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QUANTITATIVE ASPECTS OF THE METABOLISM OF 7, 12–DIMETHYLBENZ[a]ANTHRACENE BY LIVER HOMOGENATES FROM ANIMALS OF DIFFERENT AGE, SEX AND SPECIES

P. SIMS and P. L. GROVER

The Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, S.W.3.

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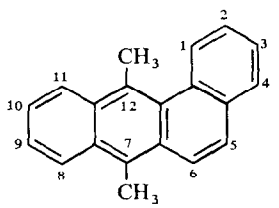
Abstract—The metabolism of DMBA *in vitro* has been examined using liver homogenates from rats, mice, hamsters and guinea pigs. Estimation of the known end-products of DMBA metabolism has shown that transient increases in hepatic enzyme activities occur in rats and mice in the post-weaning period, which are probably due to dietary components first ingested at weaning. Differences in hepatic DMBA metabolism do not appear to account for the inability of the hydrocarbon to cause adrenal necrosis in adult mice, hamsters and guinea pigs.

THE EARLIER observations of Boyland and Sims[1] that the carcinogen, DMBA* (I), is converted by rat-liver homogenate into 7-OHM-12-MBA and 12-OHM-7-MBA have been confirmed by other workers.[2–5] 7,12-DiOHMBA is also formed.[2–4, 6] It has also been shown[6] that DMBA is metabolized by processes involving ring-hydroxylation to yield products believed to be 3-OHDMBA, 4-OHDMBA and 8,9-DiHOHDMBA. 7-OHM-12-MBA and 12-OHM-7-MBA are further metabolized to phenols and dihydrodihydroxy compounds: the latter compounds are probably 8,9-DiHOH-7-OHM-12-MBA and 8,9-DiHOH-12-OHM-7-MBA. The formation of 4-OHDMBA as a metabolite of DMBA in rat-liver homogenate has recently been confirmed.[4]

The involvement of 7-OHM-12-MBA in the induction of adrenal necrosis is well-established[7–9] and it is likely that the ability of DMBA itself to induce adrenal necrosis is due to the formation of this metabolite in the body.[10] Thus any process that either reduces the formation of 7-OHM-12-MBA or leads to its further metabolism should delay or prevent the induction of adrenal necrosis. It was found[11] that the amounts of the 8,9-dihydrodihydroxy compounds formed from DMBA by rat-liver homogenates under standard conditions varied according to the age and sex of the animals used.

* The following abbreviations are used: DMBA, 7,12-dimethylbenz[a]anthracene; 7-OHM-12-MBA, 7-hydroxymethyl-12-methylbenz[a] anthracene; 12-OHM-7-MBA, 12-hydroxymethyl-7-methylbenz[a]anthracene; 7,12-DiOHMBA, 7,12-dihydroxymethylbenz[a]anthracene; 3-OHDMBA, 3-hydroxy-7,12-dimethylbenz[a]anthracene; 4-OHDMBA, 4-hydroxy-7,12-dimethylbenz[a]anthracene; 8,9-DiHOHDMBA, 8,9-dihydro-8,9-dihydroxy-7,12-dimethylbenz[a]anthracene; 8,9-DiHOH-7-OHM-12-MBA, 8,9-dihydro-8,9-dihydroxy-7-hydroxymethyl-12-methylbenz[a]anthracene; 8,9-DiHOH-12-OHM-7-MBA, 8,9-dihydro-8,9-dihydroxy-12-hydroxymethyl-7-methylbenz[a]anthracene; 12-MBA-7-COOH, 12-methylbenz[a]anthracene-7-carboxylic acid.

Increases in the formation of these metabolites in the body should reduce the effective concentration of 7-OHM-12-MBA. These observations have now been extended to include the other known metabolites of DMBA. DMBA metabolism by liver homogenates prepared from animals of other species has also been examined since it has been reported[12] that this hydrocarbon does not induce adrenal necrosis in mice, hamsters or guinea pigs.



EXPERIMENTAL

Materials. 7-OHM-12-MBA, 12-OHM-7-MBA, 7,12-DiOHMBA and 12-MBA-7-COOH were prepared as described.[1, 6] 4-OHDMBA was the gift of Dr. J. W. Flesher. DMBA (generally labelled with tritium, sp. act. 400 mc/mM) was obtained from the Radiochemical Centre, Amersham, Bucks.

