compound, m.p. 131°, was clearly shown to be a carboxylic acid, because of its positive hydroxamic acid test and infrared spectrum. Analysis of this compound gave also a good agreement with a carboxy-meprobamate. *Anal.* Calcd. for $C_9H_{16}O_6N_2$: C, 43.55, H, 6.45, N, 11.29. Found : C, 43.71, H, 6.54, N, 11.24.

The neutralization equivalent by titration with *N*/100 NaOH was 249 (Calcd. value for $C_9H_{16}O_6N_2$: 248).

Furthermore, it could be esterified easily with CH_2N_2 and then methyl ester of metabolite (III), colorless prisms, m.p. 152~153°, recrystallized from MeOH, was obtained quantitatively. *Anal.* Calcd. for $C_{10}H_{18}O_6N_2$: C, 45.80, H, 6.86, N, 10.68. Found : C, 45.51, H, 7.13, N, 10.47.

Characteristic absorption peaks of the metabolites, (I), (II), and (III) including meprobamate ware summarized in Fig. 2.

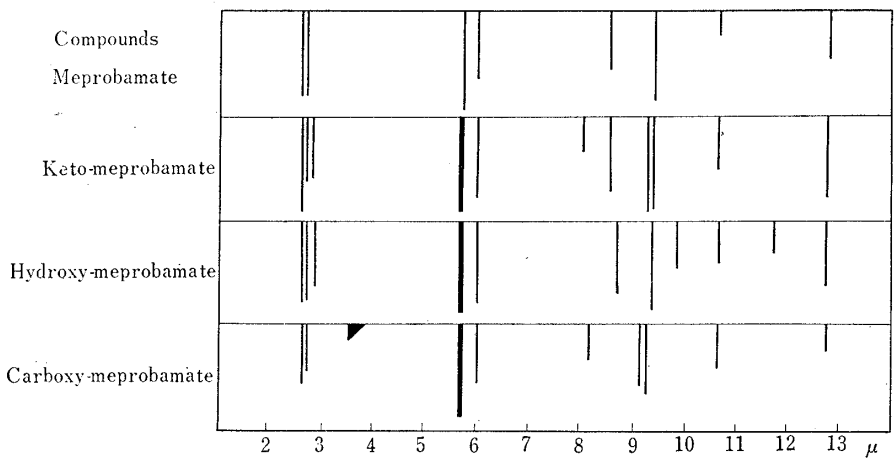

Fig. 2. Infrared Absorption Peaks of Metabolites (Nujol mull)

Metabolite (IV): This was isolated as syrup and failed to crystallize, however, it was shown to be paper chromatographically pure, It was purified by paper chromatography with solvent (1) using 40×40 cm. filter paper. The suitable section was then cut out, extracted with H_2O, H_2O was evaporated *in vacuo*, and oily substance was thus obtained. It gave no aniline hydrogenphthalate reaction before acid hydrolysis, but a positive naphthoresorcinol reaction for glucuronic acid.

After hydrolysis of 0.1 g. of purified metabolite (IV) with 5 cc. of 5% HCl for 50 min. on a boiling water bath, it was neutralized with $NaHCO_3$, and extracted 3 times with 10 cc. portions of AcOEt. Evaporating the solvent from this extract, 25 mg. of colorless crystalline powder, m.p. 102~104°, was obtained. This hydrolysis product, after recrystallization from H_2O, was identical with authentic meprobamate by its melting point on admixture and its infrared spectrum.

Metabolite (V) and (VI): Both were oily, but paper chromatographically pure. They were also purified by paper chromatography as mentioned in characterization of metabolite (IV) and both eluates of (V) and (VI) from paper chromatogram gave a positive naphthoresorcinol test.

When 0.2 g. of metabolite (V) was hydrolyzed with 10 cc. of 5% HCl for 1 hour on a boiling water bath, or enzymatically with β-glucuronidase,[8] it yielded metabolite (II) which was identified with authentic sample by the comparison of infrared spectrum and by paper chromatography.

Metabolite (V) was also found paper chromatographically in the urine of rabbits administered metabolite (II).

Metabolite (VI) was isolated only in a small amounts just enough for an examination of carbamate grouping and glucuronic acid moiety by color reaction. It was considered to be surely one kind of the glucuronides by above experiment, but no more evidences could not be obtained.

Routs of metabolism of meprobamate were summarized in Chart 1.

