Journal of Steroid Biochemistry, 1978, Vol. 9, pp. 1027–1032. Pergamon Press. Printed in Great Britain

POTENTIATION OF STEROID BINDING TO PROTEINS BY 7,12-DIMETHYLBENZ(α)ANTHRACENE*

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(*Received* 16 *January* 1978)

SUMMARY

In cytosols prepared from mammary gland parenchyma of adrenalectomised, ovariectomised 50-day old rats, DMBA (10^{-6} M) did not affect the binding of [^{3}H]-oestradiol or [^{3}H]-dexamethasone, but increased ~ten-fold the binding of 10^{-8} M [^{3}H]-progesterone [^{3}H]-P. Subsequent studies have shown (i) such potential binding is steroid specific, being displaceable by excess radioinert P (ii) no strict protein specificity exists; in addition to mammary gland and uterine cytosol, DMBA potentiates [^{3}H]-P binding to BSA, ovalbumin, catalase and rat plasma (iii) of a range of steroids studied, DMBA potentiates the binding of [^{3}H]-P > [^{3}H]-R 5020 (17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione) ≫ [^{3}H]-deoxycorticosterone; minimal effects were seen with other [^{3}H]-steroids (iv) 3-methylcholanthrene has a similar but much less marked potentiating effect on steroid binding (v) over the range of protein and DMBA concentrations studied, potentiation of [^{3}H]-P binding increased with increasing DMBA concentrations, and decreased with increasing protein concentrations (vi) binding of [^{3}H]-P as influenced by DMBA involves co-operative phenomena; under given conditions a ten-fold increase in DMBA concentration leads to a hundred-fold increase in bound [^{3}H]-P. Parallel studies on [^{3}H]-DMBA binding demonstrate no departure from linearity.

Classically the carcinogenic action of DMBA has been thought to involve an interaction with cell nuclei. Since DMBA may increase albumin binding of progesterone a thousand-fold, it may have activity at a precellular level via modification of the effector actions of progesterone.

INTRODUCTION

The aromatic hydrocarbon 7,12-dimethylbenz(α)-anthracene (DMBA) is a highly potent carcinogen, a single 20 mg intragastric dose producing breast tumours within 4–6 weeks of administration to immature female Sprague–Dawley rats [1]. Tumours produced in this fashion are characterised by their marked hormone dependence [2]. In 100% of cases, either ovariectomy plus adrenalectomy, or simply ovariectomy alone, results in complete tumour regression [3, 4]. Tumours which have regressed after such ablation can be restimulated by administration of oestrogen and progesterone [2, 4].

The carcinogenic mechanism of DMBA is unknown. Aromatic hydrocarbons are, in general, planar structures known to intercalate between strands of DNA [5]. This is true for noncarcinogenic as well as carcinogenic aromatic hydrocarbons. DMBA, however, is a non-planar molecule and although binding to DNA has been demonstrated, the exact mechanism has not been delineated at a molecular level [6]. As well as binding to DNA, the hydrocarbon has also been shown to bind, apparently covalently, to cytoplasmic proteins [7].

Tumours produced as a result of DMBA administration have been widely studied as a model for human breast cancer. The molecular mechanism of DMBA tumour induction has as yet not been fully elucidated. In the main, published reports concentrate on DMBA interaction with nucleic acids [5, 6, 8] possibly at the expense of the cytoplasmic and extracellular environment of the target cell. The need for ovarian steroids to be present for tumorigenesis has been well documented. In addition, the molecular structure of DMBA is such that it closely resembles that of progesterone (Fig. 1).

We therefore examined the possibility that the carcinogenic action of DMBA may be through an interaction with target tissue receptors for steroid hormones. Our study shows that DMBA enhances the

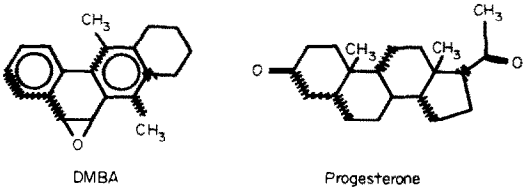

Fig. 1. Molecular structures of progesterone and DMBA. Those areas of each molecule which show structural homology are shaded [from Glusker *et al.*, 15].

