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ARYL HYDROCARBON (BENZO[a]PYRENE) HYDROXYLASES IN LIVER FROM RATS OF DIFFERENT AGE, SEX AND NUTRITIONAL STATUS

DISTINCTION OF TWO TYPES BY 7,8-BENZOFLAVONE*

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Abstract—Two types of aryl hydrocarbon (benzo[a]pyrene) hydroxylase have been distinguished in liver from rats of different sex and age by their sensitivity to the synthetic flavonoid, 7,8-benzoflavone. One type, which is stimulated by the 7,8-benzoflavone, is found in newborn rats and predominates in the liver of adult male rats. This type is inducible by phenobarbital. A second type, which is inhibited by 7,8-benzoflavone, comprises a larger fraction in the liver of adult female rats and is inducible by polycyclic hydrocarbons in immature and mature animals of either sex. The presence of this form in adult female liver is also indicated by the kinetics of the hydroxylase reaction. Removal of solid food for 18 hr not only decreases hepatic aryl hydrocarbon hydroxylase activity in female rats, but also lowers the degree of inhibition by 7,8-benzoflavone. Kinetic data suggest that at low concentrations 7,8-benzoflavone acts as a competitive inhibitor but at higher concentrations inhibits the hydroxylation reaction by a more complex mechanism.

Aryl hydrocarbon (benzo[a]pyrene) hydroxylase (AHH)† belongs to the group of microsomal mixed-function oxygenases, which metabolize numerous xenobiotics such as drugs, pesticides and carcinogens as well as endogenous steroids and fatty acids [1–4]. The AHH enzyme system is found in most mammalian tissues and in a variety of cells in culture [3, 5, 6]. Enzyme activity is inducible by a large number of compounds that comprise at least two major groups, typified by phenobarbital and 3-methylcholanthrene [2, 3, 7, 8].

The enzymatic oxidation of polycyclic hydrocarbons leads to their detoxification as well as to their activation to carcinogenic or toxic derivatives [3, 4, 9, 10]. The biological effects of the hydrocarbons will depend on the properties of the enzyme system by which it is metabolized. Constitutive enzyme levels, tissue-specific enzyme forms, and the state and type of enzyme induction may be important factors in the metabolism of the hydrocarbons to biologically active derivatives.

Hepatic constitutive AHH and AHH induced by polycyclic hydrocarbons can be distinguished by their susceptibility to the inhibitory effect of the synthetic flavonoid, 7,8-benzoflavone (7,8-BF) [11, 12]. 7,8-BF strongly inhibits the hepatic AHH from polycyclic hydrocarbon-treated rats but has no inhibitory effect on the constitutive enzyme from untreated rats. Among many flavone derivatives, 7,8-BF was the most effective in distinguishing two forms of AHH [12].

In the present study 7,8-benzoflavone was used to determine the distribution of the different forms of hepatic AHH in rats in different biological states. The results indicate that rat liver contains at least two types of AHH which vary in their relative amounts in males and females, during the development of the rat, in different nutritional states, and after treatment with different types of enzyme inducers.

EXPERIMENTAL PROCEDURE

Sprague–Dawley rats were kept on a Wayne Blox diet *ad lib.* 3-Methylcholanthrene (MC) (40 mg/kg of body weight) was injected intraperitoneally in 0·3 ml corn oil/100 g of body weight. Control animals received corn oil only. After 18 hr animals were decapitated. Postmitochondrial supernatant and microsomes were prepared from 0·25 M sucrose homogenates as described earlier [13].

Aryl hydrocarbon hydroxylase activity was assayed by the method of Wattenberg *et al.* [14] with previously described modifications [15]. Each incubation flask contained, in a volume of 1·0 ml: 50 μmoles Tris–HCl, pH 7·5; 3 μmoles $MgCl_2$; 0·5 μmole NADPH and 0·1 ml of postmitochondrial supernatant or 0·1 ml microsomes suspended in sucrose–Tris buffer. The amounts of protein per flask used in various experiments are given in legends to tables and figures. One hundred nmoles substrate, benzo[a]pyrene, were added in 0·04 ml methanol. 7,8-BF was added in 0·01 ml methanol. Controls received 0·01 ml methanol. Incubation time was 10 min if not stated otherwise. The reaction was stopped by addition of 1·0 ml

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† Aryl hydrocarbon (benzo[a]pyrene) hydroxylase = AHH; 7,8-benzoflavone (α-naphthoflavone) = 7,8-BF; benzo[a]pyrene = BP; 3-methylcholanthrene = MC.

