Chemical Oxidative Binding of Natural Oestrogens to Deoxyribonucleic Acid

G. Michael Blackburn,* Brian Kellard, and Lawrence Orgee
Department of Chemistry, The University, Sheffield S3 7HF
Michael H. Thompson
Public Health Laboratory Service, CAMR, Salisbury, Wiltshire SP4 OJG

The natural oestrogens, oestrone and oestradiol, are covalently bound to calf thymus deoxyribonucleic acid by the action of iodine and hydrogen peroxide. For oestrone, dual isotopic labelling indicates that these processes involve position 4 and/or 2 of the steroid.

A strong causal linkage has been established between both natural and synthetic oestrogens and a variety of human and animal cancers.[1] The majority of chemical carcinogens are believed to exert their carcinogenic activity *via* covalent interactions with the deoxyribonucleic acid (DNA) of their target tissues.[2,3] Such interactions have been particularly well characterised in the case of the polycyclic aromatic hydrocarbon benzo[*a*]pyrene.[4,5] It is noteworthy that benzo[*a*]pyrene, which has been shown to exhibit oestrogenic properties,[6] can bind physically to DNA.[7] Moreover, diethylstilboestrol, which is a synthetic oestrogen with established carcinogenic properties, has been bound to DNA by chemical[8] and by metabolic[9] oxidative processes.

In vitro studies involving the physical association of the natural oestrogens with DNA,[10–14] coupled with the above work, prompted an investigation into the covalent binding of these compounds to DNA. In particular, the availability of oestrone tritiated in positions 2, 4, 6, and 7 and of oestradiol tritiated only in positions 6 and 7 offered the possibility of regioselective identification of the steroidal site of binding to DNA as a consequence of radiochemical analysis of covalent adducts. We present here a full account of our studies on such chemical binding, early results having been published in a preliminary communication.[15]

Experimental

Materials and Methods.—DNA was supplied by Sigma (calf thymus Type V, average molecular weight $> 10^6$), and by Koch–Light. All enzymes, oestrone, oestradiol, and NADPH were supplied by Sigma. TCPO, 3,3,3-trichloropropene 1,2-oxide, was obtained from Aldrich.

[4-^{14}C]Oestrone, [4-^{14}C]oestradiol, [2,4,6,7-^{3}H]oestrone, and [6,7-^{3}H]oestradiol were purchased from the Radiochemical Centre, Amersham, Bucks. Unisolve 1 liquid scintillant was obtained from Koch–Light, and Sephadex LH-20 powder from Pharmacia.

Determination of Protein.—The protein content of DNA solutions was assayed by the Folin–Ciocalteu method using the procedure due to Lowry.[16]

Preparation of DNA–Oestrogen Physical Complexes.—Radioactive and non-radioactive oestrogen were admixed and applied to silica t.l.c. plates which were developed with chloroform–acetone (7:3, v/v). The oestrogen was eluted with chloroform, evaporated in a stream of dry nitrogen, and dissolved in the minimum quantity of pure dioxane. The solution was dispersed into a solution of DNA (2.5 mg DNA per ml HMP buffer) to give activities of 0.1—1.0 μCi [^{3}H]oestrogen, 0.1—0.2 μCi [^{14}C]oestrogen, and 0.1 μmol non-radioactive oestrogen per ml DNA solution. The solution was then equilibrated in the dark for 72 h at 4 °C on an inclined rotating turntable, filtered through Whatman No. 1 paper, and kept at 4 °C prior to use. Portions (1 ml) were removed for acid hydrolysis and radioactive assay.[17]

Preparation of Covalently Bound DNA–Oestrogen Complexes.—(1) *Iodine-mediated binding.* Varying amounts of iodine, dissolved in pure dioxane (0.01 ml), were added to portions (2 ml) of the filtered DNA–oestrogen solutions. These were then incubated in the dark at room temperature for 24 h. The mixture was then treated with saturated ethanolic ammonium acetate (10 ml) and the precipitated DNA pellet washed thrice with absolute ethanol (4 ml) and twice with ether (4 ml) and air-dried. The pellet was dissolved in HMP buffer (1 ml), acid hydrolysed, and assayed for radioactivity.[14]

