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Studies on Absorption, Biotransformation and Excretion of Drug. (1). Metabolites of Clofexamide in Rat

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The metabolites of clofexamide (2-(*p*-chlorophenoxy)-N-(2-diethylaminoethyl)-acetamide) (I) have been investigated in rat urine and feces. When rats were given a single 200 mg/kg dose of clofexamide (I), thirteen metabolites were found in the urine and only unchanged clofexamide in the feces. Twelve metabolites were identified by their spectrometric data (infrared (IR), mass spectrum, ultraviolet (UV) and nuclear magnetic resonance (NMR) spectra) and by thin-layer chromatography (TLC). The following compounds were found to be present in the urine: unchanged clofexamide (I), 2-(*p*-chlorophenoxy)-N-(2-diethylaminoethyl) acetamide N-oxide (II), 2-(*p*-chlorophenoxy)-N-(2-ethylaminoethyl) acetamide (III), 2-(*p*-chlorophenoxy)-N-(2-aminoethyl) acetamide (IV), *p*-chlorophenoxyacetic acid (V), *p*-chlorophenoxyaceturic acid (VI), 2-(4-chloro-3-hydroxyphenoxy)-N-(2-diethylaminoethyl) acetamide (VII), its glucuronide (VIII), 2-(4-chloro-2-hydroxyphenoxy)-N-(2-diethylaminoethyl) acetamide (IX), 2-(4-chloro-3-hydroxyphenoxy)-N-(2-ethylaminoethyl) acetamide (X), its glucuronide (XI) and 2-(4-chloro-2-hydroxyphenoxy)-N-(2-ethylaminoethyl) acetamide (XII).

Clofexamide, (2-(*p*-chlorophenoxy)-N-(2-diethylaminoethyl) acetamide) (I), of which structure is shown in Chart 1, was first synthesized in 1960 and its action as a plant growth regulator was investigated by Thuillier and Rumpf.[2] Afterward, many investigators studied on pharmaceutical properties of this compound. Thus, in 1964, Thuillier and others reported its antidepressant activity,[3] and in 1966, the noteworthy analgetic and antiinflammatory activity of clofexamide (I) was also reported.[4] Moreover, it was found that when this compound was used with vitamine U, a remarkable antiulcer effect without anticholinergic activity was obtained.[5] This investigation showed the possibility of using this drug as antiulcer agent and in recent years, the application of its antidepressant and local anesthetic activities to inhibit the ulceration tendency of phenylbutazone was successfully attempted.[6] However, there have been no reports about the metabolism of clofexamide (I) except Thuilliers' one presuming 2-(4-chloro-2-hydroxyphenoxy)-N-(2-diethylaminoethyl) acetamide (IX) and 2-(4-chloro-2-methoxyphenoxy)-N-(2-diethylaminoethyl) acetamide as its metabolites in micro organisms.[4,7] Therefore, it was thought to be significant to investigate whether these pharmaceutical properties are due to this compound itself or due to its metabolites, and studies on the metabolism of clofexamide (I) has been carried out. Thus, present report will deal with its biotransformation.

Material and Method

p-Chlorophenoxyacetic acid (V) was obtained from a commercial source. Clofexamide (I) was synthesized according to the method described by Thuillier.[4]

1) Location: *2-12-3, Sakurashinmachi, Setagaya-ku, Tokyo.*
2) G. Thuillier and P. Rumpf, *Bull. Soc. Chim. France*, **1960**, 1786.
3) *a*) G. Par and G. Thuillier, *Arzneimittel-Forsh.*, (*Drug Res.*), **14**, 556 (1964); *b*) Von E. Kreppel, *ibid.*, **14**, 559 (1964); *c*) Von K. Karzel, *ibid.*, **14**, 561 (1964).
4) G. Thuillier, *Chim. Therap.*, **1966**, 82.
5) S.L. Grand and Cie, Fr. patent M 4526 (1964) [*C.A.*, **62**, p 3982a (1965)].
6) *a*) P.J. Ottin-Pecchio, P. Bession, J. Thuillier, Thuong Cong-Trieu and A. Lemonnier, *Chim. Therap.*, 3, 46 (1968); *b*) H. Onodera, unpublished work in this laboratories.
7) W.C. Evans and B.S.W. Smith, *Biochem. J.*, **57**, xxx (1954).

