Biochemical Pharmacology, Vol. 30, No. 13, pp. 1849–1853, 1981.
Printed in Great Britain.

0006-2952/81/131849-05 $02.00/0

INCREASED INHIBITION OF HEPATIC UROPORPHYRINOGEN DECARBOXYLASE BY HEXACHLOROBENZENE IN MALE RATS GIVEN THE OESTROGENIC DRUGS DIETHYLSTILBOESTROL AND CHLOROTRIANISENE

ANDREW G. SMITH and JEAN E. FRANCIS

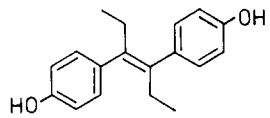
Biochemical Pharmacology Section, MRC Toxicology Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey SM5 4EF, U.K.

(*Received* 18 *December* 1980; *accepted* 17 *February* 1981)

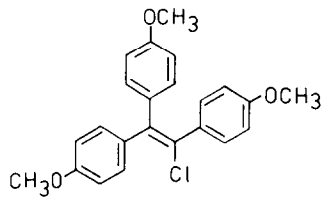
Abstract—Male rats fed hexachlorobenzene (HCB) in their diet for more than 100 days showed only small changes in hepatic porphyrin concentrations and uroporphyrinogen decarboxylase activity. However, when the animals received multiple doses of the oestrogenic drugs diethylstilboestrol (DES) as the dipropionate or chlorotrianisene (CTA)during the later period of feeding HCB, there was a large increase in porphyrin levels and uroporphyrinogen decarboxylase activities were severely depressed. DES and CTA did not produce porphyria in the absence of HCB. The induction of porphyria by oestrogens in male rats fed HCB, which had been proposed as a model for the latent porphyria cutanea tarda triggered by oestrogens in some humans, is therefore caused by a marked increase in the inhibition of uroporphyrinogen decarboxylase leading to the accumulation of uroporphyrin. Rats treated with DES or CTA had lower hepatic levels of HCB than those not given oestrogens, perhaps due to a greater metabolism of HCB lowering tissue concentrations. Thus the induction of porphyria in this system by oestrogens may be caused by a stimulation of HCB metabolism.

Familial and sporadic porphyria cutanea tarda are both due to decreased activity of uroporphyrinogen decarboxylase [EC 4.1.1.37] leading to the accumulation of uroporphyrin [1–4]. The association between oestrogen therapy and the onset of porphyria cutanea tarda in some people has long been recognised [5–8]. The precipitating oestrogens have usually been prescribed to women as contraceptives [9–11] or as a treatment following hysterectomy [11] and to men, often as diethylstilboestrol (DES), for prostatic carcinoma [6–8,11]. The induction of porphyria only occurs in some of the people given the oestrogen therapy suggesting that these drugs cause the unmasking of an underlying latent defect.

Hexachlorobenzene (HCB) causes a similar chronic hepatic porphyria in animals and humans with a concomitant decrease in uroporphyrinogen decarboxylase activity [12–17]. In male rats the condition develops much more slowly than in females [18, 19] and Ippen *et al.* proposed that the feeding of HCB at intervals to males served as a model for latent porphyria cutanea tarda in humans [20, 21]. Indeed they showed that a large increase in urinary porphyrins occurred when male rats treated in this way were repeatedly dosed with 17β-oestradiol. Steroids are known to induce porphyrin synthesis in chick embryo liver cell cultures [22] and 17β-oestradiol will stimulate hepatic 5-aminolaevulinate synthetase activity [EC 2.3.1.37] and other haem biosynthetic enzymes in ovariectomized female rats [23] including those fed HCB [24]. However, we are not aware of any studies to show whether the porphyria observed when male rats fed an HCB-containing diet are given oestrogens can be accounted for by greater inhibition of uroporphyrinogen decarboxylase rather than increased porphyrin biosynthesis. Consequently we have investigated the effects of the oestrogenic drugs DES and chlorotrianisene (CTA) (Fig. 1) on the induction of porphyria in male rats fed HCB especially with regard to uroporphyrinogen decarboxylase activity.



DIETHYLSTILBOESTROL



CHLOROTRIANISENE

Fig. 1. Structures of diethylstilboestrol and chlorotrianisene.