Animal experiments. Chester–Beatty strain rats and C-mice were used. The litters had access to solid food from birth and were fully weaned onto a diet of rat cubes (Diet 86, Plowco Feeds Ltd., South Godstone, Surrey) by 21 days old for rats and 25 days old for mice. Guinea pigs and hamsters were adult stock animals.

In each experiment, livers from at least two animals were pooled and homogenized in 5 vol. of ice-cold 1·15% (w/v) KCl using a Potter–Elvehjem type of homogenizer with a Teflon pestle. The homogenate was centrifuged at 0° for 20 min at $1480g_{av.}$ in an Angle 50 centrifuge (Measuring and Scientific Equipment Ltd., London) and a volume of supernatant equivalent to 1 g of liver was diluted with an equal volume of 0·1 M-phosphate buffer, pH 7·4, prepared from NaH_2PO_4 and Na_2HPO_4. $NADP^+$ (2·55 mg) and glucose 6-phosphate (18·65 mg) [both obtained from the Boehringer Corporation (London) Ltd., London, W.5.] were added and the mixture was heated to 37°. An ethanolic solution of DMBA (0·1 ml, $3{\cdot}83 \times 10^6$ cpm) was added and the mixture incubated at 37° for 30 min. The reaction was stopped by the addition of 2 vol. of acetone and the mixture extracted twice with ethyl acetate (10 ml). The combined extracts were washed with water (20 ml) and dried (Na_2SO_4) and the solution was evaporated. One drop of a stock ethanolic solution of unlabelled 7-OHM-12-MBA, 12-OHM-7-MBA, 7,12-DiOHMBA and 12-MBA-7-COOH was added to the residue and the mixture applied as a 9 cm line to the bottom of a TLC prepared by coating a glass plate with a film of silica gel G (E. Merck A.-G., Darmstadt, Germany) of 0·25 mm thickness. The chromatogram was developed for 15 cm with benzene containing 9% (v/v) of ethanol and examined in u.v. light whilst still wet.

Six fluorescent bands were usually seen on the chromatograms: unchanged DMBA, the four added markers and 8,9-DiHOHDMBA. The bands were marked off and the spaces between them subdivided into a number of other bands marked parallel to the main bands. In this way 23 fractions were obtained from each plate: the metabolites probably present in these fractions are indicated in Table 1.

TABLE 1. FRACTIONATION OF THE PRODUCTS OF THE METABOLISM OF TRITIATED 7,12-DIMETHYLBENZ[a]ANTHRACENE BY LIVER HOMOGENATES ON TLC's

Fraction No.	R_f	Fluorescence in u.v. light	Products probably present in fraction	Remarks
1	0·97	Violet	DMBA	The photo-oxide, 7,12-epidioxy-7,12-dimethyl benz[a]anthracene is also present in this fraction
2	0·92	None	None	
3	0·90	None	None	
4	0·86	None	None	
5	0·81	Violet	12-OHM-7-MBA	
6	0·79	None	Phenols	Probably 3- and 4-OHDMBA. Synthetic 4-OHDMBA runs in this fraction
7	0·77	None	Phenols	As for fraction 6
8	0·75	None	None	
9	0·71	Violet	7-OHM-12-MBA	An unidentified product was sometimes present in this fraction (see text)
10	0·65	None	None	An unidentified oxidation product of 7-OHM-12-MBA was sometimes present in this fraction (see ref. 1)
11	0·65	None	None	
12	0·53	None	None	
13	0·49	None	None	
14	0·45	Violet	7,12-DiOHMBA	
15	0·40	None	Phenols	These are probably the 3- and 4-hydroxy derivatives of 7-OHM-12-MBA and 12-OHM-7-MBA
16	0·34	None	Phenols	As for fraction 15
17	0·28	None	None	Synthetic *trans*-5,6-dihydro-5,6-dihydroxy-7,12-dimethylbenz[a]anthracene runs in this fraction
18	0·24	Dark violet	8,9-DiOHDMBA	The fluorescence was more easily seen after the plates were allowed to dry in the air for a few min.
19	0·20	None	None	
20	0·18	None	8,9-DiHOH-7-OHM-12-MBA and 8,9-DiHOH-12-OHM-7-MBA	
21	0·16	None	As for fraction 20	
22	0·12	None	As for fraction 20	
23	0·06	Violet	12-MBA-7-COOH and 7-MBA-12-COOH	

The products were chromatographed as described in the text. The R_f values quoted are typical: some variations in R_f values between individual chromatograms were noted, but the order in which the products ran was always the same.

