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Metabolic Fate of 6,6,9-Trimethyl-9-azabicyclo[3,3,1]non-3β-yl α,α-Di(2-thienyl)glycolate Hydrochloride Monohydrate (PG-501)

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The isolation and identification of urinary metabolites of PG-501, a new antiparkinsonian agent, has been investigated in rats.

PG-501 was extensively metabolized by rat since only less than 0.1% of the drug was recovered unchanged in the 24 hour urine. A part of PG-501 was first N-demethylated and then hydrolyzed, and almost all of the remainder was directly hydrolyzed to granatane base and α,α-di(2-thienyl)glycolic acid (DTGA). A part of granatane base was further metabolized by glucuronic acid conjugation. Most of DTGA was further metabolized by dethienylation, decarboxylation, glucuronic acid conjugation and mercapturic acid conjugation. The major metabolites of granatane base were 6,6,9-trimethyl-3-hydroxygranatane, 6,6-dimethyl-3-hydroxygranatane and their glucuronic acid conjugates whereas major metabolites of DTGA were glucuronic acid conjugate of DTGA, glucuronic acid conjugate of 2-thiophenecarboxylic acid, N-acetyl-S-[5-(2-thiophenecarbonyl)-2-thienyl]-L-cysteine, and 2-thiopheneglyoxylic acid.

Studies of urinary metabolites of rats given N-$^{14}CH_3$-PG-501 showed that a part of formaldehyde produced by N-demethylation of the drug was excreted in urine as methionine, N-formylcysteine, N,N′-diformylcystine, formic acid and urea.

6,6,9-Trimethyl-9-azabicyclo[3,3,1]non-3β-yl α,α-di(2-thienyl)glycolate hydrochloride monohydrate (PG-501) has been shown by Nose, *et al.*,[2] to have a potent antiparkinsonian activity and a low potency of side effects of mydriasis, dry mouth, which are commonly observed in the treatment of parkinsonism. The present investigation was undertaken to elucidate the metabolic fate of PG-501 in rat. This paper described not only the metabolic pathways of PG-501 but also those of formaldehyde produced by N-demethylation of this drug.

Experimental

^{14}C-PG-501 and Reference Compounds——^{14}C-Glycolyl-PG-501, which is shown in Fig. 1, was synthesized with use of methyl ^{14}C-oxalate as a starting material in this laboratory. The specific activity of the product was 2.4 μCi/mg, and the radiochemical purity was more than 98% as determined by thin-layer chromatography (TLC). N-$^{14}CH_3$-PG-501 with a specific activity of 11.0 μCi/mg was prepared by methylation of 6,6-dimethyl-3-hydroxygranatane with $^{14}CH_3I$, followed by esterification with methyl di(2-thienyl) glycolate. The following compounds of established structure, which had been synthesized previously in this laboratory,[2] were used as reference standards: 6,6,9-trimethyl-3-hydroxygranatane (TMHG), 6,6-dimethyl-3-hydroxygranatane (DMHG) and di(2-thienyl)glycolic acid (DTGA).

Isolation of Urinary Metabolites of ^{14}C-Glycolyl-PG-501——Male Wistar rats weighing about 200 g were fasted for 16 hours before dosing. ^{14}C-Glycolyl-PG-501 which was diluted with 10 amounts of nonradioactive PG-501 was administered orally in a dose of 50 mg/kg to 20 rats. (The oral LD_{50} for PG-501 is more than 800 mg/kg in rat and the toxic dose is more than 100 mg/kg.) The urine was collected separately from the feces for 24 hours. The procedure for the separation of urinary metabolites is outlined in Chart 1. The urine sample was adjusted to pH 8 with NH_4OH and extracted three times with equal volume of chloroform. Chloroform extract (fraction A) was concentrated to a small volume and spotted on Silica gel GF_{254} plates (250 μm thick), activated at 110° for 60 min. The plates were developed with the solvent systems described in the legend to Table I.

1) Location: *Kawagishi, Toda-shi, Saitama.*

2) T. Nose, M. Kojima, R. Ishida, K. Shintomi, and Y. Kowa, *Folia Pharmacol. Japan*, **67**, 387 (1971).

The aqueous solution above mentioned was acidified to pH 3 to 4 with acetic acid and then extracted with chloroform. Chloroform extract (fraction B) was concentrated to a small volume and subjected to TLC. The residual aqueous solution was passed through the column (3 × 30 cm) of Amberlite XAD-2. After washing the column, methanol was used to eluate metabolites. Methanol eluate (fraction C) was concentrated to a small volume and subjected to TLC. The effluent (fraction D) from the column was concentrated to