Synthesis of the Proposed Metabolites——1) 2-(*p*-Chlorophenoxy)-N-(2-diethylaminoethyl) acetamide N-Oxide (II): Three ml of 10% hydrogen peroxide was slowly added to a solution of clofexamide (I) (284 mg, 1 mmole) in ethanol (4 to 5 ml) and stirred at room temperature for 24 hr. Then 0.8 ml of 10% hydrogen peroxide was added and the solution was stirred for another 24 hr at room temperature. The solution was evaporated under reduced pressure below 20°. The residue solidified immediately to give white crystals which were dissolved in ethanol (1 ml) and recrystallized by adding ether (9 ml) to afford 290 mg of white needles, mp 110—112°. IR ν_{max}^{KBr} cm^{-1}: 1680 (-CO-NH-), 1490 (phenyl), 955 (-NO-$(CH_2$-$CH_3)_2$). NMR ($CDCl_3$)[8] ppm: 1.03 (6H, triplet, $J=4.0$ Hz, -CH_2-CH_3), 3.25 (6H, quartet, $J=4.0$ Hz, -CH_2-NO-$(CH_2$-$CH_3)_2$), 3.80 (2H, broad triplet, $J=4.0$ Hz, -NH-CH_2-), 4.17 (1H, broad singlet, -NH-), 4.50 (2H, singlet, -O-CH_2-CO-), 7.10 (4H, $A_2'B'_2$ pattern, aromatic protons). UV λ_{max}^{EtOH} mμ: 226, 285. *Anal.* Calcd. for $C_{14}H_{21}O_3N_2Cl$: C, 55.90; H, 7.03; N, 9.03. Found: C, 55.52; H, 7.25; N, 8.58.

2) 2-(*p*-Chlorophenoxy)-N-(2-ethylaminoethyl) acetamide (III): Method 1: A solution of *p*-chlorophenoxyacetyl chloride (410 mg, 2 mmole) in dry chloroform (5 ml) was added dropwise to a vigorously stirred solution of N-ethylethylenediamine (350 mg, *ca.* 4 mmole), which was synthesized by the method of Woodburn and Russel, in dry chloroform (10 ml) at 5° or below.[9] The mixture was stirred for 48 hr at room temperature and extracted with acidic aqueous solution. After alkalization with 2N sodium hydroxide solution, the aqueous phase was reextracted with chloroform and dried over sodium sulfate. The organic solvent was evaporated under reduced pressure to give 100 mg of 2-(*p*-chlorophenoxy)-N-(2-ethylaminoethyl) acetamide (III).

Method 2: An aqueous solution (30 ml) of tartaric acid (21 g, 100 mmole) was added to 2-(*p*-chlorophenoxy)-N-(2-diethylaminoethyl) acetamide N-oxide (II) (1 g, *ca.* 3 mmole) followed by addition of aqueous solution (10 ml) of ferric nitrate (4.04 g, 10 mmole). This mixture was adjusted to pH 6 with a saturated sodium carbonate solution and heated at 80—85° for 40 min. After cooling, the mixture was alkalized with sodium hydroxide solution and extracted with chloroform. The organic solvent was evaporated under reduced pressure to give brown residue which was developed with solvent system 1 (benzene–AcOEt–MeOH–conc. NH_3 (80: 20: 20: 2)) on thin-layer chromatography (TLC) to give two spots. The lower spot was found to be the desired secondary amine, 2-(*p*-chlorophenoxy)-N-(2-ethylaminoethyl) acetamide (III) and the upper was clofexamide (I) which was produced by reduction of N-oxide (II). The isolation of secondary amine (III) from the mixture was carried out by using TLC (solvent system 1). The obtained secondary amine (III) (300 mg) did not crystallize. IR $\nu_{max}^{Liquid\ film}$ cm^{-1}: 1670 (-CO-NH-), 1600 (phenyl), 1480 (phenyl). NMR ($CDCl_3$) ppm: 1.09 (3H, triplet, $J=6.0$ Hz, -CH_2-CH_3), 1.94 (2H, singlet, -NH-), 2.47—2.88 (4H, multiplet, -CH_2-NH-CH_2CH_3), 3.36 (2H, triplet, $J=6.0$ Hz, -NH-CH_2-), 4.44 (2H, singlet, -O-CH_2-), 7.02 (4H, $A_2'B'_2$ pattern, aromatic protons). UV λ_{max}^{EtOH} mμ: 226, 285. *Anal.* Calcd. for $C_{12}H_{17}O_2N_2Cl$: C, 56.14; H, 6.62; N, 10.92. Found: C, 55.71; H, 6.64; N, 10.51.

3) 2-(*p*-Chlorophenoxy)-N-(2-aminoethyl) acetamide (IV): A solution of *p*-chlorophenoxyacetyl chloride (410 mg, 2 mmole) in dry chloroform (20 ml) was added dropwise to a vigorously stirred solution of excess ethylenediamine (*ca.* 10 ml) in dry chloroform (20 ml) at 5° or below. The solution was stirred for 24 hr at room temperature and extracted with hydrochloric acid. After alkalization with 2N aqueous sodium hydroxide, the solution was reextracted with chloroform, which was washed with water and dried over sodium sulfate. Evaporation of the solvent under reduced pressure afforded 230 mg of white crystals. To the ether solution of this primary amine (IV) was added a saturated ethereal solution of picric acid to give immediately yellow crystals which were filtered and recrystallized from methanol, mp 208—209°. IR ν_{max}^{KBr} cm^{-1}: 1680 (-CO-NH-), 1610 (phenyl), 1500 (phenyl). UV λ_{max}^{EtOH} mμ: 225, 355. *Anal.* Calcd. for $C_{16}H_{16}O_9N_5Cl$: C, 42.20; H, 3.50; N, 15.30. Found: C, 42.52; H, 3.88; N, 14.99.

