PARTIAL PURIFICATION AND PROPERTIES OF A NON-LIGANDIN (^{3}H) 3-METHYLCHOLANTHRENE BINDING PROTEIN FROM LIVER CYTOSOL*

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SUMMARY

(^{3}H) 3-Methylcholanthrene binds in vivo to a macromolecule in addition to the previously reported binding to ligandin in liver cytosol. The properties of this second molecule are identical to those of the glucocorticosteroid receptor (Binder II) through 400 fold purification over the cytosol proteins (elution position from DEAE-Sephadex A-50 columns, molecular weight by gel filtration and pI value by isoelectrofocusing). The carcinogen, probably a metabolite, binds very strongly or covalently to the macromolecule in vivo, but non-covalently in vitro in the absence of microsomes. Large amounts of unlabeled carcinogen administered in vivo do not compete significantly with subsequent (^{3}H) dexamethasone binding to the hormone receptor fraction in vitro. Methylcholanthrene and dexamethasone do not compete for binding sites in vitro on isolated unlabeled Binder II leading to the conclusion that the glucocorticosteroid receptor and the methylcholanthrene binding protein are distinct entities.

The interaction of carcinogens with proteins of liver cytosol may be important events in the mechanism of action of these substances. Previous work from our laboratory has shown that the carcinogen, 3-methylcholanthrene, binds to ligandin (1-3), a liver protein present in high concentration which may serve to sequester small hydrophobic molecules, such as steroids and bilirubin

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and aid in their intracellular metabolism (3-5). It seems possible that carcinogens which are planar and steroid-like may interact at the molecular level in the normal pathways of steroid hormones or by analogous pathways. This idea should be explored in view of the established promotion by the adrenal hormones of liver tumor production by certain carcinogens which are steroids or have potential steroidal structures (6-10).

In this paper we describe the binding in vivo and in vitro of (^{3}H) 3-methylcholanthrene to a non-ligandin binding protein. This new binder occurs in the fraction containing the cytosol glucocorticosteroid receptor (Binder II) (11) through 400 fold purification.

MATERIALS AND METHODS

In a typical in vivo experiment 5 male adrenalectomized Fisher strain rats weighing about 115 g each (Charles River Breeding Labs.) were used four to ten days following surgery. (^{3}H) 3-Methylcholanthrene (500 mCi/mmole; Amersham-Searle) was suspended in sesame oil so that 200 μCi were injected intraperitoneally and the animals were guillotined 14 hours later followed by liver perfusion in situ with cold sterile isotonic saline. Perfused livers were homogenized and cytosols prepared by previously described methods (1). 0.6 Percent of the radioactive methylcholanthrene administered in this way was recovered in the homogenate in agreement with prior observations (1,12). About 18% of the radioactivity in the cytosol fraction was bound to macromolecules when binding was determined by gel filtration on columns of Sephadex G-25 by previous methods (1,5,13,14). Purification of the ligand-bound macromolecule was done by gel filtration chromatography on a column of Sephadex G-75 or Sephadex G-100, ion-exchange chromatography on a column of DEAE-Sephadex A-50 and isoelectofocusing in a pH 3-10 gradient (15).

In vitro binding experiments were carried out by suspending 200 μCi (^{3}H) 3-methylcholanthrene in 10% DMSO. When (^{14}C) 3-methylcholanthrene was used it was obtained from New England Nuclear and had a specific radioactivity of

0.02 Ci/mg. (1,2-^{3}H) Dexamethasone (specific activity, 29 Ci/mMole) was obtained from Amersham-Searle. 13 ml liver cytosol (400 mg protein) (11) was added and the mixture incubated with gentle stirring at 4° for 90 min. Approximately 98% of the radioactivity of the carcinogen was bound by the gel filtration analysis described above.