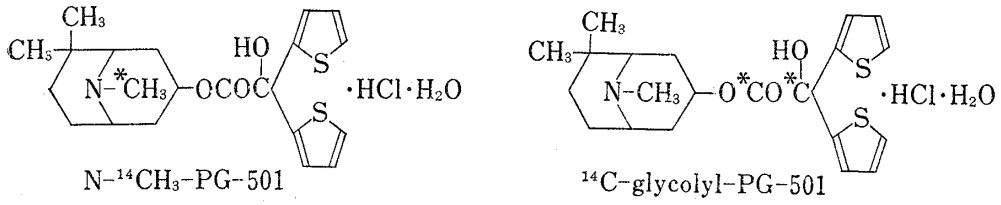

Fig. 1. Structures of N-$^{14}CH_3$-PG-501 and ^{14}C-Glycolyl-PG-501

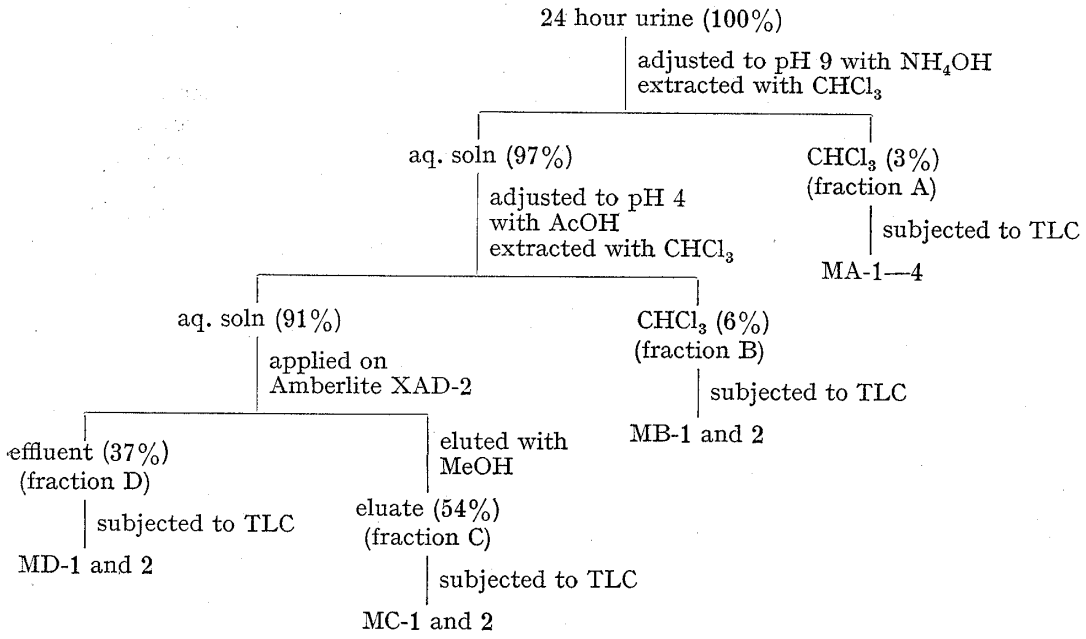

Chart 1. Major Steps used for Isolation of Metabolites of ^{14}C-Glycolyl-PG-501

Recoveries are expressed in terms of ^{14}C.

TABLE I. *Rf* Values of ^{14}C-Glycolyl-PG-501 and Its Metabolites

	Solvent I	Solvent II	Solvent III
PG-501	0.94	0.85	1.0
DTGA[a)]	0.30	0.40	0.79
Fraction A of urine[b)]	1.0 (MA-1)		
	0.94 (MA-2)		
	0.82 (MA-3)		
	0.12 (MA-4)		
Fraction B of urine		0.40 (MB-1)	0.86
		0.20 (MB-2)	0.78
Fraction C of urine		0.14	0.68 (MC-1)
		0.0	0.56 (MC-2)
Fraction D of urine		0.18 (MD-1)	
		0.11 (MD-2)	

TLC was performed on Silica gel GF_{254} with solvent systems: I, benzene–chloroform–ethyl acetate–*n*-propanol (4: 2: 4: 2); II, chloroform–methanol–NH_4OH (75: 20: 5); III, *n*-butanol–acetone–diethylamine–water (30: 30: 6: 15). PG-501 and DTGA were detected under UV light. Metabolites were detected by thin-layer chromatogram scanning or by examination of plates under UV light.

a) α,α-di(2-thienyl)glycolic acid

b) Urine sample was collected for 24 hr after oral administration of ^{14}C-glycolyl-PG-501. Separation of urine sample into fraction A, B, C and D was described under method.

about 20 ml and again passed through the column (1×20 cm) of Amberlite XAD-2 to remove most of urea. The first 30 ml of effluent was discarded and next 100 ml was collected. The effluent was concentrated to a small volume and then subjected to TLC.

Radioactive metabolites on the chromatograms were detected with an Aloka thin-layer chromatogram scanner TRM-1B. Color development of metabolites were carried out with 0.02% methyl red in ethanol, naphthoresorcinol, and $K_2Cr_2O_7$–$AgNO_3$.[3] After scanning the chromatograms, the various radioactive zones were separately scraped from the plates, and the metabolites were eluted from the silica gel with methanol. The eluates were concentrated to dryness under vacuum, and the residues were extracted with chloroform or methanol–chloroform (1 : 1). After removal of the solvent, the extracts were used for ultraviolet (UV), infrared(IR), nuclear magnetic resonance (NMR) and mass spectrometry.

Enzymatic hydrolysis of conjugated metabolites was made with use of β-glucuronidase (Sigma Chem. Co.).

Isolation of Urinary Metabolites of N-$^{14}CH_3$-PG-501—— The urine sample was collected for 24 hours after oral administration of N-$^{14}CH_3$-PG-501 diluted with nonradioactive PG-501 in a dose of 50 mg/kg to 20 rats. The major steps used for the separation of urinary metabolites are shown in Chart 2. The urine sample was adjusted to pH 9 with NH_4OH and extracted three times with chloroform. Chloroform extract (fraction E) was concentrated to a small volume and subjected to TLC with solvent systems described in the legend to to Table II. The residual aqueous solution was passed through the column (1×30 cm) of Dowex 50 (H^+ form). The effluent (fraction G) was retained for analysis of metabolites of one carbon compound formed by N-demethylation of PG-501. After washing the column with distilled water, 1N NH_4OH was used to elute metabolites. The eluate (fraction F) was concentrated to a small volume and subjected to TLC.

The effluent (fraction G) above mentioned was divided into two equal portions. To one of them was added 0.5 ml of formic acid and the mixture was distilled off to one-half of the original volume. After determining the radioactivity in the distillate, to which an equimolar amount of quinine was added and the mixture was concentrated to dryness. The residue which contained quinine formate was recrystallized from ethanol–ether, and each crystallized product was determined for the radioactivity.

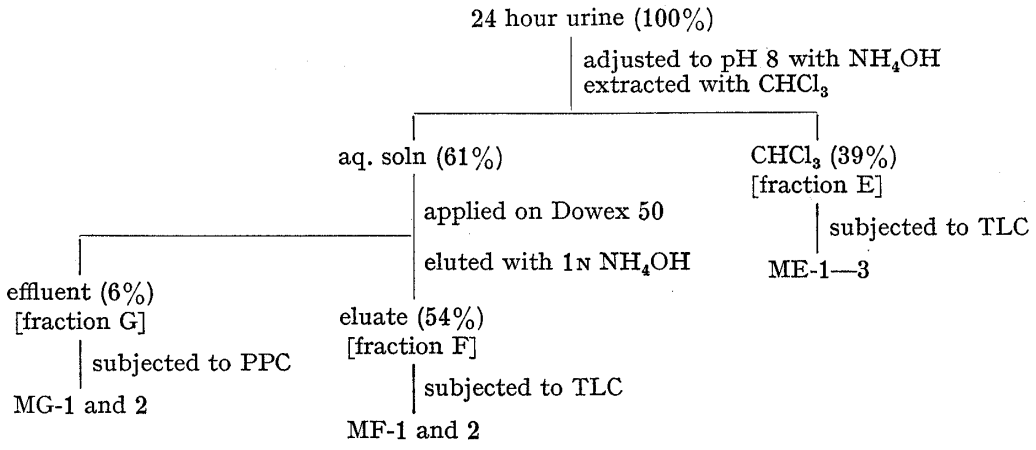

Chart 2. Major Steps used for Separation of Metabolites of N-$^{14}CH_3$-PG-501

Recoveries are expressed in terms of ^{14}C.