(2) *Hydrogen peroxide-mediated binding.* Sodium dihydrogen citrate (0.122 g) was dissolved in filtered solutions of DNA–oestrogen (35 ml). Hydrogen peroxide was then added (final concentration 30mM) and the solutions were incubated at 37 °C in the dark for 3 days. Portions (3 ml) were removed at varying times for DNA precipitation and washing, as described above, prior to radioactive assay. In some incubations citrate was omitted while in other incubations it was replaced by iron(II) sulphate (1.7 mg per 3 ml).

Enzymic Hydrolysis of Covalent Complexes.—This was based on the procedure of Rapaport and Ts'o.[18] A portion of a DNA–oestrogen washed, covalent complex was dissolved in neutral TRIS–$MgCl_2$ buffer (2 ml, 5 mg DNA; 0.01M-TRIS; 0.01M-$MgCl_2$) and treated with DNase 1 (650k units) at 37 °C for 5 h. The solution was then diluted 1:1 with alkaline TRIS buffer (0.1M; pH 9.1) and incubated at 37 °C with snake venom phosphodiesterase (0.22 units) for 24 h, followed by incubation at 37 °C with alkaline phosphatase (6 units) for 20 h.

The solution was then lyophilised to dryness and taken up in water (3.5 ml). Centrifugation at 250 × *g* deposited insoluble material which was extracted with methanol (1.5 ml). The combined supernatants were applied to a column (1.5 × 85.0 cm) of Sephadex LH-20 gel filtration medium. A gradient of 30% to 100% methanol in water (1 l total) was pumped through the column at a rate of 29 ml h^{-1}, 4.8 ml fractions being monitored for u.v. absorption (260 nm) and radioactivity.

Acid Hydrolysis of DNA–Oestrogen Complexes.—Portions (1 ml) of DNA–oestrogen washed, covalent complexes, prepared as outlined above, were placed in thick-walled glass tubes together with HCl (1 ml, 8M), the contents mixed, and the tubes sealed. After hydrolysis at 120 °C for 2 h, the contents were cooled and neutralised at < 10 °C with ammonia solution (1 ml; *d* 0.88).[19] Finally, portions (1 ml) of the hydrolysate were added to scintillation vials containing Unisolve 1 (14 ml) and assayed for radioactivity (Packard Tricarb 3385) using an automatic external standardisation facility.

By means of carbon tetrachloride quenching of radioactive

Table 1. Iodine-mediated binding of oestrone to DNA

	^3H (c.p.m.)	^{14}C (c.p.m.)	^3H (d.p.m.)	^{14}C (d.p.m.)	$^3H/^{14}C$	^3H retention %	μmoles oestrone per mole DNA base pair: gross	μmoles oestrone per mole DNA base pair: net
$[I_2]$ 0 (control)	72	45	193.8	131.5	1.47 ± 0.13	177.1 ± 15.7	15.0 ± 0.7	
$[I_2]$ 10^{-7}M	64	42	170.5	124.8	1.37 ± 0.13	165.1 ± 15.3	14.3 ± 0.7	(−0.7 ± 1.4)
$[I_2]$ 10^{-6}M	70	52	180.8	152.0	1.19 ± 0.10	143.4 ± 12.3	17.4 ± 0.8	2.4 ± 1.5
$[I_2]$ 10^{-5}M	108	115	255.3	338.0	0.76 ± 0.05	91.6 ± 5.7	38.8 ± 1.1	23.8 ± 1.8
$[I_2]$ 10^{-4}M	201	267	438.3	789.0	0.55 ± 0.02	66.3 ± 2.8	90.6 ± 1.8	75.6 ± 2.5
$[I_2]$ 10^{-3}M	408	612	838.0	1 841.8	0.45 ± 0.01	54.2 ± 1.6	211.3 ± 2.7	196.3 ± 3.4

Initial DNA solution $^3H/^{14}C$ ratio 0.83 ± 0.01; Samples counted for 10 min; Net binding = (gross binding − control); Steroid–DNA binding calculated from ^{14}C d.p.m.; DNA, Sigma type V.