* Supported by a Grant-in-Aid from the Anti-Cancer Council of Victoria. Presented in part at the 59th Meeting of the Endocrine Society, Chicago, June 1977, and at the 11th Annual Meeting of the European Society for Clinical Investigation, Rotterdam, 1977.

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binding of steroids, in particular progesterone, to both intracellular and extracellular proteins. The possible contribution of such an effect to the mechanisms of DMBA carcinogenesis is discussed.

MATERIALS AND METHODS

The following tritiated compounds were obtained from Amersham-Searle (U.K.): aldosterone ([^{3}H]-A, 51 Ci/mmol) corticosterone ([^{3}H]-B, 45 Ci/mmol), deoxycorticosterone ([^{3}H]-DOC, 44 Ci/mmol), 5α-dihydrotestosterone ([^{3}H]-DHT, 48 Ci/mmol), dexamethasone ([^{3}H]-DM, 26 Ci/mmol), cortisol ([^{3}H]-F, 41 Ci/mmol), oestradiol-17β ([^{3}H]-E_2, 42 Ci/mmol), progesterone ([^{3}H]-P, 42 Ci/mmol) and DMBA ([^{3}H]-DMBA, 13.1 Ci/mmol). Radioinert steroids were obtained from Calbiochem (Los Angeles). Radioinert DMBA was obtained from Eastman-Kodak. BSA (Cohn V fraction, Commonwealth Serum Laboratories, Melbourne), ovalbumin (Grade A, Egg Marketing Board, N.S.W.) and catalase (Boehringer Mannheim, Germany) were all dissolved in buffer (0.1 M Tris-HCl, 0.003 M $CaCl_2$, pH 7.4). Plasma from adrenalectomised, ovariectomised rats was diluted in the same buffer. Mammary gland and uterine cytosols were prepared as described previously [9].

Incubations were performed in micro test tubes (Eppendorf 3810) in ice in a reciprocating shaker bath. Protein solutions were incubated for 90 min with radiolabelled tracer, either alone, with DMBA and/or with unlabelled steroid, in a final incubation volume of 1 ml.

To separate protein bound tracer from that unbound, the incubate was passed at 4°C through 3.6 ml of G-50 (fine) Sephadex (Pharmacia, Sweden) in 5 ml glass pipettes. Under such conditions, molecules M.W. > 25,000 elute in the 1.6 ml excluded volume.

Radioactivity was determined by adding 1 ml of the excluded volume to 10 ml of scintillation fluid (POPOP, 3.77 g; PPO, 0.25 g; toluene 1667 ml; teric x-10, 833 ml) and counted in a liquid scintillation spectrometer (Packard Tri-Carb, Model 3375) for 10 min or 10,000 total counts. The remainder of the excluded volume was used to determine protein concentration [10].

RESULTS

A synopsis of initial results using mammary gland and uterine cytosol preparations is shown in Figs 2–4. Cytosols were prepared from 45 day old adrenalectomised, ovariectomised rats which were either untreated or oestrogen primed (oestradiol benzoate 0.4 mg/100 g s.c. in 0.1 ml maize oil on days −3, −2, −1 before sacrifice). After incubation for 90 min with [^{3}H]-P 10^{-8} M, alone or with DMBA 10^{-6} M, bound [^{3}H]-P was separated from residual free [^{3}H]-P by gel chromatography. Figure 2 shows binding of [^{3}H]-P in cytosols prepared from mammary gland and uterus of control or E_2 primed animals. In unprimed uterus, unprimed mammary gland and primed mammary gland, the level of [^{3}H]-P bound is greatly increased in the presence of 10^{-6} M DMBA. In contrast with the lack of effect of E_2 priming on mammary gland cytosols, [^{3}H]-P binding is elevated in uterine cytosols prepared from oestrogen primed animals. In addition DMBA has no further potentiating effect on [^{3}H]-P binding in oestrogen primed cytosols.

