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Is the Human Hyperplastic and Malignant Prostate a Target for Retinoids?

Douglas David Boyd

PhD

University of Edinburgh

1985
The binding of vitamin A alcohol (retinol) and acid (all trans isomers) in human hyperplastic prostate was investigated. Saturable cytosolic binding sites for [3H] retinol and [3H] retinoic acid sedimented to the 2S position on sucrose gradients and were protease/heat inactivated. In addition to the saturable peaks, a second retinoic acid binding component unaffected by excess unlabelled ligand and therefore regarded as non-specific was observed at the 4.6S position of the gradient. Experiments using plasma from patients with benign prostate hyperplasia indicated that these saturable binding sites were prostate derived and not blood contaminants. Competition studies established the specificity of [3H] retinol and [3H] retinoic acid binding; all trans retinol and retinoic acid were recognised preferentially. In contrast, various steroids showed little affinity for either retinoid binding site. Metabolism studies revealed the absence of radioligand conversion in vitro. These data suggested that [3H] retinol and [3H] retinoic acid were associating with separate cytosolic components. Maximal binding of [3H] retinol was achieved after 4h of incubation at 40°C and maintained for up to 24h. The enzyme inhibitors, aprotinin, PMSF and sodium molybdate were without effect on retinol binding. Under optimal assay conditions, the binding of [3H] retinol was determined and found to be of high affinity (Kd = 33 ± 6 nM). Various attempts (immuno-precipitation, dye affinity chromatography, ultrafiltration and DEAE ion exchange) to remove the non-specific component for retinoic acid in prostate cytosol were made without success and precluded the development of a quantitative assay for specific retinoic acid binding. For this reason, further characterisation of the vitamin A acid binding protein was not possible.

Although specific association of retinol and retinoic acid could not be detected in microsomes or mitochondria, pronase sensitive sites which bound [3H] retinol and [3H] retinoic acid in a saturable manner were extracted with high salt from prostate nuclei. Radioligand association was both temperature and time dependent and only observed in charcoal pretreated salt extracts from sonicated nuclei. Steroid hormones including testosterone and dihydrotestosterone showed no affinity for the extracted protein (S); in contrast transfretinol and retinoic acid were equally active in displacing either radioligand. Analysis of the binding of both ligands by the Scatchard method (1949) gave Kd values of 12.5 ± 1.0 nM for retinol and 15.0 ± 2.0 nM for retinoic acid. Under the assay conditions, metabolism of vitamin A alcohol and acid was not evident. Although aprotinin and PMSF did not augment the binding of [3H] retinol in nuclear salt extracts, sodium molybdate (10 mM) increased specific ligand association by 18%. These data are in accordance with the view that the extracted nuclear protein recognises both vitamin A alcohol and acid. Fractionation of cytosol and nuclear salt extract on an Ultrogel ACA 34 column suggested that nuclear and cytosolic retinoid binding components were separate.

The binding of [3H]retinol was compared in hyperplastic and malignant prostate cytosols. The assay for retinol binding was linear with protein concentration and reproducible (inter, intra assay variations were less than 15%). Freezing and storage of tissue at -70°C for up to 8 weeks did not adversely affect the binding of retinol in the cytosol fraction. Less [3H] retinol was specifically bound in cytosol from malignant gland compared with hyperplastic tissue (1.7 ± 1.6; 4.0 ± 1.6 pmoles/mg protein respectively). These differences were statistically significant (P < 0.01 Mann Whitney U-test). The inability of malignant prostate cytosol to metabolise [3H] retinol and the similar binding characteristics (Kd values for hyperplastic and malignant tissue were 33.6 ± 6 and 31 ± 15 nM respectively) suggests a reduced expression of retinol binding protein in cancer.
Dedication

I dedicate my PhD thesis to my parents, David and Jeanne Boyd, who made it all possible.
Acknowledgements

I am most grateful to Dr Fouad Habib, my supervisor, for his excellent advice throughout this project on both academic and non-academic matters. Just as important, Fouad was instrumental in making my quality of life, in Edinburgh, acceptable over the period 1982 - 1985.

I would also like to extend my gratitude to other workers in our laboratory including Dr Brian Houston, Alan Leake, Rose Robinson and Fiona Sutherland for providing technical advice and amusements! The latter were certainly a necessity in times of difficulty.

I would also like to thank Dr Mary Norval, Bill Neill, Anne Grail and Jean Maingay in the Department of Bacteriology for their help and hospitality at the beginning of this project.

My appreciation also extends to Dr Keith James and his group within this Department for academic advice and the provision of various supplies in times of need.

Last but by no means least is Joan Cowper, my typist, who in spite of the hieroglyphic manuscript managed to decipher and type the text in record time!
Declaration

In accordance with rule 2.4.15 of Edinburgh University (Postgraduate Study Programme 1984/85) I, Douglas David Boyd, do hereby declare that:-

(a) this thesis has been composed by myself

(b) the data presented herein is entirely my own.
Publications

In accordance with rule 2.4.11 of Edinburgh University, I hereby state that the contents of this thesis have been published as 3 papers:

(1) Boyd D, Beynon L, Chisholm G and Habib F (1984)  
    Cancer Res 44, 5532 - 5537.

(2) Boyd D, Chisholm G and Habib F (1985)  
    J. Endocr. in press.

(3) Boyd D, Copestake P, Chisholm G and Habib F (1985)  
    Br J Cancer in press.