Table 1. Effect of 7,8-benzoflavone on hepatic aryl hydrocarbon hydroxylase—Age and sex dependency*

Age and sex of rats	Aryl hydrocarbon hydroxylase (pmoles product × mg protein^{-1} × min^{-1}) Addition of 7,8-benzoflavone *in vitro*			
	Control animals		MC-treated animals	
	None	7,8-BF	None	7,8-BF
Newborn (6 days)				
Female	5·6	21·3 (+280)	182	31·0 (−83)
Male	5·6	20·3 (+262)	202	35·7 (−82)
Adult (30 days)				
Female	18·6	4·7 (−75)	306	42·0 (−86)
Male	67·0	56·3 (−16)	313	71·7 (−77)

* AHH activities were determined in triplicates of the 8000 *g* supernatant of pooled livers from 3–4 rats. Amounts of protein per incubation mixture were 0·85 to 1·20 mg. 7,8-BF (50 pmoles) was added in 0·01 ml methanol. Solvent controls received 0·01 ml methanol. Other conditions were as in Experimental Procedure. In two parallel experiments with microsomes as source of the enzyme, 7,8-BF showed virtually the same effects as in the postmitochondrial supernatant. Numbers in parentheses give the per cent stimulation (+) or inhibition (−) in the presence of 7,8-BF.

acetone, and the mixture was shaken with 3·0 ml hexane for 10 min. A 1·0-ml aliquot of the organic layer was extracted with 2 ml of 1 N NaOH, and the fluorescence of the extract was measured immediately at 396 nm excitation and 522 nm emission (Aminco–Bowman spectrophotofluorometer) and was compared to the fluorescence of a 3-hydroxy-benzo[a]-pyrene standard solution. Aryl hydrocarbon hydroxylase activity is expressed as the amount of phenolic metabolites × mg protein^{-1} × min^{-1}. Protein concentrations were determined by the method of Lowry *et al.* [16] using ribonuclease as standard.

RESULTS

The constitutive level of hepatic AHH increases several-fold during the development of rats from birth to maturation depending on the sex of the animal (Table 1). AHH activity is low in newborn rats and is identical in males and females. Enzyme activity increases 12-fold in males, but only 3-fold in females during the first 4 weeks post-partum.

Pretreatment with a polycyclic hydrocarbon (MC) induces the hydroxylase to similar levels in animals of either sex or age group. The MC-induced hepatic enzyme in both sexes of both newborn and adult rats is inhibited more than 70 per cent by 7,8-BF. In contrast, constitutive enzyme activity is increased 2- to 3-fold by the flavone in newborn animals of both sexes, is inhibited 75 per cent in adult females and is slightly inhibited in adult males. The degree of inhibition by 7,8-BF varied somewhat between different groups of adult animals and enzyme preparations, but the difference in susceptibility to 7,8-BF inhibition between male and female rats was consistent. Thus, with respect to 7,8-BF inhibition, the hepatic enzyme from untreated adult female rats behaves like that from MC-treated rats of both sexes.

Figure 1 shows the kinetics of the AHH inhibition by 7,8-BF in microsomes from MC-treated adult male animals in a Lineweaver–Burk plot. The apparent Michaelis constant and maximum velocity of the benzo[a]pyrene hydroxylation are 2×10^{-6} M and 1·8 nmoles phenolic product/mg protein/min respectively. At an inhibitor concentration of 1·7 μM, the Lineweaver–Burk plot is linear over a substrate range of 2–20 μM and intercepts the ordinate close to the control activities. This suggests that 7,8-BF acts as a competitive inhibitor at low substrate concentrations.