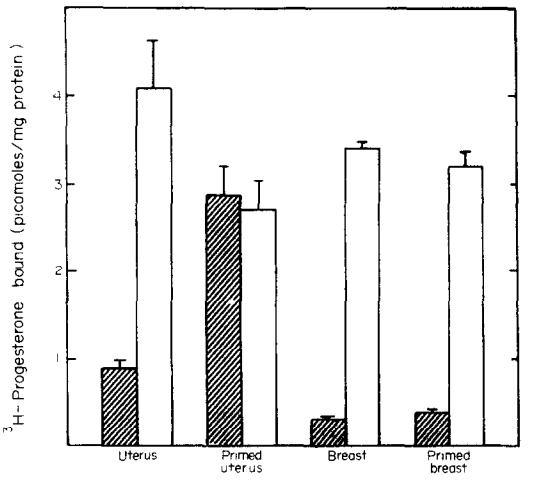

Fig. 2. Binding of [^{3}H]-P to cytosol proteins; influence of DMBA. [^{3}H]-P 10^{-8} M was incubated alone (stippled bars) or with DMBA 10^{-6} M (open bars) with cytosols prepared from uterus and mammary gland from rats either untreated, or primed with 0.4 μg/100 g oestradiol benzoate daily dissolved in 0.1 ml oil for 3 days prior to sacrifice. Values are plotted as Mean ± S.D., $n = 4$.

That the enhanced binding of [^{3}H]-P in the presence of DMBA is specific in the limited sense of being displaceable by excess unlabelled progesterone is

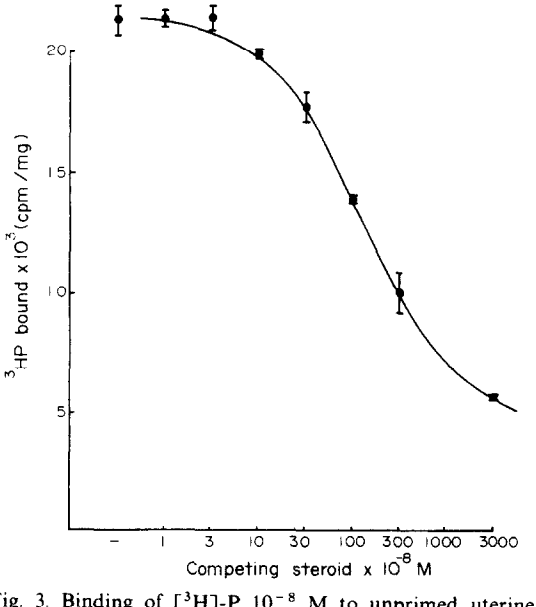

Fig. 3. Binding of [^{3}H]-P 10^{-8} M to unprimed uterine cytosol in the presence of DMBA 10^{-6} M; displacement by increasing concentrations of unlabelled progesterone, ($n = 4$).

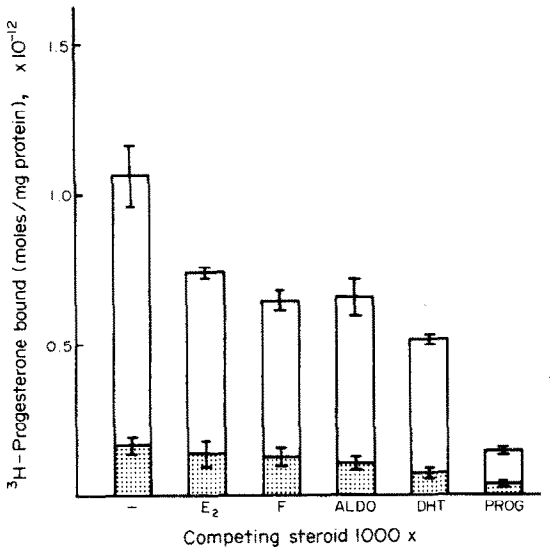

Fig. 4. Binding of [^{3}H]-P to uterine cytosol proteins: displacement by competing steroids. [^{3}H]-P 10^{-8} M was incubated alone (stippled bars) or with DMBA 10^{-6} M (open bars) either alone or with 1000 fold molar excess of unlabelled steroids: oestradiol-17β cortisol, aldosterone, dihydrotestosterone and progesterone. (n = 4).