The remaining portion of the effluent was concentrated to a small volume under reduced pressure and spotted on Toyo Roshi No. 51 for paper chromatography (PPC). The paper were developed by the ascending technique with the solvent systems described in the legend to Table II.

Identification of Urinary Metabolites——Identification of isolated metabolites was accomplished by co-chromatography, co-crystallization with the authentic samples and by spectrometric analyses including UV, IR, NMR and mass spectrometry. UV spectra were taken of PG-501 and the isolated metabolites dissolved in methanol. The mass spectra of PG-501 and its metabolites were taken on a RMS-4 mass spectrometer (Hitachi Co.) with a direct solid inlet operating at 100—200°. NMR spectra of PG-501 and its metabolites were taken on JEOL C-60 NMR spectrometer (Japan Electron Optics Laboratory Co.) in D_2O solution, with tetramethylsilane as an internal standard.

Quantitative Determination of Urinary Metabolites——Twenty-four hour urine samples of four rats given ^{14}C-glycolyl-PG-501 (1 mg/kg) or N-$^{14}CH_3$-PG-501 (1 mg/kg) were used for quantitative determination of metabolites. Separation of metabolites was carried out according to the procedure described for the isola-

3) R.H. Knight and L. Young, *Biochem. J.*, **70**, 111 (1958).

Table II. *Rf* Values of N-$^{14}CH_3$-PG-501 and Its Metabolites

	Thin-layer chromatograms		Paper chromatograms	
	Aluminium oxide solvent IV	Cellulose solvent V	solvent VI	solvent VII
PG-501	0.96			
TMHG[a]	0.62			
DMHG[b]	0.16			
Glucuronic acid[c]		0.45	0.10	0.28
Methionine[d]		0.55	0.44	0.63
N-Formylcysteine[e]		0.30	0.29	0.30
Urea[f]		0.46	0.51	0.58
Fraction E of urine[g]	0.96 (ME-1)			
	0.62 (ME-2)			
	0.16 (ME-3)			
Fraction F of urine	0.0	0.55 (MF-1)	0.44	0.63
		0.45 (MF-2)	0.27	0.67
Fraction G of urine			0.51 (MG-1)	0.58
			0.29 (MG-2)	0.30

TLC was performed on aluminium oxide PF_{254} and MN-cellulose with solvent systems: IV, chloroform–ethanol (10: 1); V, *n*-propanol–1M acetate pH 5.0—H_2O (7: 1: 2), and PPC was done on Toyo Roshi No. 51 with solvent systems: VI, *n*-butanol–acetic acid–water (12: 3: 5); VII, *n*-butanol–ethanol–NH_4OH (1: 1: 1).

PG-501 and its related compounds were visualized by spraying with dragendorff reagent.

a) 6,6,9-trimethyl-3-hydroxygranatane
b) 6,6-dimethyl-3-hydroxygranatane
c) detected by spraying with a mixture of 2% $NaIO_4$ and 1% $KMnO_4$
d) detected by spraying with ninhydrin
e) detected by spraying with iodoplatinate
f) detected by spraying with 5% phenol and then 5% NaClO
g) Urine sample, which was collected for 24 hr after oral administration of N-$^{14}CH_3$-PG-501, was separated into fraction E, F, and G according to the procedure described under method.

tion of urinary metabolites. After scanning thin-layer or paper chromatograms, radioactive spot areas were scraped or cut out into counting vials and extracted with 1 or 2 ml methanol. Fifteen ml of solution of 7 g 2,5-diphenyloxazole and 50 mg 1,4-bis-2-(5-phenyloxazolyl)benzene in 1 liter of 50% ethanol–toluene was added and the vials were counted in an Aloka liquid scintillation spectrometer LSC-502 equipped with an automatic quenching monitor system. This procedure resulted in quantitative detection of PG-501 and all metabolites originally spotted on the plates and papers.

Result

Isolation and Identification of Metabolites of ^{14}C-Glycolyl-PG-501

As shown in Chart 1, the urine sample of rats given ^{14}C-glycolyl-PG-501 was separated into four fractions (A,B,C and D) by means of solvent extraction and column chromatography. When fraction A,B,C and D were subjected to TLC with corresponding solvent systems, four radioactive peaks (MA-1—MA-4) were observed in fraction A, two radioactive peaks (MB-1 and MB-2) were in fraction B, two radioactive peaks (MC-1 and MC-2) were in fraction C, and two radioactive peaks (MD-1 and MD-2) were in fraction D, as shown in Table I.

MA-1, MA-2, MA-3 and MA-4

Table III shows prominent peaks of UV spectra and reaction to spray reagents for PG-501 and its metabolites. All the metabolites in fraction A were negative to methyl red. This fact suggested that the metabolites in fraction A did not have any free carboxyl group in their molecules. Table IV shows mass spectral analysis of DTGA and its metabolites.

The mass spectrum of MA-1 indicated its molecular weight to be 194 which is 46 less than that of DTGA. This fact suggested that this metabolite was a decarboxylated derivative of DTGA. Theoretically, DTGA is decarboxylated to α,α-di(2-thienyl)methanol, of which

Table III. Prominent Peaks of UV Spectra and Reaction to Color Reagents for PG-501 and Its Metabolites

	UV spectra (mμ)	Color reaction of		
		Methyl red	Naphthoresorcinol	$K_2Cr_2O_7$–$AgNO_3$
PG-501	238	−	−	−
DTGA[a]	238	+	−	−
MA-1	268, 310	−	−	−
MA-2	238	−	−	−
MA-3	236	−	−	−
MA-4	256, 279	−	−	−
MB-1	238	+	−	−
MB-2	249, 269	+	−	−
MC-1	237, 300	+	+	−
MC-2	249, 300	+	+	−
MD-1	263, 285	+	−	−
MD-2	249, 300	+	−	+

a) α,α-di(2-thienyl)glycolic acid

Table IV. Mass Spectral Analyses of DTGA and Its Metabolites

	Molecular ion composition m/e	Difference from DTGA	Prominent ion common to metabolites m/e
DTGA[a]	240 ($C_{10}H_8O_3S_2$)		195, 179, 166, 111, 97, 83
MA-1	194 ($C_9H_6OS_2$)	$-CO_2$, $-H_2$	166, 111, 83
MA-3	196 ($C_9H_8OS_2$)	$-CO_2$	179, 111, 97, 83
MA-4	112 (C_5H_4OS)	-thiophene, -COOH	111, 83
MB-1	240 ($C_{10}H_8O_3S_2$)		195, 179, 166, 111, 97, 83
MB-2	128 ($C_5H_4O_2S$)	-thiophene, $-H_2$, -CO	111, 83
MC-1[b]	240 ($C_{10}H_8O_3S_2$)		195, 179, 166, 111, 97, 83
MC-2[b]	128 ($C_5H_4O_2S$)	-thiophene, $-H_2$, -CO	111, 83
MD-1	156 ($C_6H_4O_3S$)	-thiophene, $-H_2$	128, 111, 83
MD-2	356 ($C_{14}H_{13}O_4NS_3$)	$-CO_2$, $-H_2$, $+SCH_2CH(COOH)NHCOCH_3$	193, 157, 141, 117, 111, 83

a) α,α-di(2-thienyl)glycolic acid
b) MC-1 and MC-2 were treated with β-glucuronidase before mass spectra were taken.