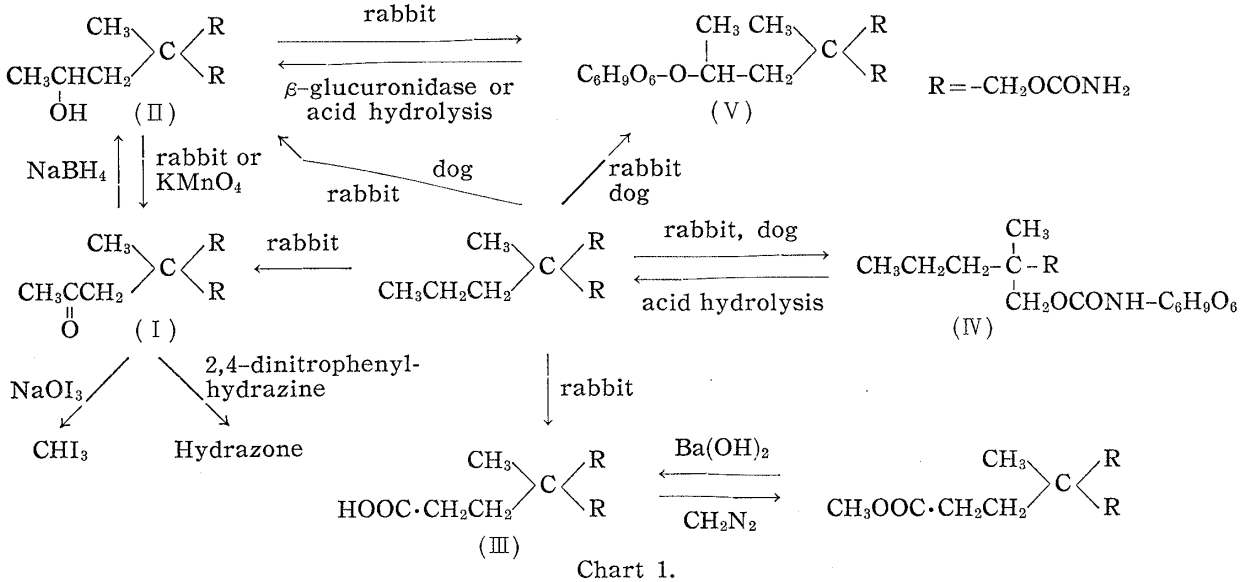

Chart 1.

Discussion

It is well known that the oxidation at ω- or (ω-1)-position of alkyl side chain in various drugs, especially in barbiturates are one of the main inactivation processes of

8) H. Tsukamoto, A. Yamamoto: This Bulletin, **7**, 434 (1959).

drugs in animal body.[9,10] The authors also expected that propyl side chain in meprobamate might be oxidized by such a reaction for its metabolic fate and successfully found that hydroxy (II), ketone (I), and carboxylic acid derivatives (III) of meprobamate were excreted with other metabolites conjugated with glucuronic acid into the urine of rabbits. Futhermore, the structure of metabolite (I) and (II) were rigorously suggested to be 2-methyl-2-acetonyl-1,3-propanediol dicarbamate and 2-methyl-2-(2-hydroxypropyl)-1,3-propanediol dicarbamate respectively, by their positive iodoform test and elemental analyses.*3 It was also proposed that the structure of metabolite (III) should be 5-hydroxy-4-hydroxymethyl-4-methylvaleric acid dicarbamate, mainly by its elemental analysis, neutralization equivalent, and infrared spectrum. Further conclusive evidences about these structures will be described in the next paper.

Recently, Walkenstein, *et al.*[3] reported that hydroxy-meprobamate (2-hydroxymethyl-2-propyl-1,3-propanediol dicarbamate) was excreted as the major metabolite into the urine of dogs received meprobamate. This was quite inconsistent with the authors' results and the authors thought that the hydroxy-meprobamate from rabbits urine might be different from those obtained from ddog urine by Walkenstein, *et al.* since species difference is encountered rather often in metabolism of drugs. Nevertheless, the facts generally known that if there are two alkyl side chains in the structure of drug, longer one is much more susceptible to oxidation in animal body, prompted us to examine the urine of dogs received meprobamate. The results were just same as in rabbit and we isolated the hydroxylated product which was oxidized in propyl, but not methyl side chain as mentioned by Walkenstein, *et al.*

They have elucidated much of facts concerning the metabolism of meprobamate with the aid of labeled drug, however, their experimental results did not seem completely clear and remained little obscurity about the structural elucidation of hydroxy-meprobamate, in which the proof was mainly based on the analysis and the identity of Rf value with synthetic sample, but no melting point and no comparison of the infrared spectra with synthetic sample were described, although they mentioned about hard distinction between infrared absorptions of NH_2 and OH stretching vibration in the metabolite. Considering these facts, it seems very likely that a true structure of hydroxy-meprobamate must be 2-methyl-2-(2-hydroxypropyl)-1,3-propanediol dicarbamate, but not 2-hydroxymethyl-2-propyl derivative. The further proofs about this structure will be described more in details in the next paper.