4) 2-(*p*-Hydroxyphenoxy)-N-(2-diethylaminoethyl) acetamide (XIV): This compound was synthesized from *p*-hydroxyphenoxyacetic acid and N,N-diethylethylenediamine by the procedure of Thuillier.[4]

5) 2-(4-Chloro-2-hydroxyphenoxy)-N-(2-diethylaminoethyl) acetamide (IX): This compound was synthesized from 4-chloro-2-hydroxyphenoxyacetic acid and N,N-diethylethylenediamine according to the method of Thuillier.[4]

6) 2-(*p*-Chlorophenoxy)-N-(2-hydroxyethyl) acetamide (XIV): This alcohol was synthesized from *p*-chlorophenoxyacetyl chloride and ethanolamine in dry benzene by the procedure of Newman, *et al.*[10]

7) *p*-Chlorophenoxyaceturic Acid (VI): Finely powdered alcohol XIII (500 mg, 3 mmole) was added to a vigorously stirred aqueous solution (10 ml) of potassium permanganate (500 mg, 3 mmole) and potassium hydroxide (57 mg, *ca.* 1 mmole) with ice cooling. The suspension was stirred for 45 min at room temperature and extracted twice with ether. After acidification of the aqueous fraction with hydrochloric acid, it was extracted with ether and the organic solvent was evaporated under reduced pressure to afford 300 mg (1.2 mmole) of white crystals which were recrystallized from ethanol, mp 138°. IR ν_{max}^{KBr} cm^{-1}:

8) NMR spectra were taken in $CDCl_3$ (internal standard TMS), CD_3COCD_3 (TMS) or D_2O(D_2O) using HITACHI-6013 spectrometer.
9) H.M. Woodburn and C.O'Gee. Russel, *J. Am. Chem. Soc.*, **73**, 1370 (1951).
10) S.M. Newman, W. Rones and M. Renoll, *J. Am. Chem. Soc.*, **69**, 718 (1947).

3500 (-NH-), 1720 (-CO_2H), 1680 (-CO-NH-), 1500 (phenyl). NMR (Na_2CO_3 in D_2O) ppm: 3.71 (2H, singlet, -CH_2-CO_2H), 4.33 (2H, singlet, -O-CH_2-), 6.50 (4H, $A_2'B_2'$ pattern, aromatic protons). *Anal.* Calcd. for $C_{10}H_{10}O_4NCl$: C, 49.28; H, 4.11; N, 5.75. Found: C, 49.00; H, 3.75; N, 5.26.

Administration of the Compounds——Clofexamide (I) and its metabolites, II, III, IV, V, VI and IX were administered orally at a dose level of 200 mg/kg to male Wistar rats (Fuji Animal Farm) weighing 300—400 g. The rats were housed in separate metabolism cages designed to permit the collection of urine separately from feces. The urine was collected for 24 hr under toluen and stored in a freezer until used. The feces were also collected for 24 hr and treated in the same way as urine.

Extraction of Metabolites——The urine, which was collected for 24 hr, was adjusted to pH 8 with 2N sodium hydroxide and extracted with chloroform. After each extraction, the aqueous fraction was tested to make sure that the solution remained basic. The chloroform extract, which contained basic and neutral compounds, was dried over sodium sulfate and evaporated under reduced pressure below 40° to give a brown residue which was dissolved in a small portion of ethanol. The aqueous solution was then adjusted to pH 2 with concentrated hydrochloric acid and was extracted with ethyl acetate. In order to separate phenolic compounds from more acidic ones, the latter was extracted with saturated sodium bicarbonate solution. The phenolic compounds remained in the ethyl acetate layer which was evaporated under recuced pressure below 40° to afford a brown residue. This was dissolved in a small portion of ethanol. The bicarbonate fraction was acidified with concentrated hydrochloric acid and was extracted with ethyl acetate. After being dried over sodium sulfate, the organic fraction was evaporated under reduced pressure below 40° to give a similar brown residue which was also dissolved in a small portion of ethanol. The extraction procedure of feces was almost the same as described above except that shown below: 1) The feces were suspended in distilled water in the first step. 2) Extraction with ethyl acetate caused an emulsion and so the emulsion was centrifuged at 15000 rpm for 2 min.

