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Metabolic Studies on Polychlorinated Biphenyls. III.[1] Complete Structure and Acute Toxicity of the Metabolites of 2,4,3′,4′-Tetrachlorobiphenyl[2]

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The present investigation was undertaken to establish the structures of two metabolites which were isolated from the feces of rats administered with 2,4,3′,4′-tetrachlorobiphenyl (2,4,3′,4′-TCB), a major component of commercial preparation of polychlorinated biphenyls. For this purpose six possible isomers of monohydroxy-2,4,3′,4′-TCB were synthesized by condensation reactions between appropriate diazotized dichloroanilines and dichlorophenols. Among these isomers, 5-hydroxy- and 3-hydroxy-2,4,3′,4′-TCB were shown to be identical with a major metabolite (M-A_2), mp 155—156°, and a minor metabolite (M-A_1), mp 92—98°, respectively.

Further study was made to know the acute lethal dose of 2,4,3′,4′-TCB and its major metabolite, 5-hydroxy-2,4,3′,4′-TCB, by *i.p.* injection using male mice of CF-1 strain. The results showed that LD_{50} of 2,4,3′,4′-TCB and 5-hydroxy-2,4,3′,4′-TCB were 2.15 g/kg and 0.43 g/kg, respectively, suggesting that acute toxicity of 2,4,3′,4′-TCB might be attributable to phenolic metabolites produced *in vivo*.

In preceding paper[1] of this series the authors reported that 2,4,3′,4′-tetrachlorobiphenyl (2,4,3′,4′-TCB), one of the representative components of KC-400,[4] was converted partly to monohydroxy-metabolites in rats. Together with unchanged compound, these metabolites were exclusively excreted into the feces, and excretion rate of a major metabolite, M-A_2, was accounted about 10% of the dose during 12 days after the treatment. Although the metabolites were well characterized to be monohydroxyl derivatives of the parent compound by various spectrometries, there were 6 possible isomers for satisfying the structure of monohydroxy-2,4,3′,4′-TCB and the complete structure was remained to be elucidated.

On the other hand, such an aromatic hydroxylation is generally recognized to be a detoxication mechanism for foreign compounds. It is also true, however, that phenolic metabolite frequently shows activity comparable to or more than the parent compound, as exemplified in the metabolism of acetanilide[5] or phenylbutazone.[6]

The present paper will report identification of a major (M-A_2) and a minor metabolite (M-A_1) of 2,4,3′,4′-TCB in rats which were described in the preceding paper[1] and also describe acute toxicity of M-A_2 in mice comparing with that of the parent compound.

Method

Synthesis of 5-Hydroxy-2,4,3′,4′-TCB——To 0.4 g of 3,4-dichloroaniline, mp 71—72°, was added 1.6 g of conc. HCl, and this solution was diluted to 3.2 ml with H_2O, being diazotized with saturated solution of 0.2 g of $NaNO_2$ at 0—5° under stirring for 30 min. This clear solution of diazonium salt was then added to 1.2 g of 2,4-dichlorophenol, mp 64—65°, under stirring and a mixture was heated on a boiling water bath for 2 hr. The resulting reaction mixture was extracted with $CHCl_3$, and the extract was then shaken with

1) Part II: H. Yoshimura, H. Yamamoto, and S. Saeki, *Chem. Pharm. Bull.* (Tokyo), **21**, 2231 (1973).
2) This work was presented at the 4th Symposium on Drug Metabolism and Action, Sendai, Sept. 1972; Abstracts of papers, p. 63.
3) Location: *Katakasu, Higashi-ku, Fukuoka.*
4) S. Saeki, A. Tsutsui, K. Oguri, H. Yoshimura, and M. Hamana, *Fukuoka Acta Med.*, **62**, 20, (1971).
5) F.B. Flinn and B.B. Brodie, *J. Pharmacol. Exptl. Therap.*, **94**, 76 (1948).
6) J.J. Burns, R.K. Rose, S. Goodwin, J. Reichenthal, E.C. Horning, and B.B. Brodie, *J. Pharmacol. Exptl. Therap.*, **113**, 481 (1955).