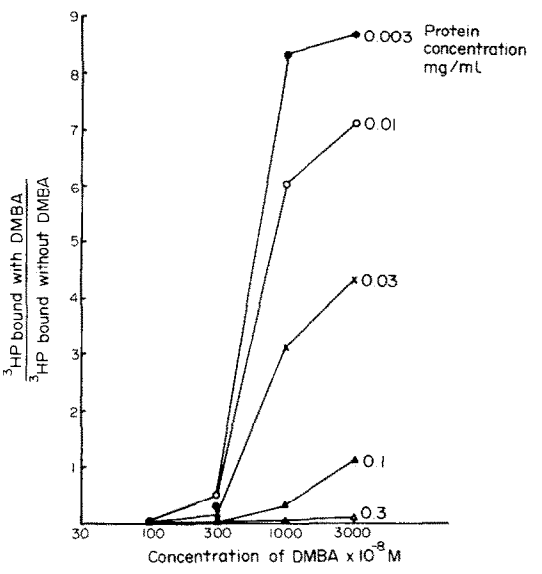

Fig. 5. Binding of [^{3}H]-P to plasma proteins; influence of DMBA and protein concentration. Adrenalectomised, ovariectomised rat plasma was diluted in buffer and incubated with [^{3}H]-P 10^{-8} M and increasing concentrations of DMBA, 1–30 μM. Parallel incubations were performed in the absence of DMBA. Each point is plotted as a ratio of [^{3}H]-P bound in the presence of DMBA to that bound in the absence, (n = 2).

shown in Fig. 3. Uterine cytosols from non E_2 primed rats were incubated with DMBA 10^{-6} M and [^{3}H]-P 10^{-8} M: binding of [^{3}H]-P is progressively lowered by progressive increases in the level of unlabelled progesterone. Similarly, the enhanced binding of [^{3}H]-P in the presence of DMBA appears steroid specific. As can be seen in Fig. 4 studies on the relative potency of a variety of steroids for [^{3}H]-P binding in the presence of DMBA showed a hierarchy similar to that seen for [^{3}H]-P receptors in uterine cytosol. In the presence of 10^{-6} M DMBA the order of displacement of [^{3}H]-P 10^{-8} M by a 1000 fold excess of unlabelled steroids is P > DHT > A > F > E_2. Identical results (not shown) were obtained using mammary gland cytosols. This hierarchy is identical to that reported by others for the progesterone receptor in the rat uterus [11] and chick oviduct [12].

That such potentiation of [^{3}H]-P binding was not confined to potential progesterone target tissues was shown when similar studies were carried out using plasma in place of target tissue cytosol preparations. Potentiation of [^{3}H]-P binding by DMBA also occurs in plasma. Increasingly dilute samples of adrenalectomised, ovariectomised rat plasma were incubated with [^{3}H]-P 10^{-8} M and increasing concentrations of DMBA (1–30 μM). The results (Fig. 5) are plotted as a ratio of [^{3}H]-P bound in the presence of DMBA to that bound in its absence. At a plasma protein concentration of 1 mg/ml the hydrocarbon is without effect over the range of DMBA concentrations studied. As the concentration of protein in the incubate decreases, however, the effect of DMBA on [^{3}H]-P binding becomes increasingly apparent. From Fig. 5, it appears that DMBA exerts its most significant effect on progesterone binding at concentrations of protein in the low end of the range tested. If an approximate mean molecular weight of 100,000 daltons is assigned to rat plasma protein, the molar concentration at the lower end of protein concentration studied is $3 \times 10^{-7} - 3 \times 10^{-8}$ M. At these plasma protein concentrations, the near-maximal potentiating effect of DMBA on [^{3}H]-P binding appears at DMBA concentrations of 10^{-5} M, i.e. a 30–300 fold molar excess. A factor limiting the effect of DMBA, particularly at low protein concentrations, may be the relative insolubility of DMBA in aqueous media.