Table 2. Iodine-mediated binding of oestradiol to DNA

	^3H (c.p.m.)	^{14}C (c.p.m.)	^3H (d.p.m.)	^{14}C (d.p.m.)	$^3H/^{14}C$	^3H retention %	μmoles oestradiol per mole DNA base pair: gross	μmoles oestradiol per mole DNA base pair: net
$[I_2]$ 0 (control)	110	51	311.8	164.5	1.81 ± 0.14	154.5 ± 12.0	18.9 ± 0.8	
$[I_2]$ 10^{-7}M	115	51	322.8	156.8	2.06 ± 0.16	167.5 ± 12.9	18.0 ± 0.8	(−0.9 ± 1.6)
$[I_2]$ 10^{-6}M	103	44	291.3	137.3	2.13 ± 0.18	173.2 ± 13.9	15.7 ± 0.7	(−3.2 ± 1.5)
$[I_2]$ 10^{-5}M	215	147	565.3	433.8	1.30 ± 0.06	105.7 ± 5.2	49.9 ± 1.3	31.0 ± 2.1
$[I_2]$ 10^{-4}M	535	383	1 389.8	1 109.8	1.25 ± 0.04	101.6 ± 3.1	127.6 ± 2.1	108.7 ± 2.9
$[I_2]$ 10^{-3}M	1 010	729	2 626.8	2 157.5	1.22 ± 0.02	99.2 ± 1.3	248.1 ± 2.9	229.2 ± 3.7

Initial DNA solution $^3H/^{14}C$ ratio 1.23 ± 0.01; Samples counted for 10 min; Net binding = (gross binding − control); Steroid–DNA binding calculated from ^{14}C d.p.m.; DNA, Sigma type V.

Table 3. Iodine-mediated binding of oestrone to DNA

	^3H (c.p.m.)	^{14}C (c.p.m.)	^3H (d.p.m.)	^{14}C (d.p.m.)	$^3H/^{14}C$	^3H retention %	μmoles oestrone per mole DNA base pair: gross	μmoles oestrone per mole DNA base pair: net
(1) DNA(K.L.)								
$[I_2]$ 0 (control)	52.5	10.0	331.0	19.0	17.40 ± 2.50	2 718.8 ± 390.6	3.6 ± 0.4	
$[I_2]$ 10^{-3}M	146.5	880.5	922.5	1 650.5	0.56 ± 0.02	87.5 ± 3.1	313.5 ± 3.4	309.9 ± 3.8
$[I_2]$ 10^{-2}M	164.5	911.0	1 037.0	1 709.0	0.61 ± 0.02	95.3 ± 3.1	324.6 ± 3.4	321.6 ± 3.8
Initial DNA solution $^3H/^{14}C$ ratio 0.64 ± 0.01								
(2) DNA (σ)								
$[I_2]$ 0 (control)	45.0	107.0	286.0	201.0	1.42 ± 0.11	218.5 ± 1.2	38.2 ± 1.2	
$[I_2]$ 10^{-3}M	89.0	504.0	562.5	947.0	0.59 ± 0.03	90.8 ± 4.6	179.9 ± 2.5	141.7 ± 3.7
$[I_2]$ 10^{-2}M	93.5	459.5	564.0	875.0	0.64 ± 0.03	98.5 ± 4.6	166.2 ± 2.5	128.0 ± 3.7