All 3 papers have been included in the appendix of this thesis.
### Abbreviations

<table>
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<th>Abbreviation</th>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine - 3, 5 - monophosphate</td>
</tr>
<tr>
<td>cRBP</td>
<td>Cellular retinol binding protein</td>
</tr>
<tr>
<td>cRABP</td>
<td>cellular retinoic acid binding protein</td>
</tr>
<tr>
<td>DCC</td>
<td>dextran coated charcoal</td>
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<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
</tr>
<tr>
<td>DHT</td>
<td>dihydrotestosterone</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>diaminoocteteta - acetic acid disodium salt</td>
</tr>
<tr>
<td>$g_{AV}$</td>
<td>unit of centrifugal force</td>
</tr>
<tr>
<td>$K_d$</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>ODC</td>
<td>ornithine decarboxylase</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>retinoids</td>
<td>collective term for vitamin A alcohol, aldehyde and acid and their synthetic derivatives</td>
</tr>
<tr>
<td>S</td>
<td>sedimentation coefficient</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
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<tr>
<td>u/v</td>
<td>ultraviolet</td>
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CHAPTER 1 (INTRODUCTION)
1.1. The Prostate Gland - Location, Structure and Function

The human prostate, a gland thought to serve a function in male fertility, is situated at the base of the bladder and partly surrounds the top end of the urethra.

Histologically, sections of the prostate show well defined concentric zones of glandular tissue interspersed with mesenchymal elements (Franks 1954 - Figure 1). For convenience, the gland may be divided arbitrarily into peripheral and periurethral zones (Figure 1).

---

**Figure 1.** Transverse Section of the Human Prostate.
The gland secretes a thin, milky alkaline fluid containing citric acid, calcium, acid phosphatase, a clotting enzyme and a profibrinolysin (Guyton 1981). During emission, the prostatic capsule contracts simultaneously with the vas deferens so that the thin fluid of the former adds to the bulk of the semen. The alkaline characteristic of the prostatic fluid serves to neutralize the acidity of the vas deferens secretions thereby achieving an optimal pH environment for the spermatozoa (pH 6.0 - 6.5). Although not yet established, the possibility exists that other aspects of the prostatic secretions play an important role in male fertility.

1.2. The Hyperplastic and Malignant Prostate

Disease of the human prostate is relatively uncommon in young males but increases in incidence with age. Of these, benign (hyperplastic) and malignant changes probably present the greatest problems to both patient and urologist.

Hyperplasia

Microscopically, most males above the age of 50 will have some evidence of benign prostate hyperplasia. The disease is characterised by an increase in stromal proliferation in the periurethral zone (Franks 1954) resulting in, in many cases, urethral obstruction. In those individuals, incontinence and difficulties with micturition may manifest requiring surgical ablation of the gland either by electroresection or open prostatectomy. Unfortunately, obstruction of the urethra may recur in a number of patients due to growth of residual gland. This requires further surgical intervention. At the present time there is no means of control of the benign disease other than surgical excision.

Malignancy

Prostate cancer rates as the third killer of United Kingdom males (Cancer Statistics 1980).
The disease is associated with the peripheral glands of the organ (Franks 1954) but may in the advanced stage extend throughout and in some cases beyond the fibrous capsule.

A combination of two factors probably accounts for the high mortality rate. Firstly the majority of patients present with the advanced disease (Hendry and Ferguson 1976). Secondly treatment focuses on hormonal manipulation (oestrogens, castration, anti-androgens) of the primary tumour and secondary metastases and presupposes that the aberrant cells are responsive to androgen deprivation.

Regression of the disease is marked in 70% of patients treated with the oestrogen stilboestrol (Chisholm 1981). Unfortunately, relapse in the oestrogen escape phase is common. The reasons underlying disease progression in oestrogen escape are not clear but may be due to:

(a) the selection and growth of hormone independent malignant cells,
(b) development of autonomy by hormone-sensitive cells.

These proposals are of course not mutually exclusive.

Clearly the outlook for those patients with advanced prostate cancer is bleak; it remains to be seen whether, in the future, new therapeutic approaches will alter this situation.

1.3. Vitamin A Absorption, Transport and Storage

\[
\begin{align*}
\text{CH}_2\text{OH} & \quad \text{RETINOL} \\
\text{CHO} & \quad \text{RETINAL} \\
\text{COOH} & \quad \text{RETINOIC ACID}
\end{align*}
\]

Figure 2.
Vitamin A alcohol (retinol - Figure 2) is obtained in the diet either as a pro-vitamin, such as β-carotene, or as long-chain fatty acyl esters of retinol. Carotene is absorbed directly into the intestinal mucosa cell where a specific enzyme oxidatively cleaves it into two molecules of retinal (Thompson et al 1950, Olson 1961, Goodman and Huang 1965, Olson and Hayaishi 1965). The retinal is then reduced to vitamin A alcohol (Fidge and Goodman 1967).

Esters of retinol are not absorbed well and first must be cleaved to free retinal either by pancreatic esterases or a brush border esterase (Mahadevan et al 1961). Once within the intestinal cell, the retinol is esterified with long-chain fatty acids (Huang and Goodman 1965) and then incorporated into chylomicrons which are released to the lymph. The chylomicrons or their remnants are taken up by the liver for storage. Although obligatory hydrolysis may be involved (Goodman et al 1965) retinol is stored in the ester form primarily with saturated long-chain fatty acids (Futterman and Andrew 1964).