Solutions prepared from homogenized tissues or from plasma are heterogeneous in protein constitution. Potentiation of [^{3}H]-P binding by DMBA is, however, similarly seen in solutions containing a single protein. In Fig. 6 the binding of [^{3}H]-P in the presence of DMBA is shown to purified BSA, ovalbumin and catalase. Protein dissolved in buffer (10 μg/ml) was incubated with [^{3}H]-P 10^{-8} M alone or with 1–30 μM DMBA. None of these proteins bound [^{3}H]-P in the absence of DMBA with affinity sufficient to withstand gel chromatography.

One hypothesis consistent with such results is that [^{3}H]-P and DMBA interact in solution, and the resultant complex has a higher affinity for protein than does [^{3}H]-P itself. To examine this possibility, DMBA and protein were incubated without [^{3}H]-P and the solution chromatographed after 90 min. Aliquots of the excluded volume were incubated with [^{3}H]-P and were found to bind [^{3}H]-P just as well as if no pre-separation had occurred, i.e. the continued presence of free DMBA is not essential for potentiation of [^{3}H]-P binding. Accordingly, a more

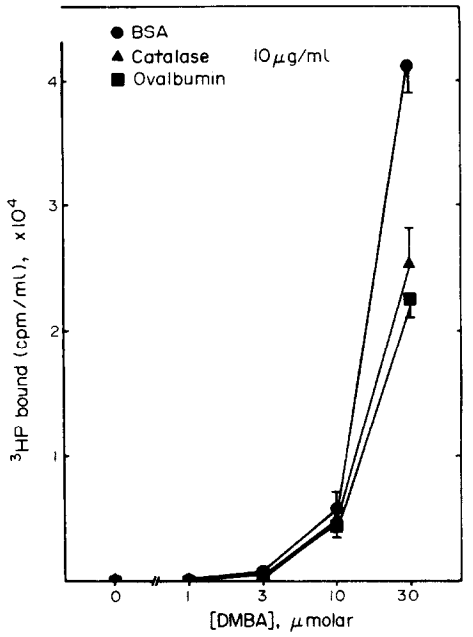

Fig. 6. Binding of [^{3}H]-P to BSA, catalase and ovalbumin; influence of DMBA. BSA (circles) catalase (triangles) and ovalbumin (squares) were dissolved in buffer to give a final concentration of 10 μg/ml. Protein solution was incubated in ice for 90 min with [^{3}H]-P 10^{-8} M and DMBA 0–30 μM. (n = 4).

likely explanation for the results shown in Fig. 6 is that DMBA induces some modification in a wide variety of proteins to increase the affinity with which they bind progesterone.

At certain concentrations of DMBA—for example, between 3 and 10 μM—the enhancement of [^{3}H]-P binding is much greater than the ~3 fold increase in DMBA concentrations (Figs 5, 6 and left hand panel of Fig. 7). To determine whether this non-linearity is a function of DMBA–protein interaction, or of a subsequent interaction of [^{3}H]-P with a DMBA–protein complex, the study shown in Fig. 7 was performed. Rat plasma, diluted to a protein content of 10 μg/ml, was incubated with [^{3}H]-P 10^{-8} M in the presence of DMBA 0.3–30 μM, or with [^{3}H]-DMBA 0.3–30 μM, in a parallel set of tubes.