the molecular weight is 2 greater than that of MA-1. This suggested that MA-1 was a dehydrogenated derivative of α,α-di(2-thienyl)methanol. The prominent fragment ions at 166 and 111, which are observed characteristically for MA-1 and DTGA, are formed probably by the loss of the carbonyl and the thiophene from MA-1, respectively. The probably structures of these peaks are depicted in Fig. 2. From these data, it seems that MA-1 is di(2-thienyl)-ketone. Incidentally, the UV and mass spectra of MA-1 were identical with those of the synthetic sample of di(2-thienyl)ketone,[4] which was prepared by treating with DTGA with chromic acid.

MA-2 had the same *Rf* values (in solvent systems I, II and III) as the authentic sample of PG-501. The UV and mass spectra of MA-2 were identical with those of PG-501. From these data, it could be concluded that MA-2 was intact PG-501.

The UV spectrum of MA-3 resembled those of PG-501 and DTGA. The mass spectrum of MA-3 gave molecular ion at *m/e* 196. The decrease in weight of 44 mass units of the molecular ion of MA-3 as compared with DTGA indicated that this compound was a decarboxylated

4) W. Steinkopf and H. Hempel, *Ann.*, **495**, 144 (1932).

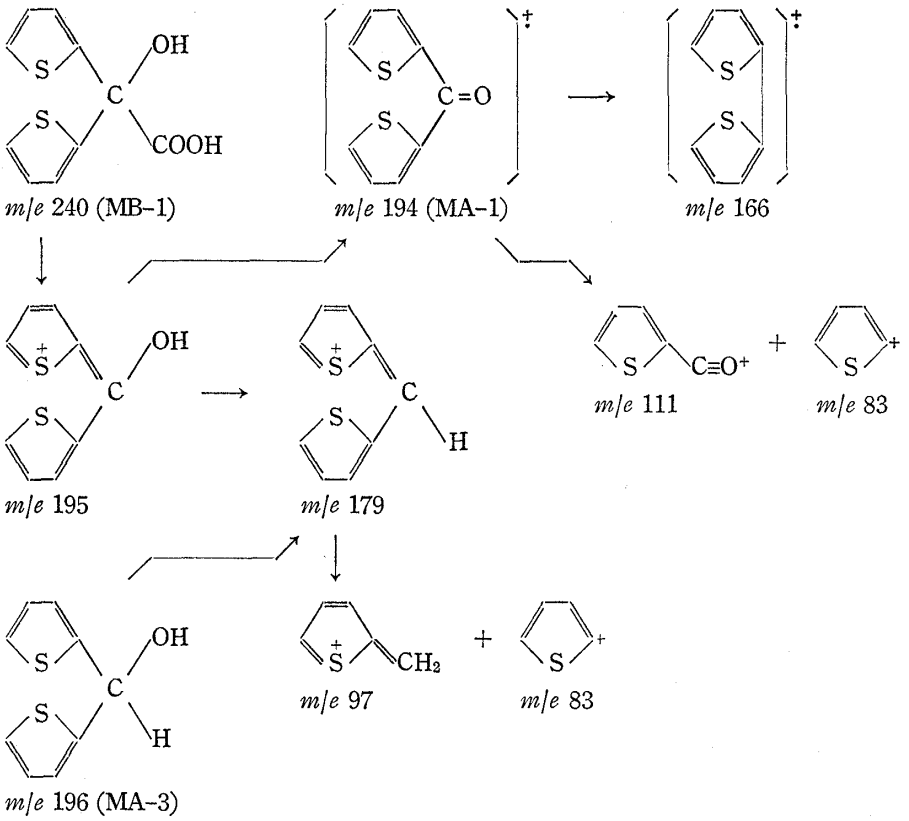

Fig. 2. Structures of Selected Fragments of MA-1, MA-3 and MB-1

derivative of DTGA. The fragment ions at *m/e* 179 and 97, which are characteristic for DTGA and MA-3, are formed probably by the loss of the hydroxyl and both hydroxyl and thiophene from MA-3, respectively, as shown in Fig. 2. From these data, it seems that MA-3 is α,α-di(2-thienyl)methanol. In fact, the UV and mass spectra of MA-3 were identical with those of the authentic sample of α,α-di(2-thienyl)methanol,[5] which was prepared by treating di(2-thienyl)ketone with $NaBH_4$.

The mass spectrum of MA-4 indicated its molecular weight to be 112 which is 128 less than that of DTGA. This fact suggested that this metabolite was formed by the loss of thiophene and carboxyl group from DTGA. The prominent fragment ions common to MA-4 and DTGA appeared at *m/e* 111 and 83, which are probably due to thiophenecarbonyl and thienyl ions, respectively. Treating MA-4 with chromic acid yielded 2-thiophenecarboxylic acid, which was identified by co-chromatography with the authentic sample. From these data, it seems likely that MA-4 is 2-thiophenealdehyde.

MB-1 and MB-2

Both MB-1 and MB-2 were positive to methyl red. This fact suggested that these metabolites had a carboxylic group in their molecules.

MB-1 had the same *Rf* values (in solvent systems I, II and III) as the authentic sample of DTGA. The UV and mass spectra of MB-1 were identical with those of DTGA. From these data, it could be concluded that MB-1 was DTGA.

MB-2 had the same *Rf* values (in solvent systems I, II and III) as the authentic sample of 2-thiophenecarboxylic acid. The UV and mass spectra of MB-2 were identical with those of 2-thiophenecarboxylic acid. From these data, it could be concluded that MB-1 was 2-thiophenecarboxylic acid.

5) T. Kralt, J.P.L. Bots, H.D. Moed, and E.J. Ariens, *Rec. Trav. Chim. Pays-Bas*, **86**, 961 (1967).