MATERIALS AND METHODS

Materials. HCB (Organic Analytical Grade) was purchased from BDH Chemicals Co. (Poole, U.K.), DES as the dipropionate and CTA were obtained from Sigma Chemical Co. (Poole, U.K.). 5-Amino[4-^{14}C]laevulinate (50 mCi/mmol) was from The Radiochemical Centre (Amersham, U.K.).

Animals and treatments. Male Fischer (F344) rats (approximately 100 g) were fed diets of powdered 41B diet (Labsure Animal Feeds, Poole, U.K.) containing 2 per cent arachis oil and in appropriate cases 0.02 per cent HCB. Animals received DES dipropionate dissolved in arachis oil (10 mg/ml) by i.p. injection; CTA was similarly given (12.5 mg/ml). Controls received oil alone. The rats were weighed every week and were killed by decapitation. Weights of liver, kidneys and testes were recorded. The livers were rinsed and blotted before homogenization of the median lobe in 0.1 M Na_2HPO_4-NaH_2PO_4 buffer (pH 6.8) containing 0.1 mM EDTA (1:4 w/v). The homogenates were then assayed as described below.

Analyses. Porphyrins were estimated (calculated as uroporphyrin) by fluorescence spectroscopy [25, 26] and non-haem iron by atomic absorption spectroscopy [26, 27]. HCB concentrations were determined as previously described using electron capture gas chromatography [28, 29]. Rats fed control diets had only traces of HCB in their livers. Uroporphyrinogen decarboxylase activity was assayed with uroporphyrinogen generated from uroporphyrin (mainly isomer III) isolated from the livers of female rats made porphyric with HCB [30]. The activity is expressed as nmoles of coproporphyrinogen formed/hr/g wet tissue. The method of Bonkowsky *et al.* [31] was employed to study the formation of haem 2 hr after i.p. injection of rats with 5-amino[4-^{14}C]laevulinate (1 μCi/animal). Significance of differences between groups was assessed by Student's *t*-test.

RESULTS AND DISCUSSION

Single and triple doses of diethylstilboestrol

To explore the influence of DES on the susceptibility of male rats to HCB, the effects of varying treatments were investigated. In the studies of Ippen *et al.* [20, 21] rats received HCB in their diet for three days a week at a level of 0.2% (w/w) and were dosed periodically with 17β-oestradiol. We chose to feed HCB continuously at a lower level (0.02 per cent), which in preliminary experiments produced porphyria by 10 weeks in females but not in males, and determined how much oestrogen was required to elicit a response.

When male rats (220–260 g) fed HCB for 9 or 12 weeks were then given one dose of DES (50–100 μmoles/kg) and left for one or two additional weeks on control diet only very slight increases in hepatic porphyrin concentrations occurred relative to animals not given DES (about 1.3 fold). No significant differences in haem biosynthesis from 5-amino[4-^{14}C]laevulinate were detected. Oestrogen-treated animals lost weight (5–10 per cent) although testes sizes did not change.

In a further experiment, rats fed HCB for 84 days were dosed with DES (1.5 mg/animal, about 16 μmoles/kg) on days 60, 68 and 77. The oestrogen again caused losses in body weight whereas those undosed animals fed control or HCB-containing diets gained weight i.e. the following changes in body weights from those at the time of initial injection were observed (− denotes loss in weight), control 16 ± 4; DES −52 ± 12; HCB 34 ± 2; HCB + DES −26 ± 6 g (±S.E.M. $N = 4$). The oestrogenic influence of DES was particularly demonstrated by the decreased size of testes (approx 50 per cent of those of undosed animals), but DES on its own had little effect on liver size or porphyrin levels. In contrast, a modest increase in hepatic porphyrin concentrations (8-fold) of the coproporphyrin/uroporphyrin fluorescence spectra type [25] occurred with the combination of HCB and triple injections of DES (Fig. 2).