The fractions were removed from the plate, the silica gel transferred to glass vials and the radioactivity present was determined by liquid scintillation counting using a Packard Tri-Carb Spectrometer, Model 314 with a counting efficiency of 15 per cent for tritium.

Experiments showed that the addition of silica gel to the scintillation fluid caused quenching of less than 5 per cent. All determinations were carried out in duplicate and the results are shown in Tables 2 and 3. All manipulations up to the end of the chromatography were carried out either in the dark or away from direct sunlight.

The solution of tritiated DMBA used in the incubations was also examined on TLC's: a 0·1 ml portion to which a drop of the solution containing the unlabelled

TABLE 2. DISTRIBUTION OF RADIOACTIVITY FOLLOWING THE CHROMATOGRAPHY OF TRITIATED 7,12-DIMETHYLBENZ[a]ANTHRACENE AND ITS METABOLITES ON THIN-LAYER PLATES

Fraction No.	Products probably present in fraction	Radioactivity in fractions from control experiments in which		Radioactivity in fractions obtained from the incubation of DMBA with liver homogenates from			
		Stock DMBA solution was chromatographed (2)*	DMBA was incubated with boiled liver homogenate from 50 day-old female rats (2)*	25 Day-old male rats (4)*	Adult male rats (5)*	15 Day-old male mice (2)*	Adult male mice (3)*
1	DMBA	> 2000	1980 (1965–1995)	218 (210–228)	980 (965–895)	705 (689–715)	1180 (1165–1209)
2	None	214 (204–224)	42 (40–44)	5 (3–7)	15 (13–16)	10 (7–12)	8 (4–9)
3	None	80 (71–89)	8 (6–10)	7 (4–9)	5 (4–6)	4 (2–5)	5 (3–7)
4	None	9 (7–11)	9 (7–11)	6 (5–8)	9 (3–12)	6 (4–7)	5 (3–7)
5	12-OHM-7-MBA	10 (8–12)	9 (7–11)	28 (25–33)	25 (19–28)	48 (42–53)	31 (27–35)
6	Phenols	3 (2–4)	3 (2–4)	12 (9–13)	4 (2–5)	15 (12–18)	7 (5–9)
7	Phenols	2 (1–3)	3 (2–4)	33 (29–35)	9 (7–12)	20 (18–22)	8 (5–10)
8	None	2 (1–3)	2 (1–2)	11 (9–13)	5 (3–7)	6 (4–8)	4 (2–6)
9	7-OHM-12-MBA	5 (4–6)	8 (7–9)	95 (90–101)	89 (86–92)	162 (152–170)	59 (53–61)
10	Oxidation product of 7-OHM-12-MBA	3 (2–4)	4 (2–4)	12 (8–15)	10 (6–12)	20 (18–22)	18 (13–23)
11	None	< 1	< 1	5 (2–7)	2 (1–4)	3 (1–4)	2 (1–3)
12	None	< 1	< 1	4 (3–5)	2 (1–4)	2 (1–4)	< 1
13	None	< 1	< 1	3 (1–3)	< 1	3 (1–3)	< 1
14	7,12-DiOHMBA	< 1	< 1	20 (18–23)	3 (1–4)	11 (9–13)	3 (1–4)
15	Phenols	< 1	< 1	4 (3–5)	2 (1–3)	4 (3–5)	2 (1–3)
16	Phenols	< 1	< 1	43 (39–48)	6 (4–7)	15 (13–17)	2 (1–3)
17	None	< 1	< 1	9 (7–12)	2 (1–3)	5 (3–7)	< 1
18	8,9-DiHOHDMBA	< 1	< 1	313 (295–321)	10 (9–11)	85 (81–89)	9 (7–11)
19	None	< 1	< 1	8 (6–10)	2 (1–3)	3 (1–5)	< 1
20	8,9-DiHOH-7-OHM-12-MBA and 8,9-DiHOH-12-OHM-7-MBA	< 1	< 1	25 (20–27)	< 1	12 (10–14)	2 (1–3)
21	As in fraction 20	< 1	< 1	46 (42–52)	< 1	25 (24–26)	< 1
22	As in fraction 20	< 1	< 1	17 (15–19)	< 1	4 (3–5)	2 (1–3)
23	12-MBA-7-COOH and 7-MBA-12-COOH	4 (3–5)	4 (3–5)	15 (13–17)	5 (4–6)	5 (3–7)	5 (3–7)