MC-1 and MC-2

Both MC-1 and MC-2 were positive to naphthoresorcinol indicative of glucuronides. When these metabolites were incubated with β-glucuronidase and then extracted with chloroform, more than 90% of the incubated radioactivity was transferred into chloroform layer. Thin-layer chromatograms of the hydrolyzed products revealed that hydrolyzed one of MC-1 had the same *Rf* values (in solvent systems I, II and III) as MB-1 and that of MC-2 had the same *Rf* values as MB-2. Furthermore, the UV and mass spectra of hydrolyzed products of MC-1 and MC-2 were identical with those of MB-1 and MB-2, respectively. From these facts, it could be concluded that MC-1 was glucuronic acid conjugate of DTGA and that MC-2 was glucuronic acid conjugate of 2-thiophenecarboxylic acid. Since MC-1 was found to be hydrolyzed by heating with 1 N NaOH, the point of attachment of glucuronic acid to DTGA was thought to be the carboxyl group.

MD-1 and MD-2

The positive reaction of MD-1 to methyl red indicated that MD-1 was acidic compound. The mass spectrum of MD-2 gave a molecular ion at 156. The decrease in weight of 84 mass units of the molecular ion of MD-1 as compared with DTGA suggested that this metabolite was formed by the loss of thiophene from DTGA. Theoretically, DTGA is dethienylated to 2-thiopheneglycolic acid, of which the molecular weight is 2 greater than that of MD-1. This suggested that MD-1 was a dehydrogenated derivative of 2-thiopheneglycolic acid. As shown in Fig. 3, the prominent ion at 128 could correspond to a loss of carbonyl from the molecular ion. Treating MD-1 with chromic acid yielded 2-thiophenecarboxylic acid. From these data, it seems that MD-1 is 2-thiopheneglyoxylic acid.

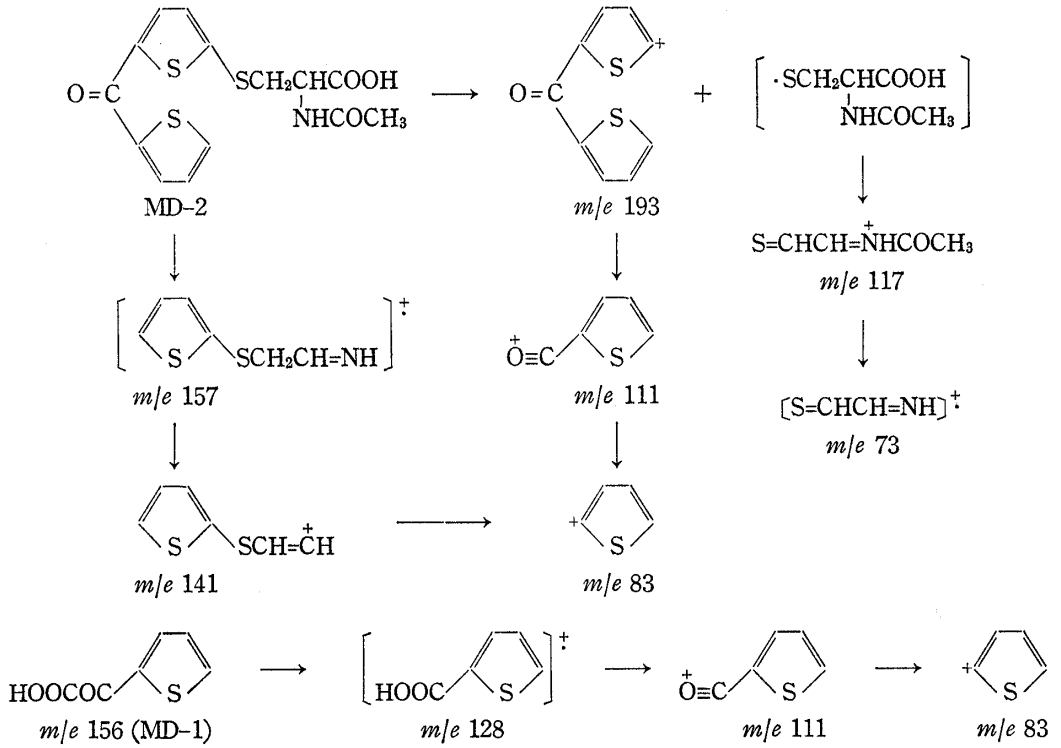

Fig. 3. Structures of Selected Fragments of MD-1 and MD-2

MD-2 responded positively to $K_2Cr_2O_7$–$AgNO_3$ indicative of bivalent sulfur-containing metabolite.[3] The mass spectrum of MD-2 did not give a molecular ion. However, prominent peaks appeared at *m/e* 193 ($C_9H_5OS_2$), 157 ($C_6H_7NS_2$), 141 ($C_6H_5S_2$), and 117 (C_4H_7ONS), the probable structures of which are depicted in Fig. 3. The fragment ion at 193,

which is formed probably by the loss of N-acetylcysteine, indicated that MD-2 was a derivative of di(2-thienyl)ketone. The fragment ion at *m/e* 117, which is formed probably by the loss of di(2-thienyl)ketone and the carboxyl group, suggested that MD-2 had N-acetylcysteine moiety in its molecule. Furthermore, the NMR spectrum of MD-2 had prominent peaks at 5.84—6.34 τ which probably belong to methine, those at 6.88—7.14 τ which probably belong to methylene, those at 8.64—8.82 τ which probably belong to methyl, and those at 2.29, 2.33 and 2.65 τ which belong to thienyl protons, as shown in Table V. This fact indicated that MD-2 was a N-acetylcysteine conjugate of di(2-thienyl)ketone. The fact that a most upfild proton of the thienyl protons is lost suggests that the point of attachment of N-acetylcysteine is 5 position of di(2-thienyl)ketone. From these data, it seems most reasonable to conclude that MD-2 is N-acetyl-S-[5-(2-thiophenecarbonyl)-2-thienyl]-L-cysteine.

TABLE V. NMR Data of MD-2

	Thiophene			Methine	Methylene	Methyl
Signal (τ)[a]	2.29,	2.33,	2.65	5.84—6.34	6.88—7.14	8.64—8.82
Number of proton	2	2	1	1	2	3

a) chemical shift in τ from tetramethylsilane, solvent D_2O

Isolation and Identification of Metabolites of N-$^{14}CH_3$-PG-501

As shown in Chart 2, urine sample of rats given N-$^{14}CH_3$-PG-501 was separated into three fractions (E, F and G). TLC of fraction E showed the presence of two radioactive spots (ME-1 and 2). One (ME-1) of them had the same *Rf* value as intact PG-501 and another(ME-2) had the same *Rf* value as TMHG. When the chromatogram of fraction E was sprayed with dragendorff reagent, two colored spots were detected. (ME-1 was too small in amount to be visualized by spraying with dragendorff reagent.) One (ME-2) of them corresponded in *Rf* value to the radioactive spot of TMHG, and another(ME-3) corresponded in *Rf* value to DMHG. The IR, NMR and mass spectra of ME-2 and ME-3 were identical with those of the authentic samples of TMHG and DMHG, respectively. From these data, it could be concluded that ME-2 was TMHG and that ME-3 was DMHG.