The release of retinol from the liver and its delivery to target organs have been described extensively by Goodman (Smith and Goodman 1979). Briefly, retinyl esters are hydrolysed and the released retinol combines in a 1 : 1 ratio with a liver protein referred to as "serum retinol binding protein" (molecular weight = 20,000). The protein bound retinol leaves the liver and in the blood forms a ternary complex with prealbumin to prevent glomerular filtration. In normally nourished man the level of serum retinol is maintained between 300 and 700 µg/l (Mandel 1975).

Target cells appear to have specific plasma membrane receptors for the circulating retinol binding protein complex (Heller 1975, Rask and Peterson 1976). Only the retinol moiety transfers across the cell membrane; apo-retinol binding protein concomitantly loses its affinity
for prealbumin, dissociates, and is degraded by the kidney after glomerular filtration.

Although retinoic acid has been shown to be an endogenous compound of human serum (De Ruyter et al 1979) its physiological significance as a source of cellular vitamin A is at question in view of its rapid metabolism and excretion (Dunagin et al 1964, Roberts and De Luca 1967). Also, in contrast to that of retinol, no cellular uptake mechanisms for retinoic acid have been reported to date.

**Metabolism of Vitamin A**

In common with other biological systems the intracellular levels of vitamin A will be subject to control mechanisms. Although cellular metabolic pathways for retinol and retinoic acid are still poorly understood the advent of high pressure liquid chromatography (HPLC) has advanced knowledge considerably in this area.

The oxidation of vitamin A alcohol to vitamin A acid has been described by Futterman (1962) and Emerick et al (1967). This pathway is present in many tissues and dependent on the presence of NAD cofactor. The conversion of retinol to retinal is reversible and indeed administration of the aldehyde results in the appearance of vitamin A alcohol in the rat (Pawson 1981). In contrast, the oxidation of retinal to retinoic acid is irreversible (Futterman 1962, Emerick et al 1967).

The metabolism of retinoic acid by microsomal and mitochondrial fractions of hamster and rat tissues including trachea, liver, and testes, is illustrated diagramatically (Figure 3) (Frolik et al 1979, Roberts et al 1979, 1980; Sietsema and De Luca 1979). The dependence of each step on cofactors is shown. It should be noted that individual tissues have different metabolic pathways and cofactor requirements. For example, testes microsomes are unable to catalyse the oxidation of all trans
4-hydroxy retinoic acid to the ketone derivative; this contrasts with a rapid conversion by the corresponding liver fraction (Roberts 1980). Also, 4-hydroxy retinoic acid is an efficient inducer of retinoic acid metabolism in liver microsomes but has little effect on this step in intestinal mucosal microsomes (Roberts et al. 1980).

Zile and colleagues (1967) detected 13 cis-retinoic acid in rat tissue extracts and reported an equal biological activity compared with the all trans isomer. However, the authenticity of this "natural" metabolite must be questioned in view of the artefactual isomerisation of trans to cis forms by extraction and HPLC methods (Frolik 1980).

Although there is little disagreement over the authenticity of 5, 6 epoxy retinoic acid as a natural metabolite of trans retinoic acid, its physiological role is a controversial issue. Krishna et al. (1970) concluded that 5, 6 epoxidation of retinoic acid lowered its biological activity, a report at variance with the findings of McCormick's group (1978). Less controversial is the physiological role of the 4-hydroxy- and 4-oxo-derivatives of all trans retinoic acid.

These agents were assessed for biological activity in 2 assay systems:

(a) ability to reverse metaplasia in explanted trachea (Frolik et al. 1979),
(b) promotion of growth in vitamin A deficient rats (Surekha et al. 1972).

In both assays, the 4-hydroxy and 4-oxo derivatives were less active than retinoic acid. These data suggested that these compounds were in fact products of elimination pathways.

1.4 Vitamin A, Cell Differentiation and Cancer

(a) Vitamin A Deficiency
Wolbach and Howe (1925) were perhaps the first workers to describe the histopathological changes manifesting in vitamin A deficiency. If rats were deprived of vitamin A by dietary means, columnar and transitional epithelium were replaced by squamous, frequently keratinizing cells. These histological features were interpreted by Wolbach and Howe to be suggestive of "neoplastic potential".

The following year, Fujimaki (1926) extended these findings, observing an increased frequency of gastric carcinomas in rats fed vitamin A deficient diets. Although Sugiura and Benedict (1930) were unable to confirm Fujimaki's findings, this may have been due to the inherent difficulties of working with low frequency spontaneous cancers.

(b) Vitamin A Deficiency and Induced Carcinogenesis

Several studies have suggested that rat bladder (Cohen et al 1976), hamster cheek pouch (Rowe and Gorlin 1959) and rat salivary gland (Rowe 1970) may be more susceptible to carcinogens in the vitamin A deficient animal. The remainder of the studies with deficient animals concern the colon in which there are mixed results. Newberne and Rogers (1973) reported that a significant number of rats on a low vitamin A intake developed colon tumours when given the liver carcinogen aflatoxin B1. In contrast, a reduced incidence of this induced cancer was observed in vitamin A deficient rats (Narisawa et al 1976). These latter findings suggested that this malignancy at least, may require vitamin A for its progression.

(c) Administration of Retinoids* and Carcinogens to Experimental Animals

There have been numerous reports on the prophylactic effects of natural and synthetic retinoids in animals treated with carcinogens.