Initial DNA solution $^3H/^{14}C$ ratio 0.65 ± 0.01; Samples counted for 10 min; Net binding = (gross binding − control); Steroid–DNA binding calculated from ^{14}C d.p.m.; DNA(KL), Koch–Light calf thymus DNA, protein content 2%; DNA (σ), Sigma type V calf thymus DNA, protein content 8%.

toluene standards a quench curve was constructed for Unisolve 1 and the data were processed using a Wang Series 700 computer. All data presented are based on multiple counts each of 10 min, of at least two portions of sample and corrected for background. The results Tables give error analyses derived from counting statistics.

Results

A number of general points deserve comment prior to an examination of the levels of oestrogen–DNA binding achieved in any specific system. (1) As a result of criticism that tritium cannot be estimated accurately in the presence of undegraded DNA,[20] all radioactive assays were carried out after acid hydrolysis of the DNA present. Although changes in the total ^3H d.p.m. on acid hydrolysis were found to be less than 5%,[21] we decided to employ this procedure routinely. (2) The level of binding of oestrogens to DNA in any particular system is conditioned by such factors as the rate of addition of DNA to the dioxane solution of the oestrogen and the degree of agitation of the mixture immediately afterwards. (3) At low counting levels there was an apparent *increase* in the ^3H:^{14}C ratio relative to that of starting material.[19]

The binding of oestrone and oestradiol to calf thymus DNA (Sigma) was first studied using iodine. Covalent binding data are given for both steroids (Tables 1 and 2). These show there to be essentially no binding above that of the controls (*i.e.* zero iodine concentration) for iodine concentrations below 10^{-5}M. In covalent complexes of oestradiol the ^3H:^{14}C ratio was

Table 4. Hydrogen peroxide-mediated binding of oestrogens to DNA

	^3H (c.p.m.)	^{14}C (c.p.m.)	^3H (d.p.m.)	^{14}C (d.p.m.)	$^3H/^{14}C$	^3H retention %	μmoles oestrogens per mole DNA base pair: gross	μmoles oestrogens per mole DNA base pair: net
(1) Oestrone								
Hydrogen peroxide	168.0	122.0	371.2	307.5	1.21 ± 0.07	77.6 ± 4.2	78.9 ± 2.3	59.6 ± 3.3
Hydrogen peroxide and sodium dihydrogen citrate (16.28mM)	115.0	50.0	251.5	106.8	2.35 ± 0.18	150.6 ± 11.7	27.4 ± 1.2	8.1 ± 2.2
Hydrogen peroxide and ferrous sulphate (2.04mM)	710.0	571.0	1 718.8	1 698.5	1.01 ± 0.03	64.7 ± 1.6	435.9 ± 5.8	416.6 ± 6.8
Initial DNA solution $^3H/^{14}C$ ratio 1.56 ± 0.01								
(2) Oestradiol								
Hydrogen peroxide	154.0	88.0	348.5	212.3	1.64 ± 0.01	101.2 ± 6.9	54.5 ± 1.8	40.3 ± 2.6
Hydrogen peroxide and sodium dihydrogen citrate (16.28mM)	114.0	52.0	248.5	115.0	2.16 ± 0.17	133.3 ± 11.2	29.5 ± 1.3	15.3 ± 2.1
Hydrogen peroxide and ferrous sulphate (2.04mM)	544.0	304.0	1 403.2	889.4	1.58 ± 0.05	97.5 ± 3.8	228.4 ± 4.1	214.6 ± 4.9
Initial DNA solution $^3H/^{14}C$ ratio 1.62 ± 0.01								

Samples counted for 10 min; net binding = (gross binding − blank); steroid–DNA binding calculated from ^{14}C d.p.m.; DNA, (Koch–Light); [hydrogen peroxide] 30mM throughout.

essentially independent of the level of steroid binding and was the same as that of the initial solution. However, at the highest levels of binding of oestrone to DNA there was a loss of *ca.* 45% of the tritium relative to carbon-14.