When fraction F was subjected to TLC in solvent V, two radioactive spots (MF-1 and 2) were detected. MF-1 responded positively to ninhydrin reagent and had the same *Rf* value as L-methionine. Co-crystallization also confirmed that MF-1 was L-methionine.

MF-2, which was purified by PPC, responded positively to naphthoresorcinol indicative of glucuronide. When MF-2 was boiled with 1 N HCl for one hour and then subjected to TLC and PPC. The chromatograms showed the presence of TMHG and glucuronic acid in the hydrolyzed products. These facts indicated that MF-2 was glucuronic acid conjugate of TMHG. However, MF-2 was scarcely hydrolyzed by treating with β-glucuronidase.

Fraction G was divided into two equal portions. When one of them was concentrated to dryness and then subjected to PPC, two radioactive spots (MG-1 and MG-2) were observed. One (MG-1) of them had the same *Rf* value as urea, and another (MG-2) had the same *Rf* value as N-formylcysteine. Co-crystallization also confirmed that MG-1 was urea and that MG-2 was N-formylcysteine. To the remaining portion of fraction G was added 0.5 ml of formic acid and then the mixture was distilled to one-half of the original volume. The distillate contained 3.5% of the radioactivity in fraction G. To the distillate was added 0.8 g of quinine and dissolved. The mixture was concentrated to dryness and the residue was recrystallized three times from ethanol–ether. Each crystallized product gave a constant specific activity. From these data it seems likely that ^{14}C-formic acid is present in fraction G.

Quantitative Determination of Urinary Metabolites of ^{14}C-Glycolyl-PG-501 and N-$^{14}CH_3$-PG-501

Table VI shows quantitative determination of urinary metabolites of ^{14}C-glycolyl-PG-501. Approximately 50% of the administered radioactivity was excreted in 24 hour urine of four

rats given ^{14}C-glycolyl-PG-501. The urinary excretion of intact PG-501 was only 0.1% of the urinary radioactivity. About 50% of the urinary radioactivity was present as glucuronic acid conjugates. Of the radioactivity excreted in the 24 hour urine of rats given ^{14}C-glycolyl-PG-501, the largest part (33%) was the glucuronide of DTGA, 21% was the glucuronide of 2-thiophenecarboxylic acid, 23% was 2-thiophenecarboxylic acid and 13% was mercapturic acid conjugate of di(2-thienyl)ketone.

Table VI. Quantitative Determination of Urinary Metabolites of Rats Given ^{14}C-Glycolyl-PG-501

	Corresponding compound	Percentages of ^{14}C in 24 hour urine
MA-1	di(2-thienyl)ketone	0.9
MA-2	PG-501	0.1
MA-3	α,α-di(2-thienyl)methanol	0.6
MA-4	2-thiophenealdehyde	1.3
MB-1	DTGA[a)]	3.9
MB-2	2-thiophenecarboxylic acid	2.2
MC-1	glucuronic acid conjugate of DTGA	32.9
MC-2	glucuronic acid conjugate of 2-thiophenecarboxylic acid	21.2
MD-1	2-thiopheneglyoxylic acid	23.4
MD-2	mercapturic acid conjugate of di(2-thienyl)ketone	12.5
	unknown[b)]	1.0

Results were expressed as percentages of the urinary radioactivity of intact PG-501 and its metabolites found in 24 hr urine of three rats given ^{14}C-glycolyl-PG-501 (1 mg/kg).
Approximately 50% of the administered radioactivity was recovered from 24 hr urine.
a) α,α-di(2-thienyl)glycolic acid
b) Fraction D contained a small amount of an unknown metabolite.

When N-$^{14}CH_3$-PG-501 was administered orally, approximately 47% of the administered radioactivity was excreted in 24 hour urine of four rats. As shown in Table VII, about 50% of the urinary radioactivity was present as glucuronic acid conjugate of TMHG, about 40% was TMHG, and the remainder was compounds derived from formaldehyde formed by N-demethylation of the drug. ^{14}C-L-Methionine, ^{14}C-N-formylcysteine and ^{14}C-urea accounted for about 3% of the urinary radioactivity, and ^{14}C-formic acid accounted for only 0.2%.

Table VII. Quantitative Determination of Radioactive Compounds in Urine of Rats Given N-$^{14}CH_3$-PG-501

	Corresponding compound	Percentages of ^{14}C in 24 hour urine
ME-1	PG-501	0.1
ME-2	TMHG[a)]	39.1
MF-1	L-methionine	2.7
MF-2	glucuronic acid conjugate of TMHG	51.1
MG-1	N-formylcysteine	2.8
MG-2	urea	2.6
	formic acid	0.2
	N,N′-diformylcystine	0.3
	unknown[b)]	1.1

Results were expressed as percentages of the urinary radioactivity of intact PG-501 and its metabolites found in 24 hr urine of three rats given N-$^{14}CH_3$-PG-501 (1 mg/kg).
Approximately 47% of the administered radioactivity was recovered from 24 hr urine.
a) 6,6,9-trimethyl-3-hydroxygranatane
b) Fraction G contained a small amount of unknown metabolites.

Discussion

The results of the metabolic study showed that very little unchanged PG-501 was excreted by rats in urine. Only less than 0.1% of a dose of PG-501 was recovered from urine unchanged. This fact suggested that PG-501 was extensively metabolized by the rat.

The fact that more than 30% of the administered radioactivity was excreted as $^{14}CO_2$ in the expired air of rats given N-$^{14}CH_3$-PG-501 indicated that PG-501 was largely N-demethylated by rats.[6] However, N-demethylated PG-501 was scarcely detected in the urine of rat given ^{14}C-glycolyl-PG-501, although the presence of DMHG, the hydrolyzed product of N-demethylated PG-501, was demonstrated. This suggested that all of N-demethylated PG-501 produced from PG-501 were hydrolyzed to DMHG and DTGA in the body of rat. In fact, all of the isolated metabolites had been hydrolyzed. Therefore, hydrolysis, the cleavage of ester linkage between TMHG and DTGA, was the main metabolic pathway.