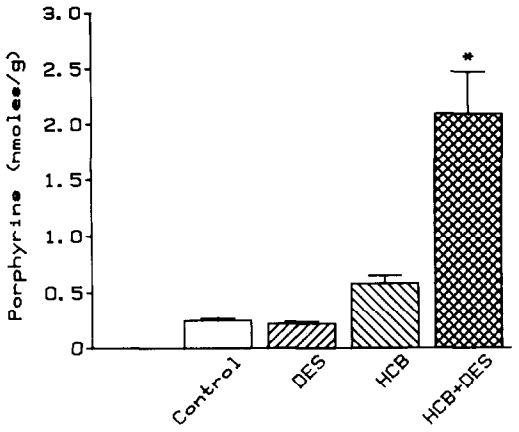


Fig. 2. Accumulation of liver porphyrins after male HCB-treated rats were dosed three times with diethylstilboestrol (DES). Rats were fed HCB in the diet (0.02 per cent) for 84 days and given three i.p. injections of DES (1.5 mg/animal, about 6 mg/kg) at days 60, 68 and 77. Control rats and those treated with DES alone received normal 41B diet. Porphyrins (determined as uroporphyrin) were estimated as described in Materials and Methods. Results are means ±S.E.M. ($N = 4$) per g of wet tissue. *Significantly different from the control $P < 0.001$. Liver weights, control 9.0 ± 0.7; DES 8.0 ± 1.0; HCB 11.7 ± 1.3; HCB + DES 11.7 ± 0.6.

These studies suggested that a longer treatment with DES was probably necessary if hepatic porphyrins were to increase to the very high levels commonly encountered in this type of porphyria.

Prolonged dosing with diethylstilboestrol

Rats fed a diet with or without HCB for 114 days were dosed with DES (2 mg/rat, about 21 μmoles/kg) every ten days from day 36 (their weights were approximately 250 g at this time) and compared with animals not treated with the oestrogenic drug. As in the previous experiments the rats given DES failed to gain weight and testes sizes were reduced, whereas those receiving control or HCB diets alone grew normally (see footnote to Table 1). Liver weights correlated with administration of HCB but no differences in kidney size were observed. A relatively

Table 1. Effects of prolonged treatment by diethylstilboestrol on hepatic porphyrins, uroporphyrinogen decarboxylase and non-haem iron in male rats fed hexachlorobenzene*

Treatment	Porphyrins (nmoles/g)	Uroporphyrinogen decarboxylase (nmoles/hr/g)	Non-haem iron (μmoles/g)
Control (5)	0.38 ± 0.04	24.1 ± 0.6	1.50 ± 0.14
DES (5)	0.26 ± 0.02	27.3 ± 0.6†‡	1.27 ± 0.03
HCB (7)	1.13 ± 0.16†	18.3 ± 0.5†	1.43 ± 0.11
HCB+DES (7)	89.2 ± 13.2†	5.7 ± 1.1†	1.39 ± 0.08

* Young male rats were fed a powdered diet containing HCB (0.02 per cent) for 114 days. From day 36 when the animals were approximately 250 g body weight, some rats on control or HCB diets were given eight i.p. injections of DES dipropionate (2 mg/rat; about 20 μmoles/kg) dissolved in arachis oil at intervals of 10 days. Controls received oil alone. The median lobes of the livers were analysed as described in Materials and Methods. Results are means ± S.E.M. per g of wet tissue (number of animals in parentheses).

† Significantly different from the control group $P < 0.001$.

‡ $P < 0.01$.

Final body, liver and testes weights respectively: control 335 ± 7, 11.0 ± 0.2, 3.2 ± 0.2; DES 211 ± 4, 12.1 ± 0.7, 0.46 ± 0.02; HCB 360 ± 8, 17.1 ± 0.3, 3.5 ± 0.1; HCB + DES 209 ± 6, 17.1 ± 0.6, 0.45 ± 0.03.

small increase in hepatic porphyrin levels occurred in HCB-fed animals (3-fold) but a far greater rise (230-fold) was observed in the HCB animals repeatedly dosed with DES (Table 1). A marked inhibition of uroporphyrinogen decarboxylase accompanied the accumulation of porphyrins in the HCB + DES group, far more than detected with HCB by itself. Although porphyrins can inhibit the enzyme [32], this did not account for the differences in estimated enzymic activity. HCB + DES homogenates gently treated with Zerolit FF (ip) ion exchange resin to remove 98 per cent of endogenous porphyrins, still showed the same degree of inhibition relative to identically tested homogenates from HCB animals. DES did not cause a rise in porphyrin levels in control diet fed rats but there was a consistent small rise in uroporphyrinogen decarboxylase activity (Table 1).