DMBA ($3{\cdot}83 \times 10^6$ cpm) was added to each incubation mixture as described in the text.
The values for the metabolites are means with the ranges in parentheses and are expressed in cpm $\times 10^{-3}$/g. liver (wet wt.).
* these figures refer to the number of experiments.

TABLE 3. ESTIMATION OF THE PRODUCTS OF THE METABOLISM OF TRITIATED 7,12-DIMETHYLBENZ[a]ANTHRACENE BY LIVER HOMOGENATES ON TLC's

Species	Sex	Age in days	Radioactivity associated with fractions containing: DMBA	12-OHM-7-MBA	3- and 4-OH DMBA*	7-OHM-12-MBA and an unidentified product	7,12-DiOHMBA	3- and 4-Hydroxy derivatives of 7-OHM-12-MBA and 12-OHM-7-MBA†	8,9-Di-HOHDMBA	8,9-DiHOH-7-OHM-12-MBA and 8,9-DiHOH-12-OHM-7-MBA‡	12-MBA-7-COOH and 7-MBA-12-COOH	Total radioactivity counted§
Rat	Male	1	1120	36	28	78	4	3	32	3	4	1343
		15	1180	30	18	51	4	4	19	4	5	1385
		25	218	28	45	95	20	43	313	88	15	939
		35	222	32	28	115	21	31	272	98	18	884
		50	690	38	25	157	11	15	87	20	5	1088
		75	920	40	12	65	5	12	17	3	5	1117
		(Adult)	980	25	13	89	3	8	10	3	5	1189
	Female	1	1180	28	36	80	3	3	28	4	6	1328
		15	1300	22	22	41	4	4	10	3	5	1467
		25	356	21	24	68	9	29	265	85	12	906
		35	360	23	26	71	14	31	205	92	18	967
		50	868	27	15	110	4	6	45	5	5	1205
		75	988	42	11	53	3	3	15	3	5	1168
		(Adult)	994	23	14	48	3	2	8	3	4	1182
Mouse	Male	1	985	53	18	96	6	7	44	3	8	1238
		10	940	39	22	94	5	8	65	2	7	1226
		15	705	48	35	162	11	19	85	32	5	1178
		20	905	41	25	75	5	10	57	14	5	1197
		25	1060	32	18	55	5	3	15	3	5	1218
		(Adult)	1180	31	15	59	3	4	9	3	5	1336
	Female	1	1050	43	15	83	4	7	31	3	7	1260
		10	950	35	15	150	7	7	60	3	5	1253
		(Adult)	1210	30	12	58	3	3	8	2	5	1330
Guinea Pig	Male	(Adult)	604	38	18	48	4	12	225	28	5	998
Hamster	Male	(Adult)	883	28	15	153	6	11	44	9	9	1192

DMBA ($3{\cdot}83 \times 10^6$ cpm) was added to each incubation mixture as described in the text.

The results, which are averages of at least two experiments, are expressed in cpm $\times 10^{-3}$/g. liver (wet wt.) and were obtained as described in the text. Deviations were no larger than those shown in Table 2.

* These results were obtained by combining the cpm measured in fractions 6 and 7.

† These results were obtained by combining the cpm measured in fractions 15 and 16.

‡ These results were obtained by combining the cpm measured in fractions 20–22.

§ The total includes radioactivity counted on the chromatograms but not given in the Table.

markers had been added was chromatographed under the conditions used above. Fractions with the same R_f values as those of the metabolic products were removed from the chromatograms and the radioactivity counted as before. Similarly, control experiments in which boiled rat-liver homogenate was used were carried out. The results of these experiments are included in Table 2.