N-Dealkylation is common metabolic reaction during drug metabolism. The excretion of $^{14}CO_2$ in the expired air of rats given N-$^{14}CH_3$-PG-501 or N-$^{14}CH_3$-TMHG showed that PG-501 was largely N-demethylated but TMHG was done very little.[6]

From these facts, it seems reasonable to conclude that a part of PG-501 is first N-demethylated and then hydrolyzed to DMHG and DTGA and almost all of the remainder are directly hydrolyzed to TMHG and DTGA.

TMHG was excreted partly free and partly conjugate with glucuronic acid.

The largest metabolite of DTGA moiety, which accounted for more than 30% of the radioactivity excreted in the 24 hour urine of rats given ^{14}C-glycolyl-PG-501, was glucuronic acid conjugate of DTGA. 2-Thiophenecarboxylic acid was excreted mainly as glucuronide, which accounted for 21% of the urinary radioactivity. The formation of 2-thiophenecarboxylic acid from DTGA indicated that the cleavage of C-C bond of DTGA occurred. At present, the pathway by which 2-thiophenecarboxylic acid is formed is uncertain. However, it may be considered that there are two pathways for the formation of 2-thiophenecarboxylic acid; one is dethienylation of DTGA to produce 2-thiopheneglycolic acid, followed by decarboxylation, and the other is first decarboxylation of DTGA to α,α-di(2-thienyl)methanol, followed by dethienylation. The results of the present study showed that a small amount of α,α-di(2-thienyl)methanol and a relatively large amount of 2-thiopheneglyoxylic acid, instead of 2-thiopheneglycolic acid, were isolated. Furthermore, the formation of 2-thiophenealdehyde suggested that 2-thiophenecarboxylic acid was formed by decarboxylation of 2-thiopheneglyoxylic acid. From these facts, it seems most likely that 2-thiophenecarboxylic acid is formed by dethienylation of DTGA, followed by decarboxylation. The fact that not 2-thiopheneglycolic acid but 2-thiopheneglyoxylic acid was isolated suggested that the dethienylation of DTGA might be oxidative reaction.

It was noted that all of 2-thiopheneglyoxylic acid were excreted as free form while most of DTGA and 2-thiophenecarboxylic acid excreted as conjugated forms.

The presence of mercapturic acid conjugate of di(2-thienyl)ketone, which accounted for more than 10% of the urinary radioactivity, was demonstrated in the urine of rats given PG-501. Bray and Carpanini[7] reported that rabbit dosed with thiophene excreted about 40% of the dose in urine as two mercapturic acids and that rabbit dosed with benzo[b]thiophene excreted about 80% of the dose in urine as mercapturic acid-like compounds. From these facts, it seems likely that thiophene and its derivatives, just like halogenated benzenes, are ready to undergo mercapturic acid conjugation.

The presence of α,α-di(2-thienyl)methanol and di(2-thienyl)ketone, which were excreted only in less than 1%, was demonstrated in the urine of rats dosed with PG-501. It is clear

6) M. Otsuka and Y. Sato, *Yakugaku Zasshi*, to be submitted. **92**, 986 (1972).
7) H.G. Bray and F.M.B. Carpanini, *Biochem. J.*, **109**, 11 (1968).

that DTGA is decarboxylated to α,α-di(2-thienyl)methanol and then oxidized to di(2-thienyl)-ketone. It is to be noted that most of di(2-thienyl)ketone is excreted as mercapturic acid conjugate while 2-thiophenecarboxylic acid is excreted mostly as glucuronic acid conjugate.

From a consideration of the facts described above, it seems most reasonable to conclude that the metabolic fate of PG-501 includes 6 different pathways of hydrolysis of ester, N-demethylation, dethienylation, decarboxylation, glucuronic acid conjugation and mercapturic acid conjugation. Therefore, ractions shown in Fig. 4 may be proposed for the metabolism of PG-501.