Iron is thought to play some role in the development of porphyria cutanea tarda [33] and there is evidence that it can influence the development of porphyria caused by HCB [34] and 2,3,7,8-tetrachlordibenzo-*p*-dioxin [35]. For this reason non-haem iron concentrations were measured in the livers to determine whether any changes occurred with DES but no significant differences could be detected following oestrogen treatment (Table 1).

Prolonged treatment with chlorotrianisene

Chlorotrianisene has also been used to treat prostatic carcinoma and two cases of porphyria cutanea tarda have been reported of patients receiving the drug [11], although oestrogenic activity by oral administration is less than that of DES [36]. When male rats fed HCB for 103 days were injected with 5 mg of CTA (65 μmoles/kg) on days 33, 44, 54, 68 and 89 (the animals were approximately 200 g body wt at the time of the first injection) similar results were obtained to those with DES. Growth was stunted and testes sizes were considerably reduced (22 per cent of control value) but there was little effect on liver and kidney weights. CTA and HCB administered separately did not cause any increase in hepatic porphyrins but when they were given jointly porphyria developed with a 120-fold increase in porphyrins and a 55 per cent inhibition of the enzyme (Fig. 3). Although the enzyme levels were lowered in the HCB group this was not yet manifested in raised porphyrin levels and again there was a tendency for the oestrogen alone to increase the activity of uroporphyrinogen decarboxylase. Estimations of non-haem iron contents showed small increases in those animals given CTA i.e. control 1.27 ± 0.07; CTA 1.97 ± 0.03*; HCB 1.16 ± 0.05; HCB + CTA 1.51 ± 0.09 μmoles/g of tissue.

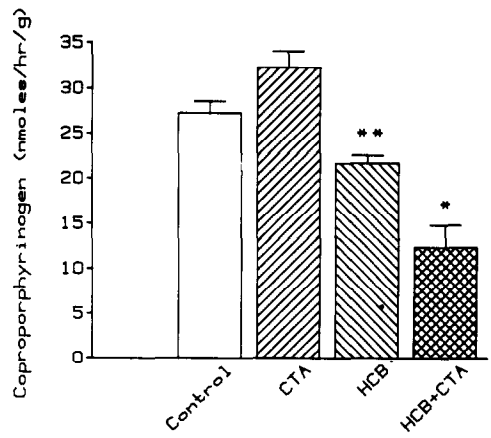


Fig. 3. Inhibition of hepatic uroporphyrinogen decarboxylase in male rats fed HCB and injected five times with chlorotrianisene. Rats were fed HCB for 103 days and injected with CTA (5 mg i.e. 65 μmoles/kg) on days 33, 44, 54, 68 and 89. Controls or animals receiving CTA alone were fed a 41B diet. Porphyrins and uroporphyrinogen decarboxylase in the livers were estimated as described in Materials and Methods. Values are means + S.E.M. (N = 5) per g of wet tissue. *Significantly different from control $P < 0.001$. **$P < 0.01$. Porphyrin levels correlated with the inhibition of the decarboxylase i.e. control 0.29 ± 0.04; CTA 0.21 ± 0.02; HCB 0.32 ± 0.02; HCB + CTA 34.8 ± 14.8† nmoles/g tissue. †Significantly different from controls $P < 0.05$.

* Significantly different from control $P < 0.001$.

Table 2. Influence of diethylstilboestrol and chlorotrianisene on the accumulation of hexachlorobenzene in male rats*

Treatment	Hepatic hexachlorobenzene (nmoles/g)	
	Diethylstilboestrol experiment	Chlorotrianisene experiment
HCB	579 ± 26	583 ± 21
HCB + oestrogenic drug	359 ± 27†	420 ± 24†

* The male rats fed HCB in their diet (0.02 per cent) and treated with the oestrogenic drugs diethylstilboestrol and chlorotrianisene were those of the experiments described in Table 1 and Fig. 3, respectively. HCB concentrations in the livers were determined as described in Materials and Methods. Results are means ± S.E.M. ($N = 5$–7).

† Significantly different from the HCB alone group $P < 0.001$.

Hepatic concentrations of HCB in diethylstilboestrol and chlorotrianisene treated rats

The rats given the oestrogenic drugs grew very slowly compared with undosed animals and it was possible that a greater accumulation of HCB in the livers explained the faster development of porphyria. However, this was not supported by the observation that the intake of food in these rats was proportional to their decreased body weights. In addition, the livers were analysed for HCB content and surprisingly those animals which received the oestrogenic drug had lower hepatic levels of HCB than undosed rats despite their greater response (Table 2).