2N NaOH. The alkaline layer was made acidic with HCl, and it was again extracted with $CHCl_3$. The extract was distilled at 200—250° under atomospheric pressure yielding 0.7 g unreacted 2,4-dichlorophenol. The residue was then distilled under reduced pressure collecting distillate of bp 180—250° (10 mmHg). The reddish-orange oil thus obtained was dissolved in a small amount of MeOH and submitted to preparative thin-layer chromatography (TLC) using solvent system of hexane–AcOEt–AcOH (40:10:1) and silica gel plates described below. The band corresponding to M-A_2 (around *Rf* 0.30) was scraped off into a flask under ultraviolet (UV) lamp and extracted with MeOH to obtain crystalline material. It was recrystallized from MeOH–H_2O (2: 1) to colorless needles, mp 155—156°. Yield was 110 mg. The mixed melting point was not depressed on admixture with a major metabolite of 2,4,3′,4′-TCB, M-A_2. Mass Spectrum *m/e*: 306 (M^+), 308 (M+2). UV λ_{max}^{EtOH}: 252, 300 mμ. IR ν_{max}^{KBr}: 3560 cm^{-1} (OH).

Synthesis of 3-Hydroxy-2,4,3′,4′-TCB——Similarly as described in synthesis of 5-hydroxy-2,4,3′,4′-TCB, 3,4-dichloroaniline (0.4 g) was diazotized with conc. HCl (1.6 g) and saturated solution of $NaNO_2$ (0.2 g), and allowed to react with 2,6-dichlorophenol (1.2 g). The reaction mixture was purified by preparative TLC same as above, and crystalline material was obtained from the band corresponding to M-A_1 (around *Rf* 0.4). It was recrystallized from MeOH–H_2O (2: 1) to colorless needles, mp 95—98°. Yield was only 5 mg. The mixed melting point was not depressed on admixture with a minor metabolite of 2,4,3′,4-TCB, M-A_1. Mass Spectrum *m/e*: 306 (M^+), 308 (M+2). UV λ_{max}^{EtOH}: 252, 290 mμ. IR ν_{max}^{KBr}: 3500 cm^{-1} (OH).

Chlorination of 5-Hydroxy-2,4,3′,4′-TCB——To 56.4 mg of phenol was added 41.6 mg of phosphorus pentachloride, and the mixture was heated at 100° for 6 hr on an oil bath. To this reaction mixture, after cooling, was added 61.2 mg of 5-hydroxy-2,4,3′,4′-TCB. The mixture was again heated at 100° for 6 hr on an oil bath and further at 300° for 10 min on a sand bath. The reaction mixture was distilled under a reduced pressure collecting a distillate of bp 170—230° (6 mmHg), which was solidified soon. This solid material was purified through silisic acid (Mallincrodt, 100 mesh) column chromatography using hexane as effluent solvent and recrystallized from MeOH to colorless needles, mp 83—85°. The mixed melting point was not depressed on admixture with authentic sample of 2,4,5,3′,4′-pentachlorobiphenyl synthesized previously.[4] Mass Spectrum *m/e*: 324 (M^+). UV λ_{max}^{EtOH}: 253 mμ. IR ν_{max}^{KBr} cm^{-1}: 1530, 1453, 1330, 1145, 1090, 1045, 885, 823, 720, 678, 634.

Thin-Layer Chromatography (TLC) and Gas-Liquid Chromatography (GLC)——These were performed similarly as described in the preceding paper.[1] TLC was conducted using silica gel plates (Wakogel B-5UA containing fluorescent indicators, 0.25 mm thick, activated at 105° for 30 min) and a solvent system of hexane–AcOEt–AcOH (40: 10: 1). The spots were visualized by UV lamp. Phenolic compounds were also revealed by Folin–Ciocalteu reagent or diazotized benzidine reagent. GLC were carried out by a Shimadzu GC-3AE gas chromatograph equipped with electron capture detector. The column was a glass spiral tube (4 mm × 2.5 m) and the column packing was 1.5% SE-30 on Chromosorb W (60—80 mesh). The column temperature was maintained at 200°. Nitrogen was used as a carrier gas with flow rate of 60 ml/min (1.5 kg/cm^2). The samples containing phenolic compounds were dissolved in dry pyridine, trimethylsilylated with N,O-bis(trimethylsilyl)acetamide and submitted to above GLC.