An early experimental system was the induction of skin papillomas

* (collective term for vitamin A alcohol, aldehyde, acid and synthetic derivatives)
by chemical carcinogens, u/v light or viruses. Several retinoid compounds including retinyl acetate, retinyl palmitate, all trans retinoic acid and all trans retinol when administered orally, parenterally or topically were effective in inhibiting the appearance or hastening the disappearance of these skin tumours (Davies 1967, Bollag 1975, Prutkin 1975).

Further interest in the therapeutic potential of these agents developed after it was shown that administration of large amounts of retinyl palmitate could reduce lung neoplasia induced by benzopyrene (Saffiotti et al 1967). Similar findings have been reported for mammary gland tumours induced by several carcinogens (McCormick et al 1983, Moon et al 1979). In addition, tumour induction at other sites including bladder, liver, tongue, forestomach have proved sensitive to the inhibitory effects of retinoids (Becci et al 1979, Sporn et al 1977, Chu and Malmgren 1965, Daoud and Griffin 1980, Shklar et al 1980).

In contrast, induction of some carcinomas at different sites appear unaffected by retinoids or in some cases even enhanced. Of particular interest is the colon because of the high incidence of this cancer in humans. Retinyl palmitate and derivatives of retinoic acid were without effect on colon tumours induced by several carcinogens (Ward et al 1978, Rogers et al 1973). Similarly, observations of retinoid-enhanced carcinogenesis have been documented for cheek pouch (McGaughey et al 1977) respiratory tract (Smith et al 1975) and mammary gland (Welsch et al 1981).

(d) Effects of Retinoids on Transplantable Tumours

Again, there is some inconsistency in the response of transplantable tumours to retinoids. Growth inhibition was achieved with leukemic L1210 (Cohen 1972), chondrosarcoma (Trown et al 1976) and melanoma S91 cells (Patek et al 1979). On the other hand, retinyl palmitate and
retinoic acid were without effect on Morris hepatoma (Cameron et al 1979) and L1210 cells (Stewart et al 1979).

(e) Effects of Administration of Retinoids to Human Cancer Patients

Successes achieved in the laboratory have provided the impetus to bring retinoids to bear on established human carcinomas - unfortunately there has been only limited success so far.

Some regression was achieved for skin basal cell carcinoma (Bollag and Ott 1971), bronchial carcinoma (Kokron et al 1982), inoperable cervical tumours (Surwitt et al 1982) and in the case of superficial bladder cancer recurrence rates were lower in Etretinate* treated patients (Alfthan et al 1983). However, Cassidy et al (1982) concluded that 13 cis retinoic acid was of no benefit to patients with advanced breast cancer and Pederson's group (1984) were unable to reproduce the findings of Alfthan et al (1983).

(f) Epidemiology

The literature reviewed so far suggests retinoids are capable of protecting against and in some cases reversing neoplastic transformation. The possibility that this situation extends to humans has been explored in a number of retrospective epidemiology studies.

In this regard possible links between cancer frequency and either dietary intake or serum levels of vitamin A have been investigated. For example, Mettlin and Graham (1979) found a higher risk of bladder cancer associated with lower intake of vitamin A; however, Shekelle et al (1981) were unable to confirm this relationship.

In a review of the epidemiological evidence, Ong and Chytil (1983) demonstrated serum levels to be a superior index to intake of vitamin A in predicting cancer risk. Of studies investigating a link between low serum vitamin A and cancer development, a relationship was established

* (Etretinate - Roche trade name for trimethylmethoxyphenylretinoic acid ethyl ester)
ten out twelve times. If vitamin A intake was used as a correlate, only five out of eight studies demonstrated an association between low intake and risk.

Perhaps more relevant to this thesis was the finding by Kark et al (1982) of a negative association between prostate cancer incidence and serum vitamin A.

(g) Vitamin A Levels in Malignancy

To the knowledge of the author, data on the levels of vitamin A in malignancy are lacking. Such information is crucial in understanding the biological actions of vitamin A in both normal and neoplastic tissues. Indeed, in view of the evidence for a link between vitamin A and cellular differentiation it might be speculated that low tissue levels predisposes to malignant transformation. Although data in support of this hypothesis are scant, Muto and Omori (1981) have observed reduced levels of vitamin A in human hepatocellular carcinoma with an unaffected tissue content in "normal" adjacent liver. These workers did comment that the malignant cells involved were vitamin A storage cells. In fact, low retinoid levels were not found in those cancers affecting non-storage liver cells. These observations suggested that suppressed vitamin A in hepatocellular carcinoma may be secondary to the disease rather than a causal factor in its pathogenesis.

1.5. The Anti-Tumour Effects of Vitamin A and Analogues - Possible Mechanisms of Action

The mechanism by which vitamin A regulates cell differentiation and exerts its anti-tumour effects is not clear. However, a number of hypotheses have been put forward and include the following:

Vitamin A may;

(a) stimulate the immune system to eliminate aberrent cells,
(b) promote lysosome labilisation and as a consequence cell
autotoxicity,

(c) stimulate cAMP dependent protein kinase thereby

1) inhibiting malignant cell proliferation
2) inducing cell differentiation

(d) prevent the induction of ornithine decarboxylase, an event
    which is prerequisite to cell cycle progression,

(e) alter the cell surface properties in such a way as to
    decrease the metastatic abilities of malignant cells,

(f) via receptors, control genomic expression governing cell
    growth and differentiation.