Mechanism of the potentiation of HCB-porphyria by oestrogens

17β-Oestradiol, on its own or in combination with other steroids can induce signs of porphyria in rats fed HCB but not previously showing overt clinical symptoms [20, 21]. It also aggravates the condition in animals already porphyric [37]. These experiments seem to suggest that HCB-fed male rats are a good model for 'latent' porphyria cutanea tarda in humans, the disease being revealed by oestrogen treatment although alcohol which is also often associated with the induction of porphyria in some patients [1], apparently has no effect in this system [20]. In those susceptible humans, oestrogens may induce porphyria cutanea tarda by increasing porphyrin biosynthesis to exacerbate a partial deficiency in uroporphyrinogen decarboxylase but this does not seem to be the mechanism of the 'latent' HCB porphyria model. Rises in aminolaevulinate synthetase activity [24] are probably the result of a feedback compensatory mechanism [34] and not the primary lesion in the manifestation of porphyria. We have demonstrated in this work that the oestrogenic drugs DES and CTA can also increase the rate of production of porphyria by HCB in male rats and that treatment over many weeks is necessary. The drugs were not capable on their own of causing hepatic porphyria. The large rises in porphyrin levels were associated with decreased activity of uroporphyrinogen decarboxylase compared with both control and HCB-fed animals so that DES and CTA must somehow be causing a greater inhibition of the enzyme. Iron has been implicated in playing a role in porphyria cutanea tarda but our analyses produced little evidence that major changes in iron levels accounted for the induction of porphyria. This does not exclude any cellular redistribution or change in the form of the iron. A consistent difference in HCB levels in the liver between DES or CTA dosed rats and untreated animals was observed (Table 2), the former group having less HCB per g of wet tissue but becoming highly porphyric. This may only reflect changes in liver size relative to intake of HCB but similar differences in HCB concentrations have been reported for slow and fast reacting lobes of female rat livers [29]. A possible explanation is that the oestrogenic drugs cause a greater metabolism of HCB thus lowering its hepatic concentration. A sex difference in rats in the metabolism of some xenobiotics has often been demonstrated [38] and although male rats usually metabolise compounds faster than females this is not always the case. For instance, ethoxyresorufin is deethylated faster in females than in males [39]. The metabolism of HCB and other chlorinated aromatic compounds has often been suggested as a requirement for the decreased uroporphyrinogen decarboxylase activity observed after their administration [40–42]. Variations in metabolism of HCB between male and female rats may well explain the susceptibility of the latter to the induction of porphyria.

Acknowledgements—We would like to thank Mrs. E. D. Tyreman and Mr. M. Clarke for their assistance with dosing of animals and Dr. F. De Matteis for his helpful comments.

REFERENCES

1. G. H. Elder, *Essays in Med. Biochem.* **2**, 75 (1976).
2. G. H. Elder, G. B. Lee and J. A. Tovey, *New Eng. J. Med.* **299**, 274 (1978).
3. H. de Verneuil, G. Aitkin and Y. Nordmann, *Human Genetics* **44**, 145 (1978).
4. H. de Verneuil, Y. Nordmann, N. Phung, B. Grandchamp, G. Aitkin, M. Grelier and J. Noire, *Int. J. Biochem.* **9**, 927 (1978).
5. C. J. Watson, *New Eng. J. Med.* **263**, 1205 (1960).
6. M. Walshe and R. P. Warin, *British J. Dermatol.* **75**, 298 (1963).
7. P. W. M. Copeman, D. J. Cripps and R. Summerly, *British Med. J.* **i**, 461 (1966).
8. J. T. Vail, *J. Amer. Med. Assoc.* **201**, 671 (1967).
9. G. Leonhardi, C. Schneider and J. Gurenci, *Deutsche. Med. Wochensch.* **102**, 160 (1977).