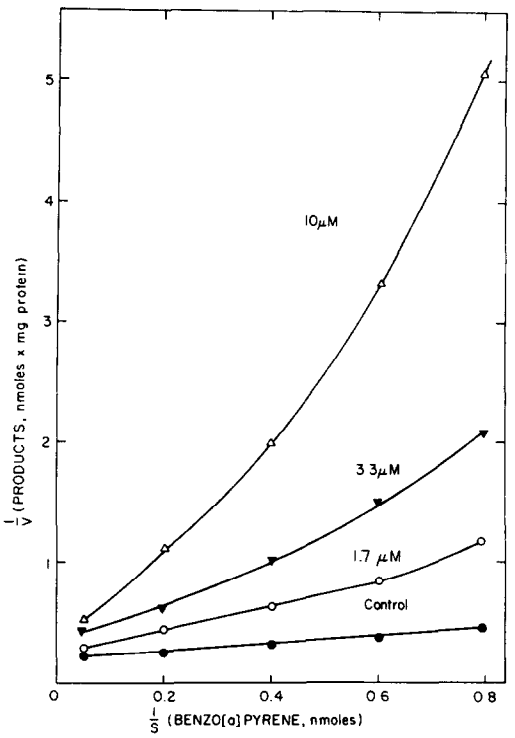

Fig. 1. Lineweaver–Burk plot for inhibition of aryl hydrocarbon hydroxylase by 7,8-benzoflavone. Incubation mixtures contained 0·06 mg of microsomal protein and 0·35 mg of 100,000 *g* supernatant protein. AHH activity is given in nmoles × mg microsomal protein^{-1} × incubation period^{-1} (3 min). BP and 7,8-BF were added in 0·02 and 0·01 ml methanol respectively. Controls received 0·01 ml methanol. Microsomes were obtained from livers of MC-treated adult male rats. Other conditions were as in Experimental Procedure. BP + 10 nmoles 7,8-BF, △; BP + 3·3 nmoles 7,8-BF, ▼; BP + 1·7 nmoles 7,8-BF, ○; BP + 0·01 ml methanol, ●.

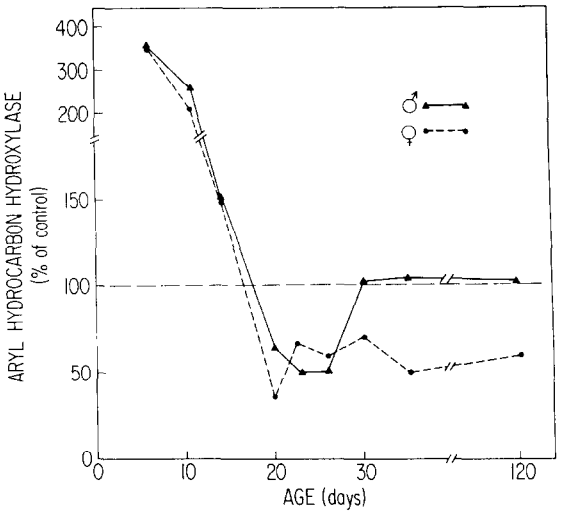

Fig. 2. Effect of 7,8-benzoflavone on hepatic aryl hydrocarbon hydroxylase—Postnatal development in male and female rats. One hundred per cent of controls refers to specific activities (pmoles × mg protein^{-1} × min^{-1}) in tissue preparations from rats of different age and sex in the absence of 7,8-BF (see Table 1). Conditions were as described under Table 1 and in Experimental Procedure. Note the change in scale of the ordinate.

However, in the presence of 3·3 or 10 μM 7,8-BF, the reaction kinetics do not give a straight line but diverge upward (Fig. 1), indicating a more complex mechanism of inhibition.

Changes in the constitutive forms of AHH during development of the rat were examined using benzoflavone as a probe (Fig. 2). The stimulation of AHH activity by 7,8-BF decreases as the animal approaches weaning. At about 20 days after birth, 7,8-BF exerts an inhibitory effect on the enzyme from both sexes. A week later the difference between the enzymes in male and female rats becomes apparent. Whereas in mature male rats the flavone has little inhibitory effect, in female rats the enzyme is inhibited by 50 per cent. This sex difference in respect to inhibition by 7,8-BF is also observed in older rats weighing 300 g.

The presence of a larger fraction of the 7,8-BF-inhibited, MC-inducible form in the liver of female rats is also suggested by the kinetics of the hydroxylase. Figure 3 shows the Lineweaver–Burk plots for benzpyrene hydroxylation by hepatic microsomes from male and female rats, and from MC-pretreated male rats. The double-reciprocal plot of activities in microsomes from untreated animals deviates from simple Michaelis–Menten kinetics in contrast to the linear plot of the MC-induced hydroxylase. The decrease in the apparent K_m after induction with the polycyclic hydrocarbon is in agreement with observations of others [17, 18].