(a) Stimulation of the Immune System

A number of workers (Dennert and Lotan 1978, Patek et al 1984,
Tachibana et al 1984) have proposed that induction of killer T cells,
activation of macrophages and stimulation of other facets of the immune
system by vitamin A can account for the tumouricidal effects of retinoids.
While evidence exists in support of this contention, the fact that many
studies are conducted in vitro in the absence of an intact immune system,
questions the exclusiveness of the theory.

(b) Lysosome Labilisation and Cell Autotoxicity

Brandes (1981) has suggested that the process of lysosome labilisa-
tion and consequently hydrolase release is promoted by vitamin A. The
proposal by this worker that tumour cell death is related to this effect
of vitamin A, however, does not provide for cell specificity. In fact,
this hypothesis would predict widespread cytotoxicity in intact animals
-treated with vitamin A; a feature clearly not evident in any studies
to date.

(c) Effect of Vitamin A on Cyclic Adenosine-3, 5-Monophosphate (cAMP)
Dependent Protein Kinase

Ludwig's group (1980) on examining B 16 - F1 mouse melanoma cells
in culture found a vitamin A induced dose-dependent inhibition of cell proliferation, accompanied by increases in both basal and cAMP-stimulated protein kinase. More important, these workers found that a B - 16 F1 variant (MR - 4) lacking in protein kinase did not exhibit growth inhibition in response to vitamin A. Plet and co-workers (1981) have speculated on the role of protein kinase activation in retinoid-differentiated teratocarcinoma cells (Martin 1980). In this regard, an early event in these cells treated with retinoid is an increased activation of cAMP dependent protein kinase in both particulate and soluble fractions.

(d) Interaction with Ornithine Decarboxylase

The induction of ornithine decarboxylase (ODC) activity in G1 of the cell cycle is thought to be a prerequisite to cell cycle progression. Evidence in support of this concept stems from several observations:

(1) a wide range of stimuli including viruses, pituitary growth factors, epidermal growth factor and insulin which induce ODC in a variety of cells including 3T3 fibroblasts and chick embryo epidermis concomitantly initiate cell replication (review - Russell and Haddox 1981),

(2) inhibitors of ODC such as α methyl ornithine, 1, 3 diaminopropane and dehydro-ornithine prevent cell replication in neuroblastoma and chick embryo muscle cells (Mamont et al 1976, Chapman et al 1978, Relyea and Rando 1975).

The mechanism by which ODC induction in G1 promotes cell transit is not clear but it is unlikely that the products of its synthetic pathway, namely putrescine, spermidine and spermine are directly involved since enzyme blockade is not always accompanied by a depletion of these organic cations - an observation probably due to reverse

The effect of vitamin A on the induction of ODC in Chinese hamster ovary cells has been investigated by Russell and Haddox (1981). The ability of retinol to block induction of the enzyme, followed by a halt in cell proliferation was clearly demonstrated. These workers argued that since \(^{3}\text{H}\) uridine uptake was reduced in the treated cells, the observed effect of vitamin A may have been due to inhibition of synthesis of mRNA coding for ODC.

(e) The Effects of Vitamin A on the Cell Surface

The effects of biologically active retinoids on cell surface adhesion have been reported for both normal (Jetten et al 1979) and transformed (Adamo et al 1978, 1979) cell lines, and may be related to their abilities to direct the:

(1) biosynthesis
(2) processing
of glycoproteins and glycolipids.

(1) Glycolipid Biosynthesis

The concept that cell-substratum attachment is via fibronectin linkage is well documented (Kleinman et al 1979). In addition, the absence or reduction in the amount of fibronectin at the surface of transformed cells is a reproducible correlate of metastatic ability (Hynes 1976). Morré and co-workers (1981) concluded that the ability of retinyl acetate to inhibit the development of secondary tumour foci in rats inoculated with a transplantable hepatoma, was a consequence of decreased ganglioside incorporation into the putative cell surface fibronectin receptor; metastasised cells affected in such a fashion
were unable to anchor at distant sites.

(2) Processing of Heparan - a Glycosaminoglycan

Shapiro and Mott (1981) detected a retinoid induced increase in the degree of sulphation of fibroblast matrix heparan sulphate, a heteropolymer required for cell adhesion. The extent of heparan sulphation has been shown to be a function of both cell differentiation and proliferation (Shapiro and Mott 1981).

In conclusion, alterations in cell surface properties induced by vitamin A may be of relevance to the anti-metastatic effects of these compounds. However, it does not explain other facets of retinoid action, e.g. induction of differentiation in teratocarcinoma (Strickland and Mahdavi 1978) or altered nucleic acid metabolism (Tryfiates and Krause 1971, Zachman 1967).

(f) Receptor Theory of Vitamin A

In recent years, interest in vitamin A has been generated by the discovery of two intracellular binding proteins which recognise the alcohol (Bashor et al 1973) and the acid forms (Ong and Chytil 1975) of vitamin A specifically and with high affinity. The two proteins are quite distinct and separate from serum retinol binding protein (Bashor and Chytil 1975).

Homogeneous cellular retinol binding protein (cRBP) is a single polypeptide of molecular weight 14,600 (Ong and Chytil 1978) which may in the apo-form be phosphorylated by $\text{Ca}^{2+}$/phosphatidylserine dependent protein kinase (Cope et al 1984). The protein binds retinol (all trans) avidly with a dissociation constant of 16 nM, and in vivo is charged with the ligand in a 1:1 ratio at a saturation level of 40 - 100% (Ong and Chytil 1974). cRBP is present in the cytosol fraction of a number of tissues including brain, eye, heart, kidney, intestinal mucosa, lung, liver, testes, uterus (Ong and Chytil 1975,
Bashor et al 1973). Competition studies reveal that cRBP recognises the alcohol but not aldehyde or acid forms of vitamin A (Bashor et al 1973).