RESULTS AND DISCUSSION

Ion-exchange chromatography of (^{3}H) 3-methylcholanthrene bound *in vivo* and separated from the free carcinogen by gel filtration is chromatographed on a column of DEAE-Sephadex A-50 as shown in Fig. 1. This separates the bound

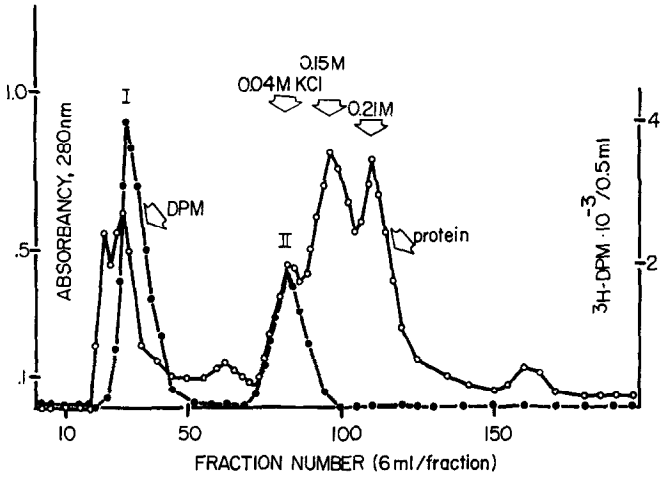

Fig. 1. Ion-exchange chromatography of macromolecular bound(^{3}H) 3-methylcholanthrene radioactivity formed *in vivo* on a column (3 x 70 cm) of DEAE-Sephadex A-50. Bound forms were separated on a column of Sephadex G-25 beforehand. A 0-1 M KCl linear gradient was used and salt concentrations were determined in eluents by conductivity measurements. Arrows and KCl concentrations indicate conductivity measurements. The buffer used is 50 mM tris-HCl, pH 7.5.

radioactivity into 2 peaks. The first peak to elute is associated with ligandin (I) and the second peak (II) elutes in the same relative ionic strength as the glucocorticosteroid receptor (11). Peak II was pooled and subjected to gel filtration on a column of Sephadex G-75 as shown in Fig. 2. Three size classes of proteins appear in this chromatogram corresponding to molecular weights in the range of 130,000, 67,000 and 31,000. The highest molecular weight size

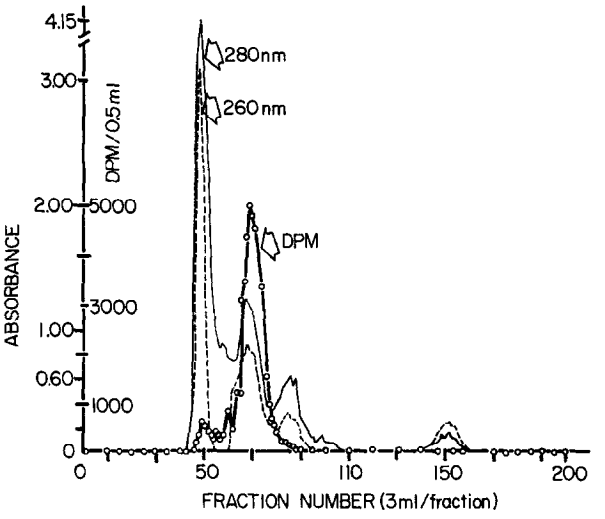

Fig.2. Gel filtration chromatography on a Sephadex G-75 column (3 x 75 cm) of the fraction II pool (in vivo binding) from ion-exchange chromatography (Fig. 1.) The buffer was 50 mM tris-HCl-1mM mercaptoethanol, pH 7.5. The load was 80 mg protein and 250,000 dpm. The recovery was 80% in protein and 75% in dpm. Similar results were obtained when (^{14}C) 3-methylcholanthrene was used for in vivo binding.

class was determined by Sephadex G-100 chromatography. The radioactive carcinogen is associated almost entirely with the middle peak corresponding to molecular weight 67,000 as determined by calibrating the column with protein markers. Of importance is the fact that very little radioactivity dissociated from this fraction as indicated by lack of radioactivity in the small molecules (fractions near number 150) of the chromatogram. This would indicate that the carcinogen is very tightly bound to the macromolecule. Probably it is covalently bound in view of the fact that subsequent in vitro experiments in the absence of microsomes give a very different picture in which the carcinogen dissociates easily from this fraction and binds loosely to many proteins (Fig. 3). Thus, when the pool from an ion-exchange chromatogram (Fig. 3) is collected in the region of Binder II (fractions 72-92) and subjected to gel filtration on a column of Sephadex G-100 (Fig. 4), 3 size classes are separated again as in in vivo experiments (Fig. 2). The non-covalently bound radioactivity is associated with the middle fraction whose molecular weight is 67,000 after cali-