RESULTS

The probable structures of most of the metabolites listed in Table 1 have been discussed previously,[1, 6] and methods for their detection described. The unidentified product in fraction 9 was not detected in earlier work:[1] its presence was recognised in large-scale incubations of unlabelled DMBA with homogenates of the livers from 25-day-old rats carried out as previously described.[6] When present, the product could be distinguished from 7-OHM-12-MBA by the differences in behaviour of the two compounds in u.v. light when TLC's were examined 24 hr after being developed in benzene. 7-OHM-12-MBA then showed a green fluorescence, presumably because of the formation of oxidation products, whereas the new product showed a violet fluorescence. It was possible to separate the two products to some extent by repeated development of these plates with benzene.

Further evidence for the presence of 4-hydroxy derivatives of 7-OHM-12-MBA and 12-OHM-7-MBA in fractions 15 and 16 was provided by the fact that 4-OHDMBA, when incubated with rat-liver homogenates by the method previously described,[6] yielded a compound (or a mixture of compounds) that was chromatographically identical with the products in fractions 15 and 16 formed both from DMBA and from the monohydroxymethyl derivates.

Table 2 shows the complete distribution of radioactivity on the chromatograms of products obtained both from the control experiments and from typical experiments with fresh homogenates of the livers of both rats and mice of two different age groups. A comparison of Table 1 and 2 shows that all the major regions of radioactivity can be associated with known metabolites of DMBA. *trans*-5,6-Dihydro-5,6-dihydroxy-7,12-dimethylbenz[a]anthracene, which, by analogy with benz[a]anthracene metabolism,[13] was an expected product, was not detected. If washed rat-liver microsomes are used in place of homogenates, however, it is possible to identify the 5,6-dihydrodihydroxy compound (P. L. Grover and P. Sims, unpublished observations). This suggests that there is some form of binding, possibly to protein, involving the 5,6-bond (the K-region) of DMBA: this binding is under investigation.

Table 3 gives the radioactivity present in fractions of the products obtained by incubating DMBA with homogenates prepared from the livers of animals of differing species, age and sex. The fractions chosen are those containing the major proportions of the recovered metabolites. The most striking feature of the results is in the increased rate of metabolism of DMBA shown by liver homogenates from rats and to a lesser extent by those from mice examined during and shortly after weaning. These increases are shown mainly by the smaller amounts of DMBA recovered and by the larger amounts of ring-hydroxylated products, particularly the 8,9-dihydro-8,9-dihydroxy compounds formed. There were no such increases in the amounts of 12-OHM-7-MBA formed: the amounts of 7-OHM-12-MBA formed were less easy to assess because of the presence of the unidentified metabolite described above. It has been shown elsewhere,[5, 6] however, that the hydroxymethyl derivatives are more readily metabolized

in liver preparations from stimulated than from normal rats. The hydroxymethyl derivatives are intermediates in the metabolism of DMBA but the amounts of the products that have probably arisen from their further metabolism (the 3- and 4-hydroxy and the 8,9-dihydro-8,9-dihydroxy derivatives of the hydroxymethyl compounds together with 7,12-DiOHMBA, 12-MBA-7-COOH and the isomeric 7-methylbenz-[a]anthracene-12-carboxylic acid) are much increased in homogenates from the livers of rats examined in the post-weaning period.

In all the experiments it was possible to account for less than one third of the added DMBA in the form of ethyl acetate-soluble compounds. Presumably, the remainder is either converted into water-soluble products or is bound either physically or chemically to cellular constituents such as proteins. It is of interest that the preparations showing the greatest metabolic activities gave the smallest recoveries of ethyl acetate-soluble compounds. Attempts to demonstrate the presence of conjugates of DMBA metabolites with glutathione, glycine or glucuronic or sulphuric acid in the aqueous fraction have so far been unsuccessful.

DISCUSSION

The results presented here, when considered in conjunction with the original observation that liver homogenates from newly weaned rats show an enhanced ability to hydroxylate the 8,9-bond of DMBA,[11] are consistent with the idea that generalized but transient increases in hepatic drug metabolizing systems occur in young animals. These large increases have been ascribed to the stimulation of enzyme activity by compounds present in the diet since it was shown that the onset of the increase can be delayed if the animals are maintained on a milk diet.[11] When these animals are subsequently transferred to a rat-cake diet the hepatic enzyme levels rise. Presumably, the smaller rises in enzyme activity which have now been found in mice are due to similar causes. It is to be expected that the size and duration of these increased enzyme levels would depend on the timing and conditions of weaning and on the nature of the diet and might well vary from laboratory to laboratory.