Table 1. Binding of [^{3}H]-steroids to BSA, influence of DMBA

[^{3}H]-Steroid (10^{-8} M)	Binding alone (c.p.m./ml)	Binding with DMBA (c.p.m./ml)
Dexamethasone (DM)	27 ± 7	40 ± 8
Cortisol (F)	25 ± 6	51 ± 9
Estradiol (E_2)	40 ± 6	210 ± 9
Corticosterone (B)	31 ± 8	222 ± 25
Aldosterone (A)	30 ± 7	325 ± 29
5α-Dihydrotestosterone (DHT)	45 ± 3	2,418 ± 25
Deoxycorticosterone (DOC)	46 ± 7	9,788 ± 484
17α,21-Dimethyl-19-nor-pregna-4,9dione (R5020)	568 ± 27	14,896 ± 2289
Progesterone (P)	41 ± 8	21,835 ± 832

A range of [^{3}H]-steroids was incubated with BSA, 10 μg/ml either alone or plus DMBA 30 μM in ice for 90 min. Bound steroid was separated from free by gel chromatography. Each value is the mean ± S.D., n = 4.

Over this range of concentrations, [^{3}H]-DMBA binding appeared to increase linearly with dose: specifically, no departure from linearity was seen between 3 and 10 μM [^{3}H]-DMBA, suggesting that the amplification of [^{3}H]-P binding over this concentration range (left hand panel, Fig. 7) is a secondary phenomenon, rather than due to a parallel non-linear increase in DMBA binding.

DMBA increases the ability of protein to bind not only progesterone, but in addition at least eight other steroids. BSA (10 μg/ml) was incubated in the presence of DMBA (30 μM) with nine tritiated steroids representing both naturally occurring and synthetic compounds (Table 1). The order of binding potentiation is progesterone > R5020 > DOC > DHT > A > B > E_2 > F > DM. Furthermore, another commonly used but less potent carcinogen (3-Methylcholanthrene) shows similar properties. In Table 2 the levels of binding of a range of tritiated steroids to BSA (10 μg/ml) is shown. Of these steroids, [^{3}H]-P is potentiated approximately 12 fold (compared with 530 fold for DMBA).

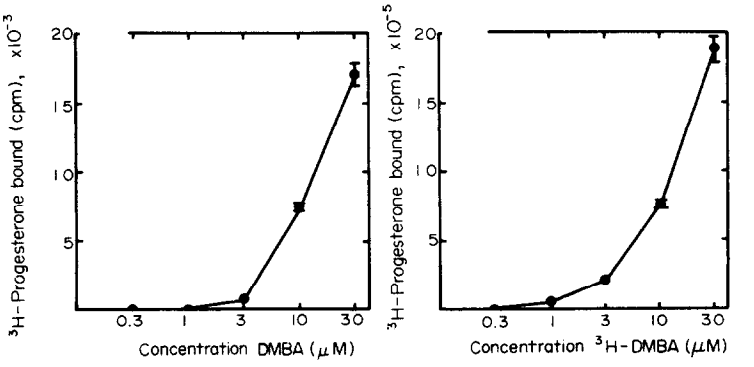

Fig. 7. Binding of [^{3}H]-P to rat plasma in presence of DMBA, and binding of [^{3}H]-DMBA to rat plasma. Adrenalectomised, ovariectomised rat plasma, 10 μg/ml, was incubated with [^{3}H]-P 10^{-8} M plus DMBA, 0.3–30 μM or with [^{3}H]-DMBA 0.3–30 μM in ice for 90 min, (n = 4).

Table 2. Binding of [^{3}H]-steroids to BSA, influence of 3MCA

[^{3}H]-Steroid (10^{-8} M)	Binding alone (c.p.m./ml)	Binding with 3MCA (c.p.m./ml)
Dexamethasone (DM)	28.6 ± 7.3	22.7 ± 1.4
Cortisol (F)	25.1 ± 5.8	17.9 ± 1.4
Estradiol (E_2)	40.2 ± 5.7	113.5 ± 8.9
Corticosterone (B)	30.9 ± 7.5	167.2 ± 10.1
Aldosterone (A)	29.9 ± 7.0	75.8 ± 7.6
5α-Dihydrotestosterone (DHT)	44.9 ± 3.0	76.6 ± 19.8
Deoxycorticosterone (DOC)	45.9 ± 6.5	342.8 ± 26.3
Progesterone (P)	40.7 ± 7.6	606.3 ± 125.2

A range of [^{3}H]-steroids was incubated with BSA, 10 μg/ml either alone or plus 30 μM 3MCA in ice for 90 min. Bound steroid was separated from free by gel chromatography. Mean ± S.D., $n = 4$.