The level of steroid binding was observed to vary somewhat with the origin of the calf thymus DNA used. The results of comparative binding experiments for oestrone (Table 3) show an increase in the level of oestrone to DNA (Koch–Light) over that to DNA (Sigma). This increase was 54% when 10^{-3}M-iodine was employed and 60% for 10^{-2}M-iodine. A protein assay[16] of both types of DNA gave the following results: calf thymus DNA (Koch–Light) 0.05 mg protein per 2.5 mg DNA sample = 2% protein; calf thymus DNA (Sigma) 0.20 mg protein per 2.5 mg DNA sample = 8% protein. The results of hydrogen peroxide-mediated binding of oestrogens to DNA are shown (Table 4). Addition of iron(II) sulphate (2.04mM) increased binding levels by *ca.* 7-fold in the case of oestrone and 5-fold for oestradiol. Conversely, addition of sodium dihydrogen citrate (16.28mM) to hydrogen peroxide (30mM) resulted in oestrogen–DNA binding levels substantially lower than those obtained with hydrogen peroxide alone. There was a loss of *ca.* 22% in the tritium content relative to the initial solution for hydrogen peroxide-induced binding of oestrone to DNA, increasing to 35% when iron(II) sulphate was also present. For oestradiol there was no significant loss of tritium.

Discussion

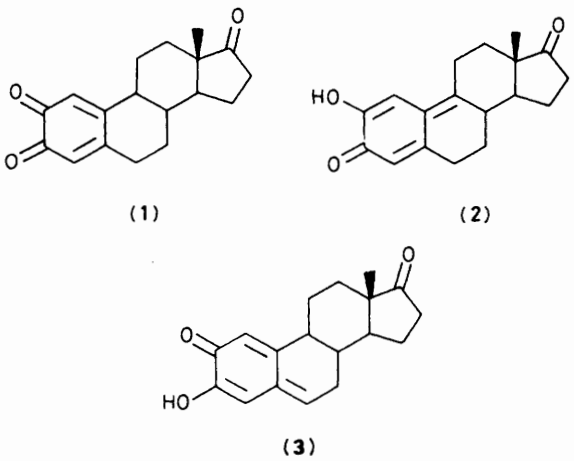
The well established iodine-mediated binding of polycyclic aromatic hydrocarbons[22,23] and of diethylstilboestrol[8] to DNA *in vitro* prompted the examination of the natural oestrogen hormones, oestrone and oestradiol, under similar conditions. The results described here (Tables 1 and 2) show that iodine concentrations of 10^{-5}M and higher cause covalent binding of both oestrogens to calf thymus DNA which attains levels up to 200 μmol of steroid per mole DNA base pair. This is about half the level of iodine-promoted binding achieved for diethylstilboestrol.[8] The relative loss of tritium for oestrone which is labelled at C-2, -4, -6, and -7 increased with the extent of covalent binding and approaches a limiting value of 50% (Table 1). By contrast, the DNA-binding of oestradiol, tritiated at C-6 and C-7, shows *no* loss of tritium (Table 2). This result clearly implicates substitution at C-2 and C-4 in the overall covalent binding process as the distribution of tritium shown by ^3H n.m.r. is 50% in positions 2 and 4 and 50% in positions 6 and 7.* Moreover, we have observed[24] that direct iodination[25] of [2,4,6,7-^3H] oestrone affords a mixture of 2-iodo-, 4-iodo-, and 2,4-di-iodo-oestrone and the latter product has lost close to 50% of the tritium of the parent steroid. The large primary kinetic isotope effect observed[26] for the iodination of phenols prevents meaningful analysis of the monoiodo-oestrones. While, therefore, it is evident that iodine-promoted binding of oestrogens to DNA involves loss of tritium from positions 2 and 4, with no loss from positions 6 and 7, it is not yet possible to say whether this result implicates steroid binding to DNA *via* C-2 or C-4 since the same loss of tritium would be observed to result either from binding pre-iodinated oestrogen to DNA or from the iodination of the steroid following its covalent attachment to DNA. While we favour the possibility that the covalent attachment of steroid to DNA results from iodine activation of the oestrogen, we also note that iodine reacts directly with DNA and modifies guanine and, more readily, cytosine bases.[27]