Cellular retinoic acid binding protein (cRABP) has a molecular weight of 14,000 and a pI (isoelectric point) value of 4.6 (Sani and Hill 1976). The protein binds all trans retinoic acid with high affinity (dissociation constant = 4nM) but not vitamin A alcohol or aldehyde (Ong and Chytil 1975, 1978). The endogenous ligand of cRABP has been identified by Saari et al (1982) to be all trans retinoic acid. In vitro phosphorylation of apo-cRABP by phosphatidylserine dependent protein kinase has been demonstrated by Cope and co-workers (1984). In addition, it has been reported that phosphorylated cRABP, at least in the case of Lewis lung carcinoma, is incapable of binding ligand (Gmeiner and Zerlauth 1982). The tissue distribution of cRABP differs to that of cRBP; positive organs (cytosol fraction) include bladder, brain, mammary gland, ovary, trachea and uterus (Ong and Chytil 1975, Futterman et al 1976, Sani and Corbett 1977).

The possibility that vitamin A is mediating its biological actions via these proteins is supported by a number of observations:

1) the binding efficacy of a series of retinol isomers to cRBP parallels their growth promoting activities in the vitamin A deficient rat (Ong and Chytil 1975).

2) the binding specificity of cRABP for a number of retinoic acid analogues correlated well with the activity of these agents as evaluated by (i) maintenance of mouse skin epidermal cultures, (ii) reversal of metaplasia in explanted hamster trachea (Chytil and Ong 1976).

Nuclear localisation of cRBP at the present time is a controversial issue. A soluble retinoic acid binding protein with similar characteristics (sedimentation coefficient) to the cytosolic component has been
extracted from nuclei of cultured retino-blastoma cells (Wiggert et al 1977). In addition, Takase et al (1979) observed saturable and specific binding of cRBP (radiolabelled with retinol) to nuclei in vitro. However, Porter's group (1983) using immunocytochemistry were unable to locate cRBP in the nuclei of several rat tissues. Also, autoradiography studies by Chader et al (1980) and a radioimmunoassay for cRBP (Adachi et al 1981) failed to show this protein in the nuclear fraction. Perhaps, noteworthy, is the observation that "positive" and "negative" results with regard to the nuclear locus of cRBP can be clearly demarked by experimental protocol; i.e. subcellular fractionation studies of Wiggert et al (1977) and Takase et al (1979) versus those utilising intact tissue sections (Chader et al (1980), Porter et al (1983), Adachi et al (1981)) for autoradiography, immunocytochemistry and radioimmunoassay. The author entertains the possibility that the discrepancy in results may be a consequence of different methodologies rather than anything else. Alternatively, a proposal by Liau et al (1981) that cRBP acts as a nuclear delivery system for retinol may resolve the issue; in this model, cRBP is not retained by the nuclei after its delivery of ligand to "acceptor" sites.

Less controversy has been generated over the nuclear locus of cRABP. In this regard, Mehta et al (1982a) and Sani and Donovan (1979) extracted the protein from nuclei of rat mammary adenocarcinoma and Lewis lung tumours. Similar results have been published by Jetten and Jetten (1979). In these studies, the presence of cRABP in the nucleus required a pre-exposure of this fraction to cytosol radiolabelled with retinoic acid. These observations indicated that the nuclear delivery of retinoic acid was dependent on cRABP. In analogy with the retinol biological system, cRABP may serve to deliver retinoic
acid to nuclear acceptor sites. In this regard, Cope's group (1984) have recently identified testicular nuclear binding proteins for retinoic acid which are separate from cRABP.

Control of genomic expression by cRBP or cRABP has not yet been proven. However, several studies have suggested that retinol, at least, is capable of influencing nucleic acid metabolism. Administration of retinol to vitamin A deficient animals results in increased incorporation of radioactive precursors into RNA (Zachman 1967, Johnson et al 1969, Zile and De Luca 1970). In addition, nuclear RNA extracted from livers of normal and vitamin A deficient rats was found to differ (Tryfiates and Krause 1971) as did RNA synthesised by hamster tracheal epithelium (Kaufman et al 1972). More recently, Omori and Chytil (1982) reported lower levels of cytoplasmic poly(A) containing RNA in retinol deficient testes, intestinal mucosa and liver. Although cRBP and cRABP have not been directly implicated in these observations, the necessity of these proteins in nuclear delivery of ligand has been discussed.

A closer examination of the literature, however, illustrates that the "receptor" theory for vitamin A action is not watertight. For example, attempts to correlate vitamin A responsiveness (inhibition of cell proliferation, induction of differentiation) in a number of cultured cell lines have led to positive relationships in the cases of 3T3 and 3T6 fibroblasts (Jetten et al 1979) MCF 7 and other human breast cancer cells (Lacroix and Lippman 1980) but not for human leukemic HL 60 cells (Douer and Koeffler 1982, Breitman et al 1980). Furthermore, Haussler and colleagues (1984) concluded that the presence of these proteins was insufficient to predict the response of neuroblastoma, myeloma cells and fibroblasts to either retinol or retinoic acid. Thus, it would seem possible that at least in cells devoid of
cRBP and cRABP, alternative mechanisms are responsible for the biological actions of retinoids.