Preliminary experiments indicated that the kinetics of the hydroxylase from male and female rats diverged below substrate concentrations of about 3×10^{-6} M. Since this could have been caused by the differences in the total hydroxylase activity (V_{max}) of these two preparations (Table 1), we varied the protein concentration or the time of incubation so that the formation of phenolic products was about equal in both preparations. In all cases, the reciprocal plots diverged at lower substrate concentrations as shown in Fig. 3, indicating the existence of qualitative and not merely quantitative differences in the hydroxylase. The enzyme activity obtained at lower substrate concentrations for microsomes from female rats suggests the presence of a hydroxylase form with a lower K_m which may correspond to the form with the low K_m found after polycyclic hydrocarbon induction.

Table 2 shows the influence of phenobarbital treatment and the state of nutrition on hepatic AHH activity from female rats and its susceptibility to 7,8-BF inhibition. Removal of solid food for 18 hr reduces hepatic AHH by 35 per cent and considerably lowers the degree of inhibition by 7,8-BF. Phenobarbital treatment causes a 3- to 4-fold induction of hydroxylase activity. This increased activity is no longer inhibited by 7,8-benzoflavone and is even stimulated in the presence of lower concentrations of the benzoflavone. These results are in agreement with earlier observations indicating that phenobarbital induces the enzyme form which is not inhibited by 7,8-BF [11].

DISCUSSION

The present observations indicate that the liver of rats contains at least two types of aryl hydrocarbon hydroxylating enzyme systems which vary in their relative amounts depending on the age, sex and nutritional state of the rat as well as their exposure to specific inducers. One type, which is stimulated by

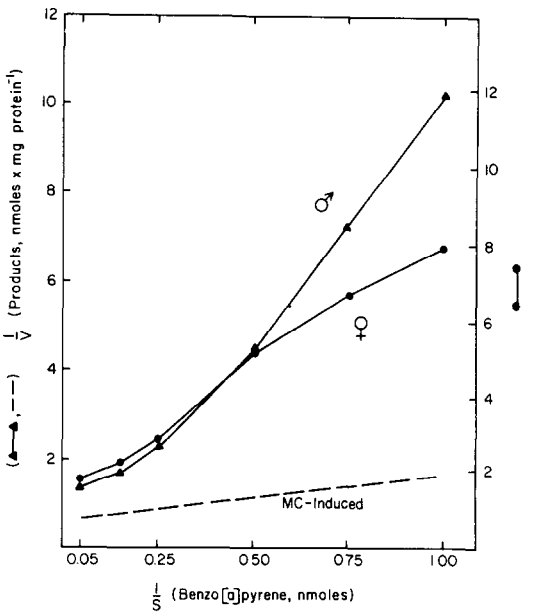

Fig. 3. Kinetics of aryl hydrocarbon hydroxylase of liver from female and male rats (Lineweaver–Burk plot). Microsomes were prepared from young adult, female and male rats (four animals/group). Incubation mixtures contained: 0·16 and 0·19 mg of microsomal protein from female and male animals, respectively, and 0·4 mg protein of 100,000 g supernatant from male liver. Enzyme activity is given in nmoles × mg microsomal protein^{-1} × incubation period^{-1} (6 and 3 min for females and males respectively). Points represent means of triplicate determinations. The phenolic products were extracted from 1 ml of the organic phase into 1 ml NaOH. Other conditions were as in Experimental Procedure.

Table 2. Effect of 7,8-benzoflavone on aryl hydrocarbon hydroxylase from female rats—Influence of starvation and phenobarbital induction*

Addition	Aryl hydrocarbon hydroxylase (pmoles × mg protein^{-1} × min^{-1})		
	Saline		Phenobarbital
	Fed	Fasted	Fasted
None	25·3 (100)	16·3 (100)	73·3 (100)
7,8-Benzoflavone, 10^{-4} M	5·0 (−80)	9·0 (−45)	76·6 (−2)
7,8-Benzoflavone, 10^{-5} M	15·6 (−38)	17·3 (+6)	120·3 (+54)

* Rats were kept without food and with water *ad lib.* for 18 hr before sacrifice. Phenobarbital (80 mg/kg body wt) was injected intraperitoneally in 0·2 ml saline daily for 3 consecutive days and the animals were sacrificed 24 hr after the last injection. Controls received saline only. AHH activity was assayed in triplicate on the 10,000 *g* supernatant of pooled livers from 3–4 female rats (130 g body wt). Amounts of protein per incubation mixture were 0·7 to 0·8 mg. Other conditions were as in Experimental Procedure. Numbers in parentheses give the per cent stimulation (+) or inhibition (−) in the presence of 7,8-BF.