We found that the extent of covalent binding of oestrone to calf thymus DNA at higher levels varied somewhat with the source of the DNA. An examination of the protein content of the two samples of DNA showed the greater degree of oestrone binding to the DNA having the *lower* protein content. While this result substantially established that the covalent binding involves the DNA itself and not DNA-associated protein, it could also be influenced by some partial denaturation of the two DNA samples.†

Hydrogen peroxide has also been employed to bind polycyclic aromatic hydrocarbons to DNA[23,28] though it is noticeably less effective in the case of diethylstilboestrol, for which binding to DNA necessitates the addition of iron(II) sulphate to the system.[8] As a model for a biological system, hydrogen peroxide is certainly more realistic than iodine oxidation. *Inter alia* it is

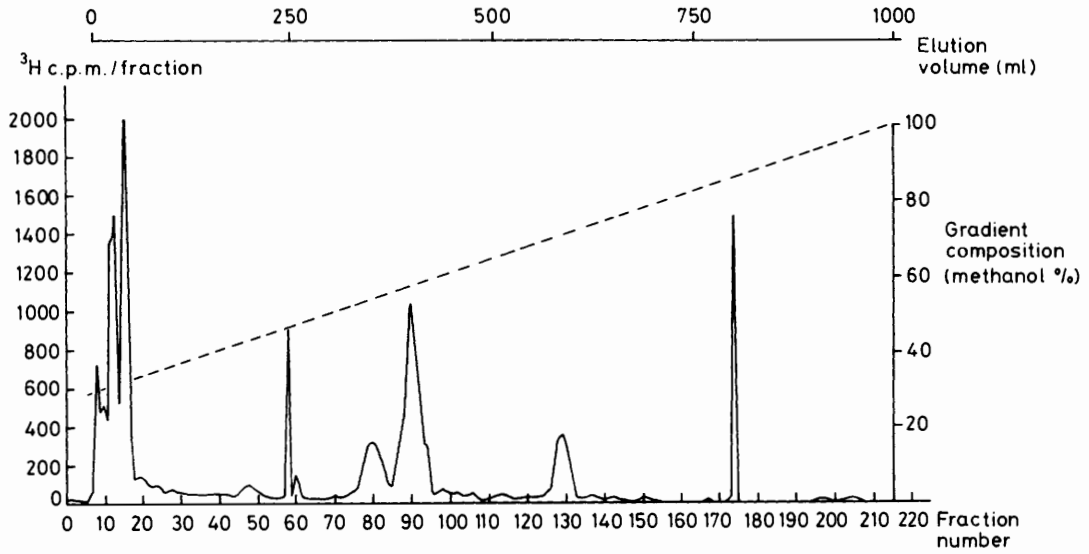
* Information provided by Amersham International plc.

† Both samples showed *ca.* 30—35% hypochromicity.



Such a pattern would be compatible, for instance, with C-2 hydroxylation followed by further oxidation to either the *ortho*-quinone (**1**) or the quinomethide (**2**) which could be expected to act as alkylating agents with respect to DNA.[32] The alternative quinomethide (**3**) would be associated with partial loss of tritium from position 6 which is not observed.