The Expression of cRBP and cRABP in Tumour Tissue

A number of studies have analyzed for intracellular retinoid binding proteins in tumour tissue. The presence of cRABP in tumours was first reported for carcinomas from human lung and breast (Ong et al. 1975). A particularly salient point was the absence of protein in histologically normal tissue adjacent to the tumour, suggesting either a dramatic increase in the number of cells which normally contain this protein, or an altered expression of the genome. Subsequent studies have in some cases shown dramatic changes in the concentration of these proteins in neoplastic tissue (Ong and Chytil 1976, Ong et al. 1982, Chytil and Ong 1979).

The levels of these binding proteins have also been determined in animal tumours both transplantable and carcinogen-induced. In a number of carcinogen-induced tumours, the amounts of binding protein were considerably higher than in adjacent grossly normal tissue. These include cRABP in mouse papilloma (Chytil and Ong 1976) and cRBP in colon adenocarcinoma (Ong et al. 1978). However, increased concentrations of cRBP and cRABP are not universal features of malignancy. Palan and Romney (1980) detected reduced levels of cRBP in lung, endometrial, ovarian and breast cancers. Furthermore, Mehta et al. (1982b) noted an increasing suppression of cRABP levels when progressing from well differentiated to anaplastic breast cancer.

Although the levels of these proteins in malignancy are well documented, other data such as ligand occupation and dissociation constants are lacking. However, at least one binding feature is conserved in neoplastic transformation - the binding affinities of
cRABP for a range of synthetic retinoids was assessed and found to be unchanged in mouse papilloma when compared with normal testes (Chytil and Ong 1978).

1.6 Retinoids, Binding Proteins and the Prostate Gland

The ability of vitamin A alcohol and vitamin A acid to reverse methylcholanthrene-induced squamous metaplasia and benign hyperplasia in cultured mouse prostate has been reported by Laznitski (1963) and Chopra and Wilkoff (1976). Further studies by Reese et al (1983) demonstrated that the sensitivity of rat prostate adenocarcinoma cell lines to various retinoids manifested as a decrease in cell saturation density. The effect of vitamin A on cultured human prostate has yet to be determined.

Perhaps relevant to these in vitro morphological observations are the findings of cRABP in the cytosol of rat prostate R3327 sublines (Brandes 1980). Parallel studies with human prostate demonstrated a progression of cRABP from non-detectable in normal to high (10.6 p MOLES/mg protein) in malignant gland. However, this worker failed to characterise the retinoic acid binding component in terms of ligand specificity, affinity and protease sensitivity, nor did he investigate ligand association in other subcellular compartments.

Also, human prostate binding systems for retinol, the transported form of vitamin A, have yet to be reported on.

1.7 Objectives

The objectives of this project were to investigate and characterise the binding of retinol and retinoic acid (all trans isomers) in the:

(i) cytosol
(ii) microsomes and mitochondria
(iii) nucleus
of the human hyperplastic and malignant prostate with the view that these data might argue for or against the retinoid sensitivity of the gland.
CHAPTER 2 (MATERIALS AND METHODS)
CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

Radiochemicals

All trans [11, 12 (n) - $^3$H] vitamin A free alcohol (specific activity 40 - 60 Ci/mmol) was purchased from Amersham International, Bucks, U.K.

All trans [11, 12 - $^3$H] retinoic acid (specific activity 23 - 39 Ci/mmol) was a gift from Hoffmann La Roche, Basle, Switzerland.

n[1, 2 (n) $^3$H] hexadecane (4 x 10$^6$ dpm/gm) was supplied by Amersham International.

Unlabelled Retinoids and Steroids

All trans - 9 (4 - methoxy - 2, 3, 6 - trimethylphenyl) - 3, 7 - dimethyl 2, 4, 6, 8 - nonatetraenoic acid (RO 10 - 1670); p - [E - 2 - (5, 6, 7, 8 - tetrahydro - 5, 5, 8, 8 - tetramethyl - 2 - napthyl) - 1 - propenyl] benzoic acid (RO 13 - 7410) and 13 - cis - retinoic acid were gifts from Roche, Herts, U.K. Retinol, retinal and retinoic acid (all trans isomers) were obtained from Sigma Chemicals, Poole, Dorset, U.K.

The steroids testosterone, 5 $\alpha$ androstan - 17$\beta$ - ol - 3 - one (dihydrotestosterone), oestradiol - 17$\beta$ and progesterone were purchased from Sigma Chemicals.

Buffer Reagents

All reagents were of "Analar" grade. The following were from Fisons, Leics., U.K.; Tris[2 - amino - 2 - (hydroxy-methyl) propane - 1, 3 - diol], EDTA (diamino-ethanetetra - acetic acid disodium salt), CaCl$_2$ 6H$_2$O, KCl, Triton x 100 and glycerol. May and Baker, Kent, U.K. supplied MgCl$_2$ and hydrochloric acid. Dextran, activated charcoal and sodium molybdate were purchased from Sigma Chemicals. Gelatin was obtained from BDH, Poole, Dorset, U.K.
Biochemicals
Bovine pancreas trypsin (type I), sucrose, deoxyribonucleic acid (polymerised calf thymus), bovine serum albumin (fraction V), myoglobin, "Pronase", micrococcal nuclease (grade VI) and anti human serum albumin were all supplied by Sigma Chemicals. Dextran Blue 2000 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Enzyme Inhibitors
Phenyl methyl sulphonyl fluoride and aprotinin were obtained from Sigma Chemicals.