Attempts to correlate the results obtained by *in vitro* experiments with results obtained in whole animals should be treated with caution, but clearly alterations in the hepatic metabolism of the hydrocarbon could affect its action at sites in the body remote from the point of application, by promoting either the formation of active metabolites or the inactivation of the hydrocarbon by detoxifying mechanisms. The property of DMBA in inducing mammary tumours in rats in well established[12] but hepatic metabolites are apparently not involved: tests with 12-OHM-7-MBA and 4-OHDMBA were negative and 7-OHM-12-MBA produced fewer tumours with longer induction periods than DMBA itself tested under the same conditions.[4, 7] There is some evidence that 30-day-old rats of the Chester–Beatty strain are more susceptible to mammary tumours induced by DMBA than are older animals,[14] but the reverse is true for mammary tumours induced with 3-methylcholanthrene,[15, 16] which is presumably metabolized by the same enzyme systems that metabolize DMBA.

The induction of hepatomas in mice after treatment in the neo-natal state with DMBA has been described.[17] The hepatomas arose mainly in male mice, whereas little sex difference was apparent in the metabolic patterns of DMBA produced by mice-liver homogenates.

It was shown[18] that adrenal necrosis could not be induced in 25-day-old rats that

had been treated with DMBA under the same conditions that induced 100% necrosis in 50-day-old animals. A possible explanation for this is to be found in the high rate of metabolism in the younger animals. The inability of DMBA to induce adrenal necrosis in adult hamsters and mice[12] cannot be explained in this way, however, since the metabolism of DMBA by liver homogenates from these species is similar to that of liver homogenates from adult rats. Adult guinea pigs are also resistant to DMBA-induced adrenal necrosis: their livers are more active in DMBA metabolism than those of adult rats, mice or hamsters but are still less active than those of 25-day-old rats. Alternative suggestions have been made to account for these age and species differences: among these are the high corticosterone contents of adult rat adrenals as compared with those of immature rats[18] and the formation of an unidentified DMBA metabolite by adult rat adrenals that is not formed by those of immature rats or of mice.[19]

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REFERENCES

1. E. BOYLAND and P. SIMS, *Biochem. J.* **95**, 780 (1965).
2. P. H. JELLINCK and B. GOUDY, *Science* **152**, 1375 (1966).
3. P. H. JELLINCK and B. GOUDY, *Biochem. Pharmac.* **16**, 131 (1967).
4. J. W. FLESHER, S. SOEDIGDO and D. H. KELLEY, *J. mednl. Chem.* **10**, 932 (1967).
5. W. LEVIN and A. H. CONNEY, *Cancer Res.* **27** 1931 (1967).
6. E. BOYLAND and P. SIMS, *Biochem. J.* **104**, 394 (1967).
7. E. BOYLAND, P. SIMS and C. HUGGINS, *Nature, Lond.* **207**, 816 (1965).
8. D. N. WHEATLEY, A. G. HAMILTON, A. R. CURRIE, E. BOYLAND and P. SIMS, *Nature, Lond.* **211**, 1311 (1966).
9. J. PATAKI and C. B. HUGGINS, *Biochem. Pharmac.* **16**, 607 (1967).
10. D. N. WHEATLEY, I. R. KERNOHAN and A. R. CURRIE, *Nature, Lond.* **211**, 387.
11. P. SIMS and P. L. GROVER, *Nature, Lond.* **216**, 77 (1967).
12. F. CEFIS and C. M. GOODALL, *Am. J. Path.* **46**, 227 (1965).
13. E. BOYLAND and P. SIMS, *Biochem. J.* **97**, 7 (1965).
14. E. BOYLAND and K. SYDNOR, *Br. J. Cancer* **16**, 731 (1962).
15. T. L. DAO, M. GREINER and H. SUNDERLAND, *Proc. Am. Ass. Cancer Res.* **3**, 14 (1959).
16. C. HUGGINS, L. C. GRAND and F. P. BRILLANTES, *Nature, Lond.* **189**, 204 (1961).
17. F. J. C. ROE and M. A. WALTERS, *Nature, Lond.* **214**, 299 (1967).
18. S. MORII and C. HUGGINS, *Endocrinology* **71**, 972 (1962).
19. P. H. JELLINCK, S. COLES and M. GARLAND, *Biochem. Pharmac.* **16**, 2449 (1967).