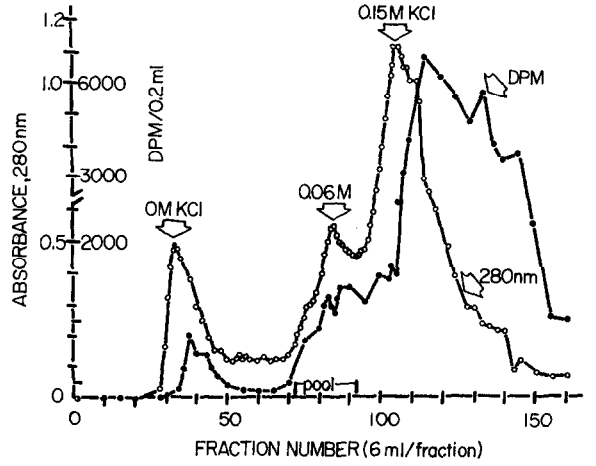

Fig. 3. Chromatography on DEAE-Sephadex A-50 column (3 x 63 cm) of macromolecular bound forms from in vitro incubation of cytosol and(^{3}H) 3-methylcholanthrene for 90 min. at 4°. Conditions of chromatography are similar to those described in Fig. 1. Load was 485 mg protein and 10 x 10^7 dpm. The noncovalently bound radioactivity is spread throughout most of the chromatogram.

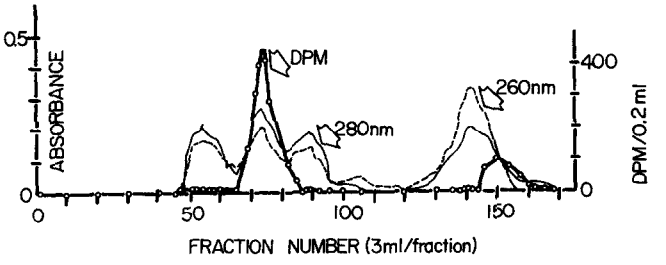

Fig. 4. Gel filtration on a column (3 x 74 cm) of Sephadex G-100 of the Binder II pool from DEAE -Sephadex A-50 (Fig. 3) derived from an in vitro labeling experiment. Elution buffer is 50 mM tris-HCl-1 mM mercaptoethanol, pH 7.5.

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bration with marker proteins. In contrast to in vivo binding (Fig. 2) there is considerable dissociation of radioactivity to the area of small molecules in the chromatogram (Fig. 4).

After in vivo binding and separation of macromolecular bound forms by ion-exchange chromatography the peak tubes of the middle peak from subsequent gel filtration corresponding to a molecular weight of 67,000 are subjected to isoelectrofocusing in a pH 3-10 gradient. A single bound peak is obtained with a pI of 6.7 (Fig. 5). The bulk of protein is co-electrophoresed with

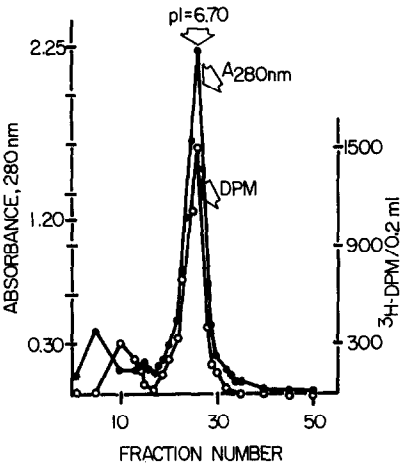

Fig. 5. Isoelectrofocusing of 67,000 dalton class of proteins from gel filtration on Sephadex G-100 columns of in vivo bound (^{3}H) 3-methylcholanthrene. A pH 3-10 gradient was used. Load was 24 280 nm absorbancy units and about 15,000 dpm. The recovery was 85% in 280 nm absorbancy units and 90% of the applied dpm.

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the bound 3-methylcholanthrene which is characteristic of the glucocorticosteroid receptor at this stage of its purification (13) and also of the estrogen receptor prepared from bovine uterus (16). After isoelectrofocusing, the 3-methylcholanthrene binding protein has been purified about 400 fold over the cytosol proteins similarly to the hormone receptor.