Now, attention may be focused on the metabolites conjugated with glucuronic acid. It was very interesting that glucuronide of meprobamate itself (metabolite IV) was isolated with other glucuronides. Chemical structure of this glucuronide was not clear so far, but it was rigorously shown that its hydrolysis with acid gave a crystalline meprobamate and also a positive naphthoresorcinol reaction for glucuronic acid. It was failed to crystallizet his metabolite, but its homogeneity or purity was proved by paper chromatography. Of course further studies will be necessary for establishment of this structure, however it may be concluded that this is a new type of N-glucuronide, because of the absence of any other functional groups except nitrogen in carbamate group for glucuronide

*3 After sending of this paper to publication, the report of Ludwig, *et al.* became available here, in which there were described the confirmation of hydroxy meprobamate as 2-methyl-2(2-hydroxypropyl)-1,3-propanediol dicarbamate by the synthesis. They also mentioned the occurrence of glucuronide of meprobamate in human urine same as described in this paper. B. J. Ludwig, *et al.*: J. Med. Pharm. Chem., **3**, 53 (1961).

9) E. W. Maynert, H. B. van Dyke: Pharmacol. Review, **1**, 217 (1949).

10) J. Raventós: J. Pharm. Pharmacol., **6**, 217 (1954).

formation of meprobamate. In recent years, a number of N-glucuronides was found in various biological origins administered sulfonamide,[11] or other amino compounds,[12,13] but no examples had been reported about N-glucuronide formation of such a carbamate groups as meprobamate.

From the results of paper chromatography, metabolite (V) should be considered as ether-type glucuronide of hydroxy-meprobamate. No evidence was obtained about the structure of last glucuronide (metabolite VI), at present time.

The authors are indebted to Messrs. E. Tamura and K. Nishiyama for technical help, to Mr. H. Yano for the determination of infrared spectra, and to Miss S. Tada for the elementary analyses. Thanks are also due to Daiichi Seiyaku Co., Ltd. for their supply of meprobamate.

Summary

The urinary metabolic end-products of meprobamate (2-methyl-2-propyl-1,3-propanediol dicarbamate) in rabbits and dogs were studied. They were isolated and characterized as keto-, hydroxy-, carboxy-meprobamate and three glucuronides containing carbamate group, but in dogs keto- and carboxy-derivatives were not isolated. Of glucuronides, one was N-glucuronide conjugated with meprobamaet itself and another was ether-type glucuronide of hydroxy-meprobamate.

(Received April 14, 1961)

11) S. Ogiya: Yakugaku Zasshi, **80**, 1538 (1960).
12) R. M. Bushby, A. J. Woiwod: Biochem. J., **63**, 406 (1956).
13) E. Boyland, D. Manson, S. F. D. Orr: *Ibid.*, **65**, 417 (1957).

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84. Ryozo Koshiura (Hirata), Yukiko Kagotani, and Toshimitsu Ujiie: Experimental Anticancer Studies. XVI.[*1] Preparation and Anticancer Activity of 4-Amino-6-hexylresorcinol on Ehrlich Carcinoma in Mice.

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As reported previously,[1] 4-hexyl-6-(2-hydroxy-3,5-dibromophenylazo)resorcinol (AZO-36)[2~4] and 2,6-bis-(2-hydroxy-3,5-dibromophenylazo)-4-propylphloroglucinol (AZO-106)[5~7] are the effective antitumor compounds among members of 2,2′-dihydroxyazobenzene derivatives so far tested in this laboratory.

Meanwhile, it became interest to test for the anticancer activity of compounds of Schiff's base type, which have chemical constitution of either R-CH=N-R′ or R—N=CH—R′ against that of R—N=N—R′ of AZO-36. The present communication describes the results of such experiments.

*1 Part XV: Ann. Rep. Tbc. Kanazawa, **19**, 225 (1961).
*2 Tsuchitoribanaga-machi, Kanazawa (越浦良三, 籠谷由紀子, 氏家俊光).
1) H. Okamoto, *et al.*: Zeitschrift für Krebsforschung, **62**, 408 (1958).
2) R. Hirata: This Bulletin, **4**, 60 (1956).
3) S. Koshimura, *et al.*: GANN, **47**, 393 (1956).
4) T. Masusaki: Juzen Igaku Zasshi, **60**, 1512 (1958).
5) R. Hirata: Japan. J. Exptl. Med., **27**, 99 (1957).
6) S. Koshimura, *et al.*: ACTA, Unio Internatl. Contra Cancrum, **15**, 154 (1959).
7) T. Miyaji: Juzen Igaku Zasshi, **63**, 278 (1959).