Solvents
All solvents were of "Analar" grade. May and Baker were the suppliers of glacial acetic acid, benzene and methanol. Diethyl ether, chloroform, perchloric acid [88% (v/v)], phosphoric acid [85% (v/v)] were obtained from Fisons. Aqueous acetaldehyde and cyclohexane were obtained from BDH. Absolute ethanol was purchased from James Burroughs, London, U.K.

Other Reagents
Comassie Brilliant Blue G and sucrose were from Sigma Chemicals. Diphenylamine was obtained from BDH and bromophenol blue from May and Baker.

Scintillation Cocktail
Triton x 100 based cocktail was purchased from Vickers Laboratories, Burley-on-Wharfedale, West Yorks., U.K.

2.2. Buffers
All buffers were made up in 11 of distilled water (20°C). Buffers were cooled to 4°C prior to pH adjustment to 7.4 with concentrated hydrochloric acid (1M). The following buffers were used throughout this investigation:-
Tris/ED  -  Tris 10mM, EDTA 1.5mM, dithiothreitol 1.0mM.
Tris/EDG  -  Tris/ED/glycerol 10%(v/v).
Tris/MgCl₂  -  Tris 10mM, MgCl₂ 5mM.
Tris/MgCl₂/TX100  -  Tris/MgCl₂/Triton x 100 1%(v/v).
Tris/MgCl₂/KCl  -  Tris/MgCl₂/KCl 0.6M.
Tris/CaCl₂  -  Tris 10mM, CaCl₂ 6H₂O 0.1mM.
Tris/CaCl₂/KCl  -  Tris/CaCl₂/KCl 0.6M.
DCC  -  activated charcoal 0.25%(w/v), dextran 0.025%(w/v), gelatin 0.1%(w/v) in Tris/ED.

Note - The half-life of PMSF in aqueous solution is about 30 minutes. For this reason, a solution of PMSF in absolute ethanol was made up on the day of the experiment and added in the appropriate amount (50 fold dilution) to the buffer just prior to its use.

2.3. Preliminary Experiments

(a) Quench Correction Curve

n[1, 2 (n) ³H] - hexadecane was weighed out into 10 counting vials. The amount of radioactivity in each vial was calculated in d.p.m. after correction for decay over the storage period.

The [³H] hexadecane was quenched by adding increasing volumes of aqueous Tris/ED buffer (0, 200, 400, 600, 800, 1000 µl) or Tris/ED (1000 µl) containing 25, 50, 75, and 100 µl of chloroform. After addition of 6 ml of scintillant, each vial was mixed well and counted on the [³H] channel of a Packard (Tri-Carb) counter with external standardisation.

Counting efficiency was calculated in the following way:

\[
\text{efficiency} = \frac{\text{c.p.m. (observed)}}{\text{d.p.m. (calculated)}} \times 100
\]

A quench correction curve was constructed by plotting counting efficiency against the external standards value for each level of quench.
A quench correction curve was constructed in the following way:-

$[^3H]$ Hexadecane in the presence of increasing amounts of quenching agent (aqueous buffer $\pm$ chloroform) was counted for radioactivity. For each level of quench, calculated counting efficiency was plotted against the "external standard ratio".
Purity Assessment of Radiolabelled Ligands

The purity of [\(^3\)H] retinol and [\(^3\)H] retinoic acid was checked fortnightly using the system recommended by the manufacturers for [\(^3\)H] retinol and by the method of Sani and Donovan (1979) for [\(^3\)H] retinoic acid.

Standard all trans retinol, or retinoic acid (1 mg/ml) solutions in ethanol) were spotted (20µg) on polysilic acid gel impregnated glass fibre sheets (Gelman Sciences Inc., Michigan, USA) in the dark at 4°C, under a stream of nitrogen. Radioactive compounds were applied in a similar fashion using a micropipette.

Chromatotanks were pre-equilibrated in the cold room with 50 ml of the cyclohexane : ether (2 : 1) or benzene : chloroform : methanol (4 : 1 : 1) solvent systems for [\(^3\)H] retinol and [\(^3\)H] retinoic acid respectively.

Chromatograms were developed in the dark for about 40 minutes. At the end of this time, solvent was evaporated and the position of standards located under ultraviolet (u/v) light. The sheet was then cut breadth-wise into 10 mm slices. Each slice was placed in counting vials and radioactivity determined.

Typical chromatograms for [\(^3\)H] retinol and [\(^3\)H] retinoic acid are shown in Figures 5a and 5b.

Both radioligands were calculated to be over 95% pure. This value could, however, have been an overestimation since thin layer chromatography (T.L.C.) on polysilic acid impregnated sheets proved unsuitable for separating alcohol and acid derivatives of vitamin A. In the next section, reversed phase T.L.C. is described. Retinol could be well separated from retinoic acid and consequently routine purity checks of radioligands were made using this method.
Figures 5a and 5b. Purity Assessment of $[^3H]$ Retinol and $[^3H]$ Retinoic Acid.

The purity of radioactive ligands was checked by thin layer chromatography. Chromatograms were developed at 4°C in the dark using a cyclohexane : ether (2 : 1) for $[^3H]$ retinol (a) and a benzene : chloroform : methanol (4 : 1 : 1) solvent system for $[^3H]$ retinoic acid (b). Plates were