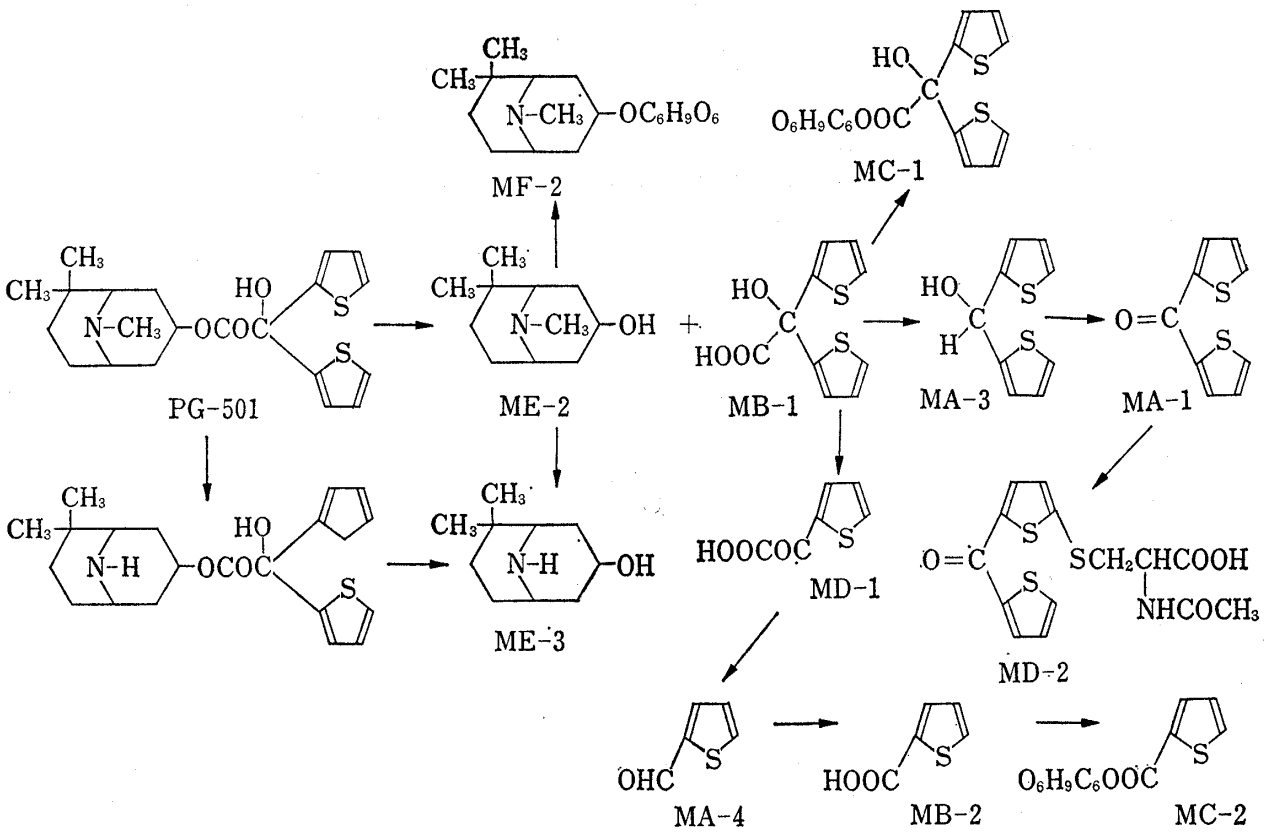

Fig. 4. Possible Metabolic Pathways of PG-501

It is well known that oxidative N-demethylation of drugs gives formaldehyde as a metabolic intermediate. Neely[8] studied the metabolic fate of ^{14}C-formaldehyde given intraperitoneally to rat and reported that 82% of the dose was excreted as $^{14}CO_2$ and 13—14% was excreted in urine as methionine, serine and an adduct formed from cysteine and formaldehyde. Edwards, *et al.*[9] reported that formaldehyde was metabolized by rats and mice to methionine, N-formylcysteine and N,N′-diformylcysteine. The results of the present study with use of N-$^{14}CH_3$-PG-501 revealed that L-methionine, N-formylcysteine, N,N′-diformylcystine, urea, and formic acid were excreted in the urine of rats as the metabolic products of formaldehyde derived from PG-501. L-Methionine, N-formylcysteine and urea accounted for about 8% of the radioactivity in the 24 hour urine of rats given N-$^{14}CH_3$-PG-501 while formic acid and N,N′-diformylcystine accounted for only 0.5%. L-Methionine must arise from a transfer of the 1-carbon compound to homocysteine by the folic acid cycle, since

8) W.B. Neely, *Biochem. Pharmacol.*, **13**, 1137 (1964).
9) K. Edwards, H. Jackson, and A.R. Jones, *Biochem. Pharmacol.*, **19**, 179 (1970).
10) A. Stevens and W. Sakami, *J. Biol. Chem.*, **234**, 2063 (1959).
11) P. Berg, *J. Biol. Chem.*, **205**, 145 (1953).

Stevens and Sakami,[10] Berg[11] and Nakao and Greenberg[12,13] have demonstrated that 1-carbon compounds including formaldehyde are converted to methionine methyl group in the presence of homocysteine and tetrahydrofolate by liver preparations. N-Formylcysteine must be formed by reaction of formaldehyde and cysteine, since Mackenzie and Harris[14] have demonstrated that liver mitochondria converts formaldehyde and L-cysteine to N-formylcysteine *via* L-thiazolidinecarboxylic acid.

Sullivan, *et al.*[15] studied oxidative N-demethylation of N-$^{14}CH_3$-N,N-dimethyl-3,5,7-trimethyladamantane-1-carboxamide and reported that 1.9% of the administered radioactivity was excreted in urine as formic acid and 1.1% was excreted as urea. In the metabolism of N-$^{14}CH_3$-PG-501 only 0.2% of the urinary radioactivity was excreted as formic acid while 2.6% was excreted as urea. It is clear that formic acid is formed by oxidation of formaldehyde. Urea must be formed from carbon dioxide by the urea cycle *via* carbamyl phosphate, since Grisolia and Cohen[16] and Jones, *et al.*[17] have demonstrated that citrulline is formed enzymatically in mammalian liver through a transfer of the carbamyl group of carbamyl phosphate to ornithine and that carbon dioxide was fixed in carbamyl phosphate. From the facts described above, the metabolic pathways of N-CH_3 group of PG-501 may be represented as shown in Fig. 5.

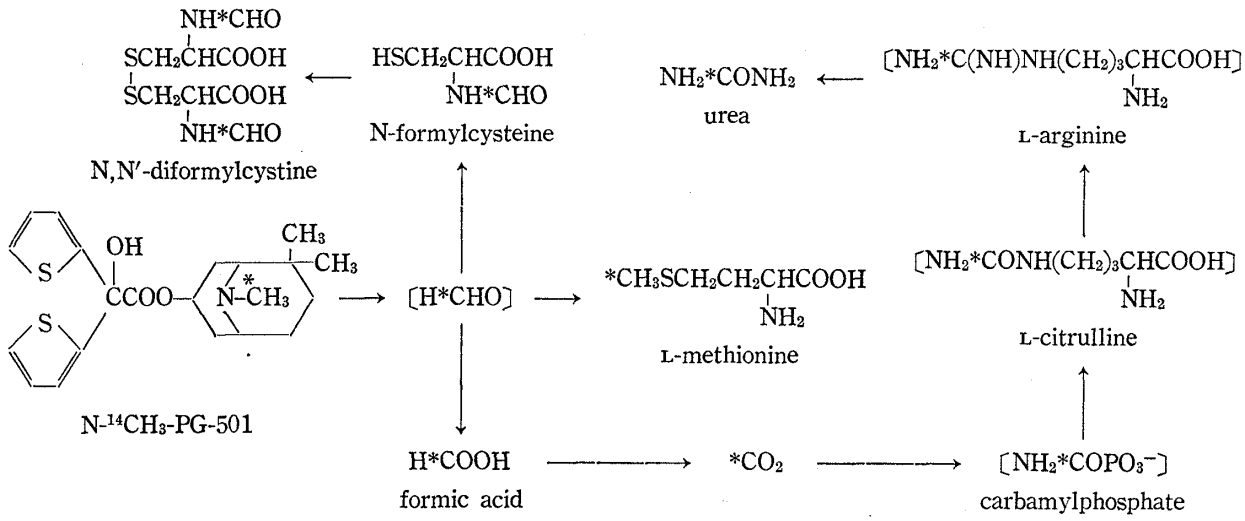

Fig. 5. Metabolic Pathways of N-$^{14}CH_3$ Group of N-$^{14}CH_3$-PG-501

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13) A. Nakao and D.M. Greenberg, *J. Biol. Chem.*, **230**, 603 (1958).
14) C.G. Mackenzie and J. Harris, *J. Biol. Chem.*, **227**, 393 (1957).
15) H.R. Sullivan, R.E. Billings, and R. E. McMahon, *J. Med. Chem.*, **11**, 250 (1968).
16) S. Grisolia and P.P. Cohen, *J. Biol. Chem.*, **198**, 561 (1952).
17) M.E. Jones, L. Spector, and F. Lipmann, *J. Am. Chem. Soc.*, **77**, 819 (1955).