Animal Experiment——Male CF-1 mice weighing 16—23 g were used for the present study. They were divided into 13 groups, each of which consisted of 8 mice having similar body weight and was injected intraperitoneally with 0.35, 0.70, 1.05, 1.40, 1.75, 2.10, 2.45 or 3.15 g of 2,4,3′,4′-TCB, or 0.2, 0.3, 0.4, 0.5, or 0.75 g of 5-hydroxy-2,4,3′,4′-TCB per kg body weight of mice, respectively. In above injection, each dose of 2,4,3′,4′-TCB and 5-hydroxy-2,4,3′,4′-TCB was dissolved in 0.2—0.5 ml of soybean oil. Animals were housed in a room maintaining at 20° and numbers of died mice were counted at 24, 48, 72, and 96 hr after administration.

Result and Discussion

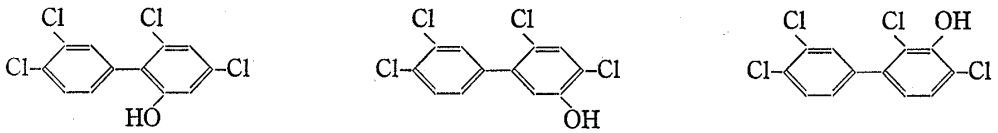
Complete Structure of the Metabolites of 2,4,3′,4′-TCB

As already described, both M-A_2, mp 155—156°, and M-A_1, mp 92—98°, a major and minor metabolites of 2,4,3′,4′-TCB in the rat, respectively, were found to be monohydroxy-2,4,3′,4′-TCB. All possible isomers satisfying this structure are illustrated in Fig. 1.

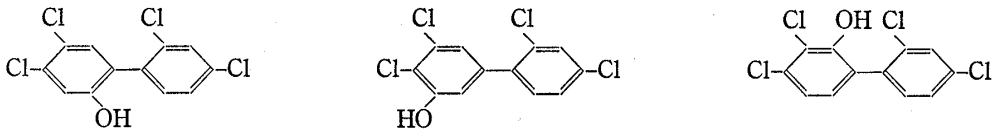
These monohydroxy-derivatives of 2,4,3′,4′-TCB could be synthesized by coupling reactions of appropriate diazotized dichloroanilines and dichlorophenols utilizing the method of Colbert and Lacy,[7] as shown in Fig. 2.

Therefore each reaction described in Fig. 2 was firstly carried out in a small scale to examine which reaction could provide the metabolites, M-A_2 and M-A_1 by GLC and TLC. Judging from the retention time of products yielded in above 5 reactions, M-A_1 was suggested to be

7) J.C. Colbert and R.M. Lacy, *J. Amer. Chem. Soc.*, **68**, 270 (1946).



I: 6-hydroxy-2,4,3′,4′-TCB II: 5-hydroxy-2,4,3′,4′-TCB III: 3-hydroxy-2,4,3′,4′-TCB



IV: 6′-hydroxy-2,4,3′,4′-TCB V: 5′-hydroxy-2,4,3′,4′-TCB VI: 2′-hydroxy-2,4,3′,4′-TCB

Fig. 1. Possible Isomers of Monohydroxy-2,4,3′,4′-TCB

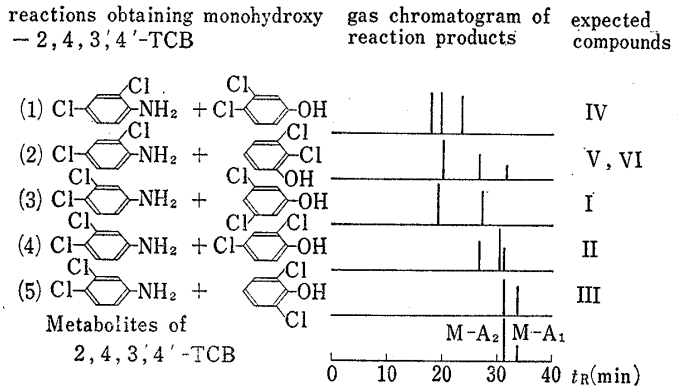


Fig. 2. Gas Chromatographic Patterns of Products Obtained in the Coupling Reactions