7,8-benzoflavone, predominates in the livers of immature animals and of normal adult male rats. This type is inducible by phenobarbital. A second form, which is inhibited by 7,8-benzoflavone, comprises a large fraction of the AHH activity in normal adult female rats and is inducible by polycyclic hydrocarbons. This form also predominates in the microsomes of extrahepatic tissues [11, 19]. The two types of AHH enzyme systems, characterized by their susceptibility to inhibition by 7,8-benzoflavone, are found in mice [20] as well as rats.

We do not know what constitutes the different forms of the AHH system and whether they are interconvertible. They are probably associated with the two closely related hemoproteins, cytochrome P-450 and P-448* [21–23]. The change from the predominance of the cytochrome P-450 in hepatic microsomes of untreated animals to a high level of cytochrome P-448 after treatment with a polycyclic hydrocarbon inducer [21–23] correlates well with the induction of the AHH form characterized by the lower K_m [17, 18] and the marked sensitivity to 7,8-BF inhibition. Similarly, the 7,8-BF inhibition of the hydroxylase in extrahepatic tissues from both untreated and MC-treated animals is paralleled by a preponderance of microsomal cytochrome P-448 [19].

Furthermore, observations with partially purified and reconstituted microsomal preparations indicate that the stimulatory and inhibitory effect of the flavone resides in the cytochrome P-450 and cytochrome P-448 component of the enzyme complex [24].

There is some evidence that benzoflavones are substrates for the microsomal mixed-function oxygenase system. Addition of the flavones to microsomal enzyme preparations causes: (1) spectral shifts typical for the binding of type I substrates to cytochrome P-450 [20], and (2) an increase in the rate of cytochrome P-450 reduction similar to that observed with the polycyclic hydrocarbons.† Thus, inhibition of AHH by 7,8-BF might be due to competition with the polycyclic hydrocarbon substrate for the enzyme system. This is compatible with the inhibition kinetics at low 7,8-BF concentrations. The nonlinear Lineweaver–Burk plots at higher 7,8-BF concentrations suggest that the inhibitor may interact with the enzyme system in more than one way [25]. A multiple interaction is not unlikely, since the microsomal hydroxylase represents a multi-component system and its terminal cytochrome may contain more than one substrate binding site. The stimulatory effect of the 7,8-BF on the enzyme may be due to "effector" type activation of the AHH system. Other possibilities to be considered are alteration of the electron flow to the P-450–P-448 system by 7,8-BF or competition with the substrate for nonspecific binding sites.

Age- and sex-dependent differences are common in the metabolism of drugs and steroids by microsomal hydroxylases [3, 26–28] and have also been observed for the metabolism of carcinogenic hydrocarbons such as 7,8-dimethylbenz[a]anthracene [29]. Our results clearly indicate that the AHH of liver differs not only quantitatively but also qualitatively in rats of different sex and age. It should be noted that AHH in the liver of female rats appears to contain relatively more of the enzyme form with the higher substrate affinity. At the low substrate concentrations that are likely to occur *in vivo*, this might compensate for the lower levels of enzyme in female rats.

The rapid decrease in AHH activity in female rats after an 18-hr fast correlates with the reduced metabolism of a number of drugs observed by Dixon *et al.* in mice [30]. Our data indicate that in adult female rats the partial loss of AHH activity during starvation is largely due to a decrease in the 7,8-BF-inhibited type. We do not know whether the absence of fortuitous inducers of the polycyclic hydrocarbon type in the diet [29, 31, 32] or other factors related to metabolism during fasting cause this phenomenon. It is worth noting that the frequently used 24-hr fasting period before the preparation of microsomes causes not only a decrease in AHH activity of female rats, but also introduces a change in the apparent properties of the enzyme complex.

Polycyclic hydrocarbons are metabolized to a number of products which include various phenols,

* This cytochrome has been referred to as P_1-450 [21], P-448 [22] or P-446 [23].

† F. J. Wiebel, B. Stripp and H. V. Gelboin, unpublished observations.

dihydrodiols, quinones, epoxides and conjugates [1, 33] with widely differing cytotoxic and carcinogenic potencies [34, 35]. Since the particular profile of metabolites might depend on the type of AHH involved, the existence and distribution of these different enzyme types may be unique determinants of an organism's susceptibility to polycyclic hydrocarbon carcinogenesis.