10. H. H. Roenigk and M. E. Gottlob, *Archs. Dermatol.* **102**, 260 (1970).
11. J. S. Taylor and H. H. Roenigk, in *Porphyrins in Human Diseases* (Ed. M. Doss) p. 328. Karger, Basel (1976).
12. R. Schmid, *New Engl. J. Med.* **263**, 397 (1960).
13. R. K. Ockner and R. Schmid, *Nature, Lond.* **189**, 499 (1961).
14. F. De Matteis, B. E. Prior and C. Rimington, *Nature, Lond.* **191**, 363 (1961).
15. G. H. Blekkenhorst, N. R. Pimstone, B. L. Weber and L. Eales, *Ann. Clin. Res.* **8**, supplement 17, 108 (1976).
16. G. H. Elder, J. O. Evans and S. Matlin, *Clin. Sci. Mol. Med.* **51**, 71 (1976).
17. L. C. San Martin de Viale, M. del C. Rios de Molina, R. Wainstok de Calmanovici and J. M. Tomio, *Biochem. J.* **168**, 393 (1977).
18. L. C. San Martin de Viale, A. A. Viale, S. Nacht and M. Grinstein, *Clin. Chim. Acta* **28**, 13 (1970).
19. D. L. Grant, F. Iverson, G. V. Hatina, D. C. Villeneuve, *Environ. Physiol. Biochem.* **4**, 159 (1974).
20. H. Ippen and D. Aust, *Klin. Wschr.* **50**, 793 (1972).
21. H. Ippen, D. Aust and G. Goerz, *Archs Derm. Forsch.* **245**, 305 (1972).
22. A. Kappas and S. Granick, *J. biol. Chem.* **243**, 346 (1968).
23. D. P. Tschudy, A. Waxman and A. Collins, *Proc. natn Acad. Sci. U.S.A.* **58**, 1944 (1967).
24. L. C. San Martin de Viale, J. M. Tomio, A. M. Ferramola, H. A. Sancovich and H. A. Tigier in *Porphyrins in Human Diseases* (Ed. M. Doss) p. 453. Karger, Basel (1976).
25. S. Granick, P. Sinclair, S. Sassa and G. Grieninger, *J. biol. Chem.* **250**, 9215 (1975).
26. A. G. Smith, J. R. P. Cabral and F. De Matteis, *Chem-Biol. Interact.* **27**, 353 (1979).
27. V. G. Cunningham, F. De Matteis and M. D. Stonard, *Biochem. J.* **158**, 105 (1976).
28. C. E. Mendoza, H. M. Vijay, J. B. Shields and G. W. Laver, *Toxicol. Appl. Pharmacol.* **41**, 127 (1977).
29. A. G. Smith, J. E. Francis and F. De Matteis, *Biochem. Pharmacol.* **29**, 3127 (1980).
30. A. G. Smith and J. E. Francis, *Biochem. J.* **183**, 455 (1979).
31. H. L. Bonkowsky, W. J. Bement and R. Erny, *Biochem. biophys. Acta* **541**, 119 (1978).
32. A. G. Smith and J. E. Francis, *Biochem. J.* **195**, 241 (1981).
33. B. F. Felsher and J. P. Kushner, *Semin. Haematol.* **14**, 243 (1977).
34. M. Louw, A. C. Neethling, V. A. Percy, M. Carstens and B. C. Shanley, *Clin. Sci. Mol. Med.*, **53**, 111 (1977).
35. G. D. Sweeney, K. G. Jones, F. M. Cole, D. Basford and F. Krestynski, *Science* **204**, 332 (1979).
36. F. Murad and R. C. Haynes, in *The Pharmacological Basis of Therapeutics* (Eds L. S. Goodman and A. G. Gilman), 6th Edition, p. 1428. MacMillan, New York (1980).
37. N. Simon, G. Berkó and A. Dobozy, *Dermatol Wochensch.* **159**, 868 (1973).
38. J. Doull, in *Toxicology: The Basic Science of Poisons* (Eds. J. Doull, C. D. Klaassen and M. O. Amdur) 2nd Edition, p. 77. MacMillan, New York (1980).
39. M. D. Burke, S. Orrenius and J. A. Gustafsson, *Biochem. Pharmacol.* **27**, 1125 (1978).
40. P. R. Sinclair and S. Granick, *Biochem. biophys. Res. Commun.* **61**, 124 (1974).
41. F. M. H. Debets and J. J. T. W. A. Strik in *Chemical Porphyria in Man* (Ed. J. J. T. W. A. Strik and J. H. Koeman) p. 181. Elsevier, Amsterdam (1970).
42. A. G. Smith and J. E. Francis. *Res. Commun. Chem. Path. Pharmac.* **28**, 377 (1980).