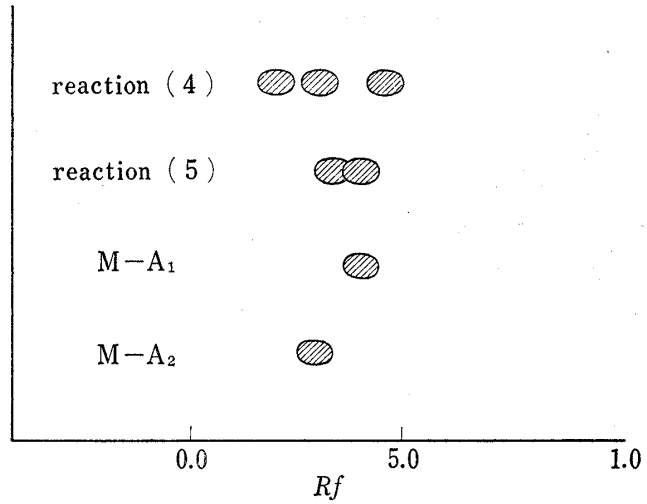


Fig. 3. Thin-Layer Chromatograms of Products Obtained in the Coupling Reactions

solvent system: hexane–AcOEt–AcOH (40: 10: 1)
●: positive to Folin–Ciocalteu reagent and UV lamp

produced only by the reaction (5). On the other hand, however, a peak having identical retention time with that of M-A_2 could be detected in the gas chromatogram of the extract from either reaction (4) or (5). Finally by TLC examination it was concluded that M-A_2 and M-A_1 could be produced only by reactions (4) and (5), respectively, as shown in Fig. 3.

By these preliminary experiments, M-A_2 and M-A_1 were presumed to correspond with the compounds (II) and (III), respectively, and therefore an attempt to isolate these as crystalline form was then carried out by larger scale of reactions (4) and (5).

The reaction (4), in which diazotized 3,4-dichloroaniline was condensed with 2,4-dichlorophenol, was found to afford three products by GLC (Fig. 2). These were also visualized as blue spots at *Rf* 0.20, 0.30 and 0.45 with Folin–Ciocalteu reagent in TLC using a solvent system of hexane–AcOEt–AcOH (40: 10: 1) (see Fig. 3). Three products described above could be isolated by preparative TLC using the same solvent system as above and all their mass spectra showed prominent peaks at *m/e* 306 (M^+) and 308 (M+2), suggesting that they should correspond to the compound (II, IIa and IIb) which were theoretically possible to form in the reaction (4). Among these, one having *Rf* value of about 0.30 gave colorless needles, mp 155—156°, on recrystallization and its melting point was not depressed on admixture with M-A_2.

The complete identity of both compounds was further shown by UV (λ_{max}^{EtOH}: 252, 300 mμ), infrared (IR) (Fig. 5) and Mass Spectra [*m/e*: 306 (M^+), 308 (M+2)]. As described above, three compounds (II, IIa and IIb), were produced in the reaction (4), however only II was

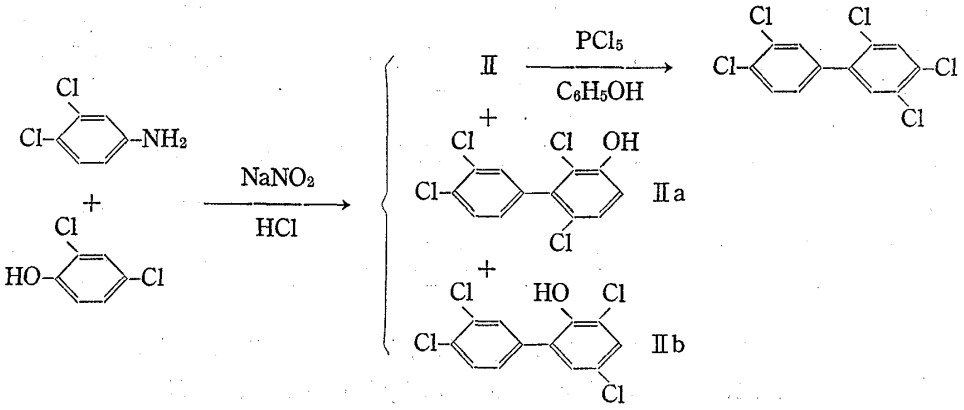


Fig. 4. Coupling Reaction of Diazotized 3,4-Dichloroaniline and 2,4-Dichlorophenol

a derivative of 2,4,3′,4′-TCB, and this indicated undoubtedly that the structure of M-A_2 should be 5-hydroxy-2,4,3′,4′-TCB (II). The structure of II was further confirmed by its conversion to the known compound, 2,4,5,3′,4′-pentachlorobiphenyl, mp 83—87°,[4] according to the method of Coe, *et al.*[8] The product, after purification by distillation, column chromatography and recrystallization, was obtained as colorless needles, mp 83—85°, and was shown to be identical with authentic sample of 2,4,5,3′,4′-pentachlorobiphenyl[4] by the mixedmelting point test, GLC and UV, IR and mass spectrometry.