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REFERENCES

1. A. H. Conney, E. C. Miller and J. A. Miller, *J. biol. Chem.* **228,** 753 (1957).
2. J. R. Gillette, *Prog. Drug Res.* **6,** 11 (1963).
3. A. H. Conney, *Pharmac. Rev.* **19,** 317 (1967).
4. A. H. Conney and J. J. Burns, *Science, N.Y.* **178,** 576 (1972).
5. D. W. Nebert and H. V. Gelboin, *Archs Biochem. Biophys.* **134,** 76 (1969).
6. L. W. Wattenberg and J. L. Leong, *J. Histochem. Cytochem.* **10,** 412 (1962).
7. H. Remmer, in *Proc. First Int. Pharmac. Meeting*, Vol. 6, p. 235. MacMillan, New York (1962).
8. G. J. Mannering, in *Pharmacological Testing Methods* (Ed. A. Burger), pp. 51–119. Marcel Dekker, New York (1968).
9. H. V. Gelboin, F. Wiebel and L. Diamond, *Science, N.Y.* **170,** 169 (1970).
10. H. V. Gelboin, N. Kinoshita and F. J. Wiebel, *Fedn Proc.* **31,** 1298 (1972).
11. F. J. Wiebel, J. C. Leutz, L. Diamond and H. V. Gelboin, *Archs Biochem. Biophys.* **144,** 78 (1971).
12. F. J. Wiebel, H. V. Gelboin, N. P. Buu-Hoi, M. G. Stout and W. S. Burnham, in *Model Studies in Chemical Carcinogenesis* (Eds. P. O. P. Ts'o and J. A. DiPaolo), p. 249. Marcel Dekker, New York (1974).
13. H. V. Gelboin, *Biochim. biophys. Acta* **91,** 130 (1964).
14. L. W. Wattenberg, J. L. Leong and P. J. Strand, *Cancer Res.* **22,** 1120 (1962).
15. D. W. Nebert and H. V. Gelboin, *J. biol. Chem.* **243,** 6242 (1968).
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
17. A. P. Alvares, G. R. Schilling and R. Kuntzman, *Biochem. biophys. Res. Commun.* **5,** 588 (1968).
18. H. L. Gurtoo and T. C. Campbell, *Biochem. Pharmac.* **19,** 1729 (1970).
19. N. G. Zampaglione and G. J. Mannering, *J. Pharmac. exp. Ther.* **185,** 676 (1973).
20. F. M. Goujon, D. W. Nebert and J. E. Gielen, *Molec. Pharmac.* **8,** 667 (1972).
21. N. E. Sladek and G. J. Mannering, *Biochem. biophys. Res. Commun.* **24,** 668 (1966).
22. A. P. Alvares, G. Schilling, W. Levin and R. Kuntzman, *Biochem. biophys. Res. Commun.* **29,** 521 (1967).
23. A. Hildebrandt, H. Remmer and R. W. Estabrook, *Biochem. biophys. Res. Commun.* **30,** 607 (1968).
24. A. Y. H. Lu, R. Kuntzman, S. West, M. Jacobson and A. H. Conney, *J. biol. Chem.* **247,** 1727 (1972).
25. M. Dixon and E. C. Webb, *Enzymes*, p. 324. Academic Press, New York (1964).
26. M. Jacobson and R. Kuntzman, *Steroids* **13,** 327 (1969).
27. S. M. MacLeod, K. W. Renton and N. R. Eade, *J. Pharmac. exp. Ther.* **183,** 489 (1972).
28. R. Kato and J. R. Gillette, *J. Pharmac. exp. Ther.* **150,** 285 (1965).
29. P. Sims and P. L. Grover, *Biochem. Pharmac.* **17,** 1751 (1968).
30. R. L. Dixon, R. W. Shultice and J. R. Fouts, *Proc. Soc. exp. Biol. Med.* **103,** 333 (1960).
31. L. W. Wattenberg, *Cancer Res.* **28,** 99 (1971).
32. R. E. Billings and L. W. Wattenberg, *Proc. Soc. exp. Biol. Med.* **139,** 865 (1972).
33. P. Sims, *Biochem. Pharmac.* **19,** 795 (1970).
34. I. Berenblum and R. Schoental, *Cancer Res.* **3,** 145 (1943).
35. H. Marquardt, T. Kuroki, E. Huberman, J. K. Selkirk, C. Heidelberger, P. L. Grover and P. Sims, *Cancer Res.* **32,** 716 (1972).