DISCUSSION

Our initial interpretation [13] of the data shown in Figs 2–4 was that DMBA *in vitro* has the ability to expose latent progesterone receptors, and that oestrogen has a similar property *in vivo*. An hypothesis was accordingly proposed that DMBA may exert its carcinogenic action via the progesterone effector system.

In the light of the subsequent studies this would appear not to be the case. DMBA enhances progesterone binding to all proteins studied, not only to specific progesterone receptors. Potentiation *in vitro* appears restricted to experimental conditions where the protein concentration is less than 1 mg/ml, which may explain our initial misinterpretation. Uterine cytosol routinely prepared from adrenalectomised, ovariectomised rats contained 0.4 mg protein/ml; that from oestrogen primed rats 1.5 mg/ml or more. Similarly, mammary gland cytosols whether from primed or unprimed animals contained approximately 0.6 mg protein/ml. The lack of DMBA effect in oestrogen primed uterine cytosols is therefore a function of high protein concentration, rather than the pre-emptive *in vivo* opening up of progesterone receptors.

The findings described in this paper suggest further avenues for investigation. In Fig. 6, for example, a 10 fold increase in DMBA concentration (3–30 μM) is followed by a ~ 100 fold increase in [^{3}H]-P binding to each of the three proteins. From the right hand panel of Fig. 7, there is no indication of co-operative phenomena in DMBA binding to protein. Finally, at equivalent concentrations of DMBA (Fig. 5), increases in protein concentrations are followed by decreases in [^{3}H]-P binding. The model which appears to us simplest in attempting to accommodate these observations is as follows. DMBA may bind to more than one site on a variety of proteins; when a single molecule of DMBA binds to a protein, there are no effects on either subsequent binding of further DMBA molecules, nor on the affinity of [^{3}H]-P binding to the affected protein. When protein concentrations are low, or those of DMBA relatively high, then binding of DMBA to more than one—perhaps contiguous—site on protein occurs; and that this phenomenon is responsible for the increase in apparent affinity of the protein for [^{3}H]-P. Such an interpretation is in accord with the absolute values for tracer binding shown in Fig. 7. Taking into account the differences in specific activity (13.1 vs 42 Ci/mmol) there are ~ 300 molecules of DMBA bound per [^{3}H]-P bound.

Our results imply that in any protein solution DMBA at high concentration binds to protein molecules and profoundly affects the steroid binding ability of these molecules. Current methods of DMBA administration involve a single 20 mg intragastric dose [1] or 5 mg directly into the tail vein [14]. Even the lower of these doses, into a 100 g rat, would give an immediate concentration of 4×10^{-3} M in plasma, i.e. almost an order of magnitude higher than the protein concentration in rat plasma. *In vitro* the effect of DMBA upon [^{3}H]-P binding becomes apparent when the concentration of hydrocarbon is similar to that of protein. At protein concentrations between 0.03 and 0.1 mg/ml (~ $0.3–1 \times 10^{-6}$ M), with DMBA at 10 fold higher concentrations ($1–10 \times 10^{-6}$ M) (Fig. 4), marked increases in [^{3}H]-P binding are observed. *In vivo*, therefore, where the concentration of hydrocarbon to protein may show a similar ratio, marked alterations in steroid binding behaviour may occur. Changes in the affinity with which plasma proteins bind steroids may produce crucial alterations in free levels, so that steroid sensitive tissues may receive inappropriate signals, and perhaps eventually respond by neoplasia. That a temporal dimension may in fact be crucial is shown by studies in which the carcinogen is maximally effective in maturing animals, and completely without effect in animals 100 days old or more [1].

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