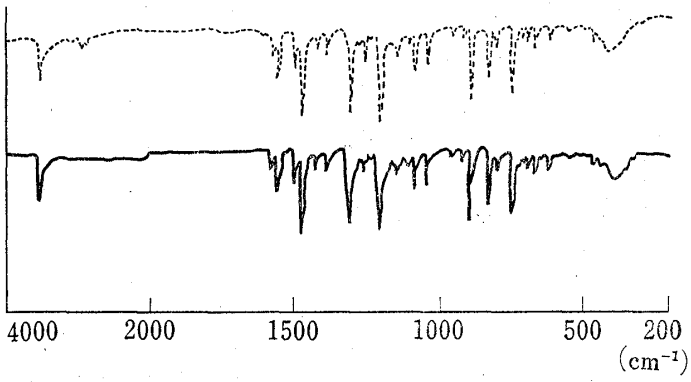


Fig. 5. IR Spectra of 5-Hydroxy-2,4,3′,4′- TCB and M-A_2[1] (KBr)

——: 5-hydroxy-2,4,3′,4′-TCB
------: M-A_2

The reaction (5), in which diazotized 3,4-dichloroaniline was condensed with 2,6-dichlorophenol, was then carried out to obtain compound (III). As shown in Fig. 2 and Fig. 3, two products could be produced in this coupling reaction, and separated by preparative TLC using hexane–AcOEt–AcOH (40: 10: 1) as a solvent system. They were revealed as blue spots at *Rf* 0.40 and 0.35 with Folin–Ciocalteu reagent in above TLC and showed prominent

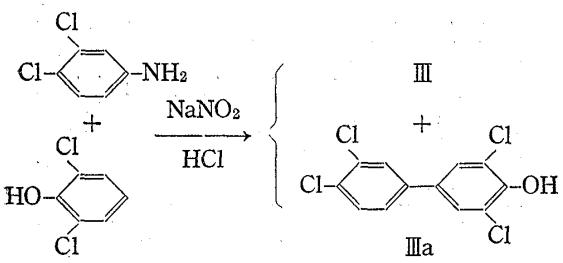


Fig. 6. Coupling Reaction of Diazotized 3,4-Dichloroaniline and 2,6-Dichlorophenol

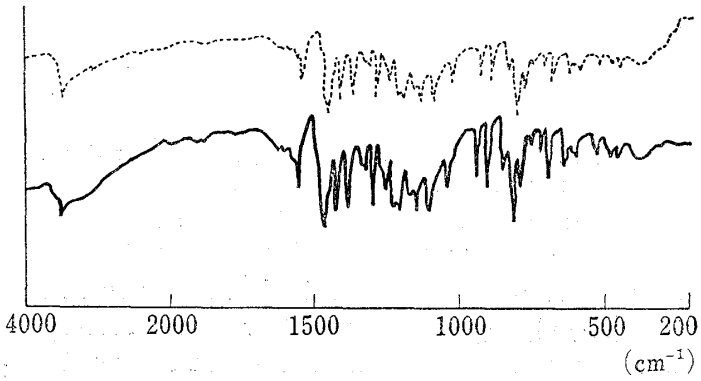


Fig. 7. IR Spectra of 3-Hydroxy-2,4,3′,4′-TCB and M-A_1[1] (KBr)

——: 3-hydroxy-2,4,3′,4′-TCB ------: M-A_1

8) D.G. Coe, H.N. Rydon, and B.L. Tonge, *J. Chem. Soc.*, **1957**, 323.

peaks at *m/e* 306 (M^+) and 308 (M+2) in the mass spectra. These findings suggested that above two products should correspond to the compounds (III and IIIa) (see Fig. 6).