In an attempt to characterise an oestrogen–DNA adduct, LH-20 chromatographic fractionation was employed on a washed covalent complex which had been enzymically degraded to phosphate-free components. In a typical result from these studies (Figure) radioactive peaks were observed in the region above 400 ml elution volume, which is the region in which Baird and Brookes have characteristically detected nucleoside–hydrocarbon adducts from similar experiments involving polycyclic aromatic hydrocarbons.[21,33,34,*] Moreover the principal products (eluted by methanol–55% water) are rather more polar than is oestradiol itself (which is eluted by methanol–35% water). This feature may also indicate that the nucleoside oestradiol species resulting from hydrogen peroxide mediated binding has been further hydroxylated on the steroid moiety in the course of the binding process.



Gel filtration pattern for DNA treated with [^{3}H]oestradiol and hydrogen peroxide after enzymic hydrolysis: LH-20 Sephadex with water–methanol gradient

thought to be a product of the respiratory burst which is required for the binding of oestradiol to phagocytosing polymorphonuclear luekocytes since such binding is inhibited by catalase yet stimulated by superoxide dismutase.[29] Moreover, the superoxide anion-radical has itself been implicated in the binding of certain oestrogens to cellular macromolecules.[30] It is also relevant that recent studies have implicated a peroxidative mechanism in the carcinogenicity of diethylstilboestrol.[31,32]

In common with earlier work, the results of the present study (Table 4) show that the hydrogen peroxide-mediated binding of oestrone and oestradiol to DNA is markedly inhibited by citrate but stongly enhanced, up to 7-fold, by iron(II) sulphate. The concomitant loss of tritium is similar in pattern to that observed for iodine-promoted binding, but of lesser degree. Thus *ca.* 35% of the tritium content of the steroid is lost on covalent binding at a level of 400 μmoles oestrone per mole DNA base pair but none is lost for oestradiol. This again identifies positions 2 and 4 as possible sites of substitution but with the clear implication that only *one* of them is involved.

While the hydrogen peroxide system provides an analogue which relates to some aspects of biological transformation of oestrogens, it is not a close model for the dominant biological oxidations of oestrogens. These are brought about by the cytochrome P-450 monoxygenases (or mixed-function oxidases)[33] which are found in the microsomal fraction of cells.[34] We shall report our conclusions on detailed studies of the microsomal activation of oestrogens in the presence of DNA in a subsequent paper.

Finally, we note that the phenomenon of DNA modification resulting from the iodine or hydrogen peroxide activation of oestrogens may have unexpected implications for the use of these agents as antiseptics for wound dressing.

* We found[21] no covalent binding of either oestrone or oestradiol to DNA as a result of the use of γ-radiation (up to 50 krad), or by superoxide ions. Apparently high binding levels achieved by the use of tyrosinase were reduced to effectively zero by phenol extraction.

Acknowledgements

We thank the Yorkshire Cancer Research Campaign for Studentships (to B. K. and L. O.).