The elution of the macromolecule at 0.04 M relative ionic strength from columns of DEAE-Sephadex A-50, the apparent molecular weight by gel filtration of 67,000 and a pI value as well as fold purification after these procedures all correspond with the characteristics of the glucocorticosteroid receptor (Binder II) (11,17). In order to test if the tight binding of 3-methylcholanthrene formed in vivo is located in the site occupied by dexamethasone on the receptor the following experiment was done. Six adrenalectomized rats (160 g each) were injected intraperitoneally with a 5 mg unlabeled 3-methylcholanthrene in corn oil per 100 g body weight. After 16 hours the animals were killed and the cytosols prepared as described in Materials and Methods. The cytosol was incubated with 2 x 10^{-9} M (^{3}H) dexamethasone (Schwarz-Mann, 16 Ci/mmole) for

TABLE 1. IN VITRO BINDING OF (^{3}H) METHYLCHOLANTHRENE AND/OR (^{3}H) OR UNLABELED DEXAMETHASONE TO UNLABELED BINDER II FRACTION (AFTER DEAE-SEPHADEX A-50).[a]

	Ligand Conc'n.	Total Protein in 3 ml incubation mixture (mg)	Number of Experiments	% Binding	p moles bound / mg protein	% recovery: Protein	% recovery: Isotope
(^{3}H) Methylcholanthrene	1×10^{-8} M	6.9	4	9.3 (8.7-12.6)[b]	5.6 (3.2-7.8)[b]	98 (96-100)[b]	10.6[c] (7.7-14.8)[b]
(^{3}H) Methylcholanthrene + dexamethasone	1×10^{-8} M 5×10^{-8} M	6.9	4	14.0 (9.2-19.0)	15.4 (7.2-20.4)	96.5 (90-100)	15.5[c] (10.2-21.4)
(^{3}H) Dexamethasone	5×10^{-8} M	6.9	4	5.3 (3.9-7.8)	0.9 (0.5-1.7)	97 (90-100)	94 (92-98)

[a] (^{3}H) Methylcholanthrene, (^{3}H) or unlabeled dexamethasone are added simultaneously to the Binder II solution (3 ml). Incubation followed for 90 min. with gentle stirring at 4°. Buffer for incubation and column elution was 50 mM Tris-HCl - 1 mM mercaptoethanol, pH 7.5. Binding was determined by gel filtration on minicolumns (10 ml plastic cyringe barrel containing 10 ml bed volume) of Sephadex G-25. Macromolecular binding was determined in the void volume (Dextran blue).

[b] Range

[c] Free (^{3}H) methylcholanthrene is bound tightly to the gel filtration bed and is not fully eluted in the small molecule region under these conditions.

90 min. in ice. Macromolecules were separated by gel filtration on a column of Sephadex G-25 and the macromolecular fraction was chromatographed on DEAE-Sephadex A-50 (Materials and Methods). In two such experiments the binding of (^{3}H) dexamethasone was decreased only an average of about 20% (5 to 35%) compared to control experiments (animals injected with corn oil alone). We conclude that either the 3-methylcholanthrene binding protein is unique but has physical properties similar to the hormone receptor or that the covalently bound 3-methylcholanthrene binds to the hormone receptor at a site different from the noncovalent binding of dexamethasone.

To resolve this question, we studied binding in vitro to Binder II isolated through the DEAE-Sephadex A-50 step (11) from adrenalectomized animals so that the Binder II fraction contained minimal or no ligand. Possible competition between dexamethasone and 3-methylcholanthrene for noncovalent binding sites is examined in the data of Table 1. From these experiments it is clear that the binding of (^{3}H) methylcholanthrene is not reduced in the presence of a 5 fold excess of dexamethasone, in fact the binding of the carcinogen is enhanced somewhat. This finding indicates that dexamethasone and the carcinogen are not competing for the same binding site and therefore the steroid and the carcinogen bind to different macromolecules in this fraction. Also it is clear that the specific binding of the carcinogen is five to fifteen times greater than for dexamethasone suggesting that there is more carcinogen binding protein in this fraction than hormone receptor. It is of great interest that the hormone receptor and the carcinogen binding protein have similar properties through 400 fold purification. Future work will be concentrated on efforts to purify and characterize each of these macromolecules.

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