Isolation Procedures of Glucuronides——From the quantitative analysis of glucuronic acid in the rat urine by Dische's method it was ascertained that the metabolites were conjugated with glucuronic acid.[11] Therefore, the glucuronides were isolated as follows. The urine collected for 24 hr was adjusted to pH 4.0 with acetic acid and the saturated normal lead acetate solution was added until precipitation was completed. The precipitates were collected by centrifugation and discarded. The supernatant was brought to pH 8.0 with ammonium hydroxide, and excess of saturated basic lead acetate (lead subacetate) solution was added. The precipitates were collected by centrifugation, suspended in water, and treated with hydrogen sulfide. The mixture was filtered and the filtrate was evaporated to dryness under reduced pressure at 40°.[12] The gummy residue (*ca.* 330 mg) was treated with β-glucuronidase (3000 units) in 0.1M acetate buffer, pH 4.6, at 38° for 24 hr.[13] The solution which contained free aglycones was treated as described above.

Isolation Procedures of Metabolites——The extracted basic compounds were separated into each component by TLC on a fluorescent silica gel (Wakogel B-5F). The solvent systems adopted were as follows: 1) Benzene–EtOAc–MeOH–conc. NH_3 (80: 20: 20: 2), 2) EtOAc–MeOH–conc. NH_3 (90: 10: 3), 3) EtOAc–MeOH–conc. NH_3 (90: 10: 1), 4) iso-PrOH–conc. NH_3 (100: 1), 5) $CHCl_3$–MeOH–conc. NH_3 (90: 2: 1). Detection of the basic metabolites was accomplished by spraying the plates with a modified Dragendorff

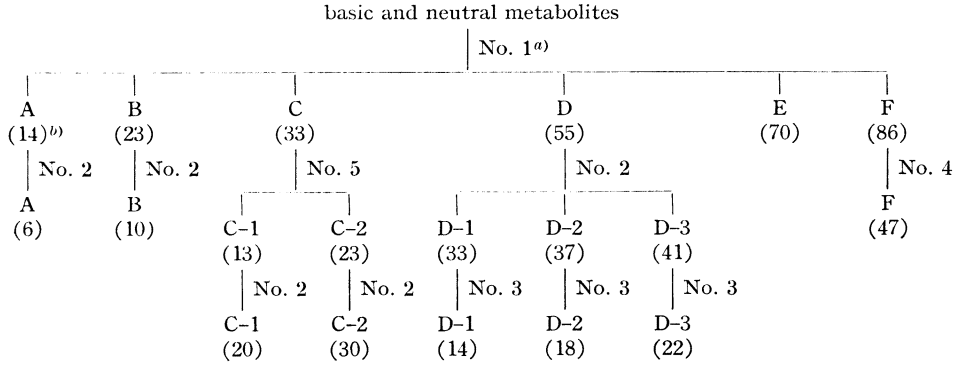

Fig. 1. Separation of Basic Metabolites on TLC

a) Solvent systems used for TLC are designated by number (see methods).
b) *Rf* ×100 values are shown in parentheses.

11) Z. Dische, *J. Biol. Chem.*, **167**, 189 (1947).
12) I.A. Kamil, J.N. Smith and R.T. Williams, *Biochem. J.*, **50**, 235 (1951).
13) P. Talalay, W.H. Fishman and C. Huggins, *J. Biol. Chem.*, **163**, 757 (1951).

reagent which was prepared according to Schwartz, *et al.*, or by spraying with ninhydrin.[14] For isolation purposes, greater aliquots were streaked across 20×20 cm chromatoplates, and after development with the solvent system 1 a narrow vertical channel on each plate was sprayed with above colouring reagents. The bands of metabolites, which also could be remarked under short-wave ultraviolet (UV) light, were scraped off and eluted with methanol (10 ml). The extracts were concentrated under reduced pressure. The metabolites in each concentrate were further separated and purified by this preparative TLC using different solvent systems. The number of solvent systems (1 to 5) used for isolation of each metabolite and their migration ($Rf \times 100$ values) are shown in Fig. 1.

The extracted acidic compounds were also separated and isolated by TLC using solvent system 6 and purified with solvent system 7 in the same way as the basic ones. The solvent systems 6 and 7 were as follows: 6) Benzene–dioxane–AcOH (200: 30: 5), 7) Benzene–MeOH–AcOH (100: 10: 5). The detection of acidic metabolites was carried out as follows: after the TLC plates were dried completely to remove acetic acid, the spots were observed under short-wave UV light or by spraying with 2% ethanolic solution of bromocresol green. When this reagent was used, yellow spots slowly became visible against a background which changed from white to blue. In the case of *p*-chlorophenoxyacetic acid (V), chromotropic acid was also used as the detecting reagent. Thus the TLC plate was dried completely and then chromotropic acid in concentrated sulphuric acid (0.5% w/v) was sprayed on the plate followed by heating at 130° to give a purple colour.

The neutral and weak acidic compounds were detected under UV light through comparison with the control sample which obtained from non-drug-treated rat urine by the same as described above.

Quantitative Analysis——After TLC separation, the main basic compounds (I, III, IV, and IX) in 24 hr urine were measured colorimetrically according to the procedure of Axelrod.[15] The amount of the acid V was also determined by the method described by Dziewiatkowski and Lewis.[16]

Result

Chromatography and Colour Reactions of Metabolites

When the urine extract containing basic and neutral compounds was chromatographed in solvent system 1, six Dragendorff-positive spots were observed, and these were designated as A, B, C, D, E and F from the lower *Rf* value to the higher one, respectively. Spots C and D were separated into several Dragendorff-positive components on the second TLC (Fig. 1). Spot C yielded C-1 and C-2 in solvent system 5. Spot D yielded D-1, D-2 and D-3 in solvent system 2. Fig. 1 shows the solvent systems used for separation and purification, and the $Rf \times 100$ values are shown in parentheses. The results of colour reactions are shown in Table I. TLC of acidic extract in solvent system 6 gave two spots, G and H, of which $Rf \times 100$ values were 15 and 40, respectively. These metabolites were purified by using the solvent system 7 ($Rf \times 100$ values: 40 and 50, respectively). With respect to the extracts of feces, neutral and acidic metabolites were not recognized. But only one basic metabolite was found and its *Rf* values in above solvent systems were identical with clofexamide (I).

Identification of Non-Conjugated-Metabolites of Clofexamide (I)

Structure of F——Metabolite F was found to be unchanged clofexamide (I), this being confirmed by mp and mixed mp and infrared (IR), UV and nrclear magnetic resonance (NMR) data.

Structure of D-3——The UV spectrum of D-3 in ethanol showed absorption maxima at 226 and 285 mμ, and it was similar to that of clofexamide (I) (maxima at 226 and 285 mμ). The NMR spectrum ($CDCl_3$) of D-3 showed a well defined $A_2'B_2'$ aromatic proton splitting pattern at the same ppm as those of clofexamide (I). These data and the results of colour reactions (Table I) suggest that the aromatic ring remains intact. The difference between metabolite D-3 and clofexamide (I) in the NMR spectra was as follows: the spectrum of metabolite D-3 showed the presence of three methyl protons (1.09 ppm, triplet, J=6.0 Hz) and four methylene protons (2.47—2.88 ppm, multiplet) assigned to the group $-CH_2-NH-CH_2-$, while that of clofexamide (I) showed the presence of six methyl protons (0.98 ppm, triplet,

14) M.A. Schwartz, F.M. Vane and A. Postma, *J. Med. Chem.*, **11**, 770 (1968).
15) J. Axelrod, *J. Pharmacol. Exptl. Therap.*, **109**, 62 (1953).
16) D.D. Dziewiatkowski and H.B. Lewis, *J. Biol. Chem.*, **158**, 877 (1945).

J=6.0 Hz) and six methylene protons (2.49—2.89 ppm, multiplet) assigned to the group -CH_2-NH-(CH_2-CH_3)$_2$. From these facts, the structure of D-3 must be monodeethylated derivative of clofexamide (I) and this assumption was confirmed by a comparison of the spectral (IR, UV and NMR) data of D-3 with those of an authentic sample III.

Structure of D-2——The UV spectrum of metabolite D-2 in ethanol showed absorption maximum at 286 mμ which shifted to 300 mμ with alkalization. The NMR spectrum of D-2 ($CDCl_3$) was almost identical with that of clofexamide (I) except that it showed three aromatic protons (6.90—6.60 ppm) and one new deuterizable proton (5.91 ppm) in stead of $A_2'B_2'$ splitting pattern of clofexamide (I). The high resolution mass spectrum of D-2 showed a base peak at 86.0973 corresponding to CH_2-N-(CH_2-CH_3)$_2$ and a molecular peak at 300.1140, corresponding to $C_{14}H_{21}O_3N_2Cl$ which differed from that of clofexamide (I) by the addition of 16 mass units. These facts and the results of colour reactions (Table I) suggest the side chain remains intact but one of the aromatic protons of clofexamide (I) must be exchanged by one hydroxy group in the structure of D-2. However, these data did not supply any information about the location of the hydroxy group present in the aromatic ring of D-2. The location was successfully determined by comparison of spectral and chemical properties of metabolite D-2 with those of synthesized amine (IX) which contains hydroxy group at *ortho*-position.

Structure of D-1——The IR spectrum of D-1 showed the presence of hydroxy (3500 cm^{-1}), amide (1660 cm^{-1}) and phenyl (1600 and 1505 cm^{-1}) groups, and its UV spectrum showed maximum at 284 mμ which shifted to 300 mμ with alkalization. A large difference between D-1 and clofexamide (I) in NMR spectra (CD_3COCD_3) was seen in the aromatic region and a typical AMX splitting pattern (7.16 ppm, 1H, doublet, J_{AB}=8.0 Hz; 6.55 ppm, 1H, doublet, J_{BC}=3.0 Hz; 6.38 ppm, 1H, quartet, J_{AB}=8.0 Hz, J_{BC}=3.0 Hz) appeared in D-1 in stead of $A_2'B_2'$ splitting pattern of clofexamide (I). The mass spectrum of D-1 showed a base peak at 86.1008, corresponding to CH_2-N-(CH_2-CH_3)$_2$ and a molecular peak at 300.1203, corresponding to $C_{14}H_{21}O_3N_2Cl$, which differed from that of clofexamide (I) by the addition of 16 mass units. From these data, it can be assumed that hydroxylation has taken place on the aromatic ring of clofexamide (I) to yield metabolite D-1. These data did not give any evidence for the location of hydroxy group in the aromatic ring. However, the structure of metabolite D-2 was determined as 2-(4-chloro-2-hydroxyphenoxy)-N-(2-diethylaminoethyl) acetamide (IX), and so only one possible hydroxylated position in aromatic ring remained if the NIH shift did not occur in D-1 and this was ascertained by the following reasons. 1) D-1 was positive to Gibb's reagent. 2) If D-1 had *para*-hydroxy group, the UV maximum absorption at 284 mμ must appear at 295 mμ as was seen in the 2-(*p*-hydroxyphenoxy)-N-(2-diethylaminoethyl) acetamide (XIV). Therefore, it was concluded that metabolite D-1 was 2-(4-chloro-3-hydroxyphenoxy)-N-(2-diethylaminoethyl) acetamide (VII).

Structure of C-2——The UV absorption spectrum of C-2 in ethanol showed maxima at 226 and 285 mμ and was similar to that of clofexamide (I). The signals of N-ethyl group of clofexamide (I) were disappeared in the NMR spectrum of metabolite C-2 (CD_3COCD_3), and in its stead, a new deuterizable signal corresponding to primary amino protons appeared at 5.90 ppm. Together with the above data, the results of colour reactions (Table I) showed that C-2 was a primary amine containing an intact aromatic ring. Metabolite C-2 was crystallized as a salt of picric acid and compared with picrate of synthesized primary amine (IV). Their spectral data were identical in all respects and had mp 208—209°, undepressed on admixture.

Structure of C-1——Since the excretion of metabolite C-1 was minor, it was impossible to isolate in sufficient quantity for IR or NMR spectra. However, from the results of colour reactions (Table I), it was assumed that the aromatic ring of C-1 was intact and its amino group was also intact or oxidated to N-oxide. The definitive structure of C-1 was established to be N-oxide (II) by comparison with the *Rf* values of TLC (Fig. 1).

Structure of B——The UV spectrum of B in ethanol showed absorption maximum at 286 mμ which shifted to 300 mμ with alkalization and it was similar to that of 2-(4-chloro-2-hydroxyphenoxy)-N-(2-diethylaminoethyl) acetamide (IX). IR spectrum of B ($CHCl_3$) showed hydroxy (3500 cm^{-1}), amide (1660 cm^{-1}) and phenyl (1600 and 1505 cm^{-1}) groups, and its mass spectrum indicated a molecular peak at 272.0870 (corresponding to $C_{12}H_{17}O_3N_2Cl$) which differed from that of clofexamide (I) by $-C_2H_4+O$. From these data and its chemical properties (Table I), it was assumed that monodeethylation and hydroxylation on the aromatic ring of clofexamide (I) occurred to give metabolite B. As for the location of hydroxy group, the UV absorption spectrum gave a good evidence of *ortho*-hydroxylation but it was not definitive. Finally, the location of hydroxy group was decided by the fact that the administration of IX to rat gave two metabolites, one was an unchanged acetamide (IX) and another was a metabolite which had same physical and chemical properties as metabolite B. Therefore, it was decided that metabolite B was 2-(4-chloro-2-hydroxyphenoxy)-N-(2-ethylaminoethyl) acetamide (XII).

Structure of A——The UV spectrum of A in ethanol gave an evidence for the presence of phenolic hydroxy group: it was almost similar to that of metabolite D-1 and showed maximum at 284 mμ which shifted to 300 mμ with alkalization. Mass spectrum of A showed a molecular peak at 272.0866, corresponding to $C_{12}H_{17}O_3N_2Cl$, which differed from that of clofexamide (I) by $-C_2H_4+O$. These data and the results of colour reactions (Table I) indicated that both monodeethylation and hydroxylation occured in clofexamide (I) to give metabolite A. The location of hydroxy group was assumed as *meta*-position from the same reasons in the case of metabolite D-1. Therefore, it was concluded that metabolite A was 2-(4-chloro-3-hydroxyphenoxy)-N-(2-ethylaminoethyl) acetamide (X).

Structure of H——This metabolite was isolated by TLC and crystallized as a salt of S-benzyl thiuronium hydrochloride by the procedure of Dziewiatkowski and Lewis.[14] Its physical data were all identical with the salt of authentic sample (V) and had mp at 194—195°, undepressed on admixture.

Structure of G——This metabolite was a minor one and could not be isolated in sufficient quantities to take physical data, but *Rf* values of authentic *p*-chlorophenoxyaceturic acid (VI) were identical with those of metabolite G in various TLC solvents. Therefore, it was assumed that metabolite G was *p*-chlorophenoxyaceturic acid (VI).

The presence of metabolites which was yielded by the NIH shift was not recognized.

Table I. Colour Reactions of Metabolites

Colour reagents	Metabolites								
	A	B	C-1	C-2	D-1	D-2	D-3	E	F
1. Dragendorff reagent	orange	orange	orange	black	orange	orange	orange	orange	orange
2. ninhydrine reagent	purple	purple	—	purple	—	—	purple	—	—
3. Simmon's reagent	blue	blue	—	—	—	—	blue	—	—
4. iodoplatinate reagent	black	black	black	black	black	black	black	black	black
5. Gibb's reagent	blue	blue	—	—	blue	blue	—	—	—
6. potassium ferricyanite ferric chloride reagent	blue	blue	—	—	blue	blue	—	blue	—

Investigation of Glucuronides

The 24 hr urine samples of five male Wistar rats (body weight: *ca.* 200 g, 8-week-old) which had received 200 mg/kg of clofexamide (I) were examined for total glucuronic acid by the method of Dische.[11] The excretion of glucuronic acid by these rats which averaged *ca.* 4 mg/day was increased to an average of 8 mg/day following administration of clofexamide (I). These results suggested that some metabolites of clofexamide (I) was excreted in urine

as glucuronides. Here, glucuronides were isolated as a mixture of lead salts by the procedure of Kamil, *et al.* and hydrolysed with β-glucuronidase.[12,13] The aglycones were extracted and separated with TLC in the same way as described above. The acidic and neutral metabolites could not be recognized, but two basic metabolites were found as aglycones. Their identification was accomplished by comparison of UV spectra, colour reactions and *Rf* values with those of identified metabolites. One of the two basic metabolites migrated as metabolite D-1 in various solvent systems, and its colour reactions and UV spectra were also identical with those of metabolite D-1 (VII). Another basic aglycone was identified as 2-(4-chloro-3-hydroxyphenoxy)-N-(2-ethylaminoethyl) acetamide (X) from UV spectra, colour reactions and *Rf* values.

Structure of Metabolite in Feces

Only one basic metabolite was found in rat feces and it was determined as unchanged clofexamide (I) through comparison with authentic sample in TLC.

Investigation of Metabolic Pathway

In order to confirm the metabolic pathway of clofexamide (I), the synthetic samples were administered to rat and their metabolic fate was investigated by using TLC and colour reactions. The administration of II to rat resulted in excretion of all the metabolites that were recognized as those of clofexamide (I). In case of III, secondary amines (X, XI and XII), primary amine (IV) and acidic metabolites (V and VI) were found. The administration of primary amine (IV) gave unchanged primary amine (IV) and acidic metabolites (V and VI). When IX was administered, two amines were found to be present in urine. They were unchanged tertiary amine (IX) and its monodeethylated amine (XII). After the administration of VI to rat, there were two acidic metabolites, unchanged acid (VI) and V in urine. When acid V was administered, glycine conjugated metabolite VI was not recognized in urine.

Amounts of Main Metabolites in Urine

In 24 hr, approximately 85% of the dosed clofexamide (I) was excreted in urine as metabolites (III=20%, IV=7%, IX=7% and V=15%) and unchanged clofexamide (I) (36%).

Discussion

From the above results, it becomes evident that the following biotransformations occurred in the metabolism of clofexamide (I): N-deethylation, oxidative deamination, hydroxylation of aromatic ring, oxidation of amino nitrogen, hydrolysis of the amide group and conjugation of phenolic hydroxy group with glucuronic acid.

On the basis of the results obtained from the administration of metabolites, the possible pathway for the metabolism of clofexamide (I) in rat may be represented in Chart 1.

However, the possibility that N-oxide may rearrange to unstable carbinolamine intermediate which in turn could form N-dealkylated metabolites can not be omitted by the present data. The possibility that the carboxylic acid V (of which formation from the acid VI was confirmed in this investigation) could also produce from amines (I, III and IV) was suggested from the amount of acids V (15%)and VI (minor). If the acid V was only produced from VI, a more amount of latter compound must be excreted in urine, since the administration of VI to rat gave a large amount of unchanged VI. The result that the acid V was not conjugated with glycine to form VI was compatible with the report of Leavey and Lewis who investigated the metabolism of acid V in rabbit.[17]

The conjugation of hydroxy group with glucuronic acid was observed in the present study. Although the metabolites D-2 and B which contained *ortho*-hydroxy group were ex-

17) S. Leavey and H.B. Lewis, *J. Biol. Chem.*, **168**, 213 (1947).

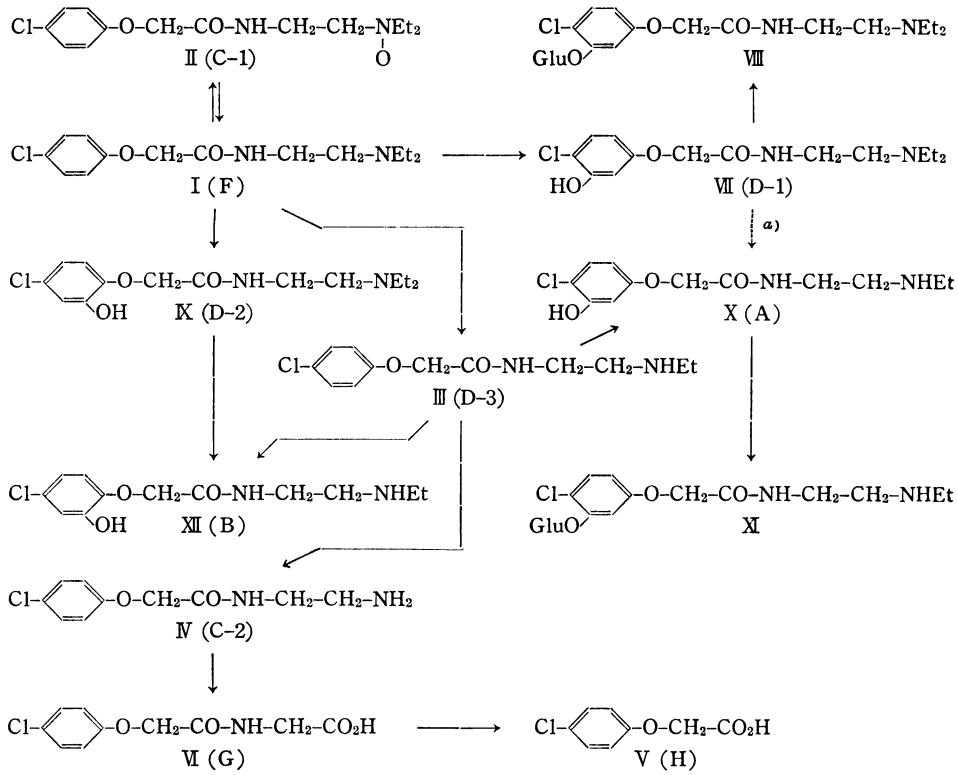

Chart 1. Proposed Metabolic Pathway of Clofexamide (I) in Rat

a) Broken line shows that it was not confirmed by experiment

creted as unconjugated form, the *meta*-hydroxylated metabolites D-1 and A were conjugated with glucuronic acid to give VIII and XI. Such selective conjugation was seen in the metabolites of gentisic acid,[18] 1-phenylazo-2-naphthol and others.[19] But, in these metabolites, the hydroxy group makes intramolecular hydrogen bond with carbonyl or azo group and it inhibits conjugation. Although such a hydrogen bond is not present in the metabolites of clofexamide (I), the *meta*-hydroxylated metabolites D-1 and A were specifically conjugated with glucuronic acid. Even if the hydroxy group of metabolites D-2 and B was conjugated with glucuronic acid, there was no large steric hindrance between the glucuronic acid and side chain, as evidenced by stereochemical studies. However, when larger molecule than glucuronic acid attached to the hydroxy group, the marked steric hindrance is occurred in *ortho*-hydroxylated ones (D-2 and B). Thus the approach of glucuronyl transferase to hydroxy group is difficult in case of metabolites D-2 and B due to the bulkiness of this enzyme, but it is rather easier in case of *meta*-hydroxylated metabolites D-1 and A. The fact that such a sterically hindered *ortho*-position can be hydroxylated by the drug metabolizing enzyme seems to be incompatible with the thought described above. But when the arene oxides as intermediates in the enzymatic formation of phenols are taken into consideration, this result could be satisfactorily explained.[20] Therefore, the selective conjugation occurred on the *meta*-hydroxy group to produce metabolites VIII and XI.

18) B.D. Still, D.W. Fassett and R.L. Rondabush, *Biochem. J.*, **42**, 451 (1947).
19) J.J. Childs and D.B. Clayon, *Biochem. Pharmac.*, **15**, 1247 (1966).
20) *a*) D.M. Jerina, J.W. Daly, B. Witkopl, P. Zaltsman-Nirenberg and Udenfriend, *Biochemistry*, **9**, 147 (1970); *b*) A. Karin, G. Garden and W. Trager, *J. Pharmacol. Exptl. Therap.*, **177**, 546 (1971).

The identification of metabolite E and the pharmaceutical properties of these metabolites are under investigation.

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