References

1 J. J. Li, S. A. Li, J. K. Klicka, J. A. Parsons, and L. K. T. Lam, *Cancer Res.*, 1983, **43**, 5200; J. A. McLachlan, A. Wong, G. H. Degan, and J. C. Barrett, *ibid.*, 1982, **42**, 3040; M. A. Kirschner, G. Schneider, N. H. Ertel, and E. Worton, *ibid.*, p. 3281; R. Hertz, *J. Steroid Biochem.*, 1979, **11**, 435.
2 P. Brookes, in 'Biological Reactive Intermediates,' eds. D. J. Jallow, J. J. Kocais, R. Snyder, and H. Vainio, Plenum, New York and London, 1977, p. 470.
3 O. Pelkonen and D. W. Nebert, *Pharm. Rev.*, 1982, **34**, 189; F. Oesch, in 'Chemical Carcinogenesis,' ed. C. Nicolini, Plenum, New York and London, 1982, p. 1.
4 P. Brookes and P. D. Lawley, *Nature (London)*, 1964, **202**, 781.
5 A. M. Jeffrey, K. W. Jennette, S. H. Blobstein, I. B. Weinstein, F. A. Beland, R. G. Harvey, H. Kasai, I. Miura, and K. Nakanishi, *J. Am. Chem. Soc.*, 1976, **98**, 5714.
6 J. M. Cook and E. C. Dodds, *Nature (London)*, 1933, **131**, 205.
7 M. Craig and I. Isenberg, *Biopolymers*, 1970, **9**, 689.
8 G. M. Blackburn, A. J. Flavell, and M. H. Thompson, *Cancer Res.*, 1974, **34**, 2015; A. J. Flavell, Ph.D. Thesis, Sheffield University, 1975.
9 G. M. Blackburn, M. H. Thompson, and H. W. S. King, *Biochem. J.*, 1976, **158**, 643.
10 P. O. P. Ts'o and P. Lu, *Proc. Natl. Acad. Sci. USA*, 1964, **51**, 17.
11 M. L. Goldberg and W. A. Atchley, *Proc. Natl. Acad. Sci. USA*, 1966, **55**, 989.
12 P. Cohen, R.-C. Chin, and C. Kidson, *Biochemistry*, 1969, **8**, 3603.
13 S. K. Arya and J. T. Yang, *Biochemistry*, 1975, **14**, 963.
14 G. M. Blackburn, B. Kellard, A. Rashid, and M. H. Thompson, *Biochem. Pharmacol.*, 1983, **32**, 2571.
15 G. M. Blackburn, L. Orgee, and G. M. Williams, *J. Chem. Soc., Chem. Commun.*, 1977, 386.
16 O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 1951, **193**, 265.
17 G. M. Blackburn, J. Buckingham, R. G. Fenwick, P. Taussig, and M. H. Thompson, *J. Chem. Soc., Perkin Trans. 1*, 1973, 2809.
18 S. A. Rapaport and P. O. P. Ts'o, *Proc. Natl. Acad. Sci. USA*, 1966, **55**, 381.
19 G. M. Blackburn, A. J. Flavell, L. Orgee, J. P. Will, and G. M. Williams, *J. Chem. Soc., Perkin Trans. 1*, 1981, 3196.
20 M. R. Osborne, M. H. Thompson, H. W. S. King, and P. Brookes, *Int. J. Cancer*, 1975, **16**, 659.
21 L. Orgee, Ph.D. Thesis, Sheffield University, 1981.
22 Y. Pascal, F. Pochon, and A. M. Michelson, *Biochimie*, 1971, **53**, 365.
23 S. A. Lesko, P. O. P. Ts'o, and R. S. Umans, *Biochemistry*, 1969, **8**, 2291.
24 G. M. Williams, Ph.D. Thesis, Sheffield University, 1978.
25 W. Brown and A. B. Turner, *J. Chromatogr.*, 1967, **26**, 518.
26 E. Grovenstein and D. C. Kilby, *J. Am. Chem. Soc.*, 1957, **79**, 2972.
27 S. L. Commerford, *Biochemistry*, 1971, **10**, 1993.
28 C. E. Morreal, T. L. Dao, K. Eskins, C. L. King, and J. Dienstag, *Biochim. Biophys. Acta*, 1968, **169**, 224.
29 S. J. Klebanoff, *J. Exp. Med.*, 1977, **145**, 983.
30 S. D. Nelson, J. R. Mitchell, E. Dybing, and H. A. Sasame, *Biochem. Biophys. Res. Commun.*, 1976, **70**, 1157.
31 M. Metzler, *Biol. Res. Pregnancy*, 1982, **3**, 103.
32 J. G. Liehr, A. M. Ballatore, J. A. McLachlan, and D. A. Sirbasku, *Cancer Res.*, 1983, **43**, 2678.
33 W. M. Baird and P. Brookes, *Cancer Res.*, 1973, **33**, 2378.
34 H. W. S. King, M. H. Thompson, and P. Brookes, *Cancer Res.*, 1975, **34**, 1263.

Received 21*st May* 1984; *Paper* 4/826