One of these two products showed identical *Rf* value (0.40) and retention time (14.5 min) with M-A_1 on TLC and GLC (TMS derivatives). It was isolated as colorless needles, mp 95—98°, after purification by preparative TLC and recrystallization. This sample showed complete identity with M-A_1 in the mixed melting point test, TLC, GLC and UV, IR (Fig. 7) and mass spectrometry. From these findings the complete structure of M-A_1 can be concluded to be 3-hydroxy-2,4,3′,4′-TCB (III), because (IIIa) could not be derived from 2,4,3′,4′-TCB.

It must be noticed that this is the first study by which complete structure of the metabolite of PCB isomers having more than two chlorine atoms was established.

Acute Toxicity of 5-Hydroxy-2,4,3′,4′-TCB, a Major Metabolite of 2,4,3′,4′-TCB

Considering high toxicity, for example, of pentachlorophenol, the question whether phenolic metabolites of PCB possess more toxicity than the parent compound is of very importance. Acute oral LD_{50} of KC-400 was already reported to be about 2.0 g/kg in mice by Tanaka, *et al.*[9] In the present study, intraperitoneal LD_{50} of 5-hydroxy-2,4,3′,4′-TCB, a major metabolite of 2,4,3′,4′-TCB, was determined together with that of 2,4,3′,4′-TCB using male CF–1 mice by the method of Litchfield–Wilcoxon.[10] The compounds dissolved in soybean oil were injected intraperitoneally and numbers of mice died within 4 days after the injection were counted as described in Method.

As shown in Table I, LD_{50} of 2,4,3′,4′-TCB and 5-hydroxy-2,4,3′,4′-TCB were 2.15 and 0.43 g/kg, respectively. This means that acute toxicity of 5-hydroxy-2,4,3′,4′-TCB, a major metabolite of 2,4,3′,4′-TCB is about 5 times as high as that of the parent compound. In addition, most of mice died at 3 rd to 4 th day after the injection of 2,4,3′,4′-TCB, while in the case of 5-hydroxy-2,4,3′,4′-TCB all the mice died within the first 2 days.

Table I. Mortality and Acute Lethal Dose of 2,4,3′,4′-TCB and M-A_2 in Mice

Dose (g/kg) (*i.p.*) 2,4,3′,4′-TCB	Number of dead mice				
	24 hr	48 hr	72 hr	96 hr	Total
0.35	0	0	0	0	0/8
0.70	0	0	0	0	0/8
1.05	0	0	0	0	0/8
1.40	0	0	0	0	0/8
1.75	0	0	1	1	2/8
2.10	0	0	1	3	4/8
2.45	0	0	2	3	5/8
3.15	1	1	3	2	7/8
	LD_{50} 2.15(1.79—2.58) g/kg ($p<0.05$)				
M-A_2					
0.20	0	0	0	0	0/8
0.30	1	1	0	0	2/8
0.40	3	1	0	0	4/8
0.50	5	1	0	0	6/8
0.75	5	1	0	0	6/8
	LD_{50} 0.43(0.34—0.55)g/kg($p<0.05$)				

Each group consists of 8 mice.

9) K. Tanaka, S. Fujita, F. Komatsu, and N. Tamura, *Fukuoka Acta Med.*, **60**, 544 (1969).
10) J.T. Litchfield, Jr. and F. Wilcoxon, *J. Pharmacol. Exptl. Therap.*, **96**, 99 (1949).

These results suggested that the acute toxicity of 2,4,3′,4′-TCB might be attributable to its phenolic metabolites produced *in vivo*. Further studies, however, are necessary to ascertain this suggestion conclusively. Recently Brodie, *et al.*[11] reported that single doses of chloro-, bromo- or iodobenzene (1 ml/kg, *i.p.*) administered to rats produced massive necrosis in the centrolobuler regions of the liver and suggested that an epoxide which produces in aromatic hydroxylation as a labile intermediate would be responsible for this hepatic necrosis. This hypothesis may be extended to PCB toxicity, however no evidence has been obtained for epoxide formation in PCB metabolism to date.

Acknowledgement This work was supported partially by a Grant-in-Aid for Scientific Research provided by the Ministry of Education and also by a research grant provided by the Ministry of Health and Welfare to which the authors are greatly indebted.

11) B.B. Brodie, A.K. Cho, G. Krishna, and W.D. Reid, *Ann. N. Y. Acad. Sci.*, **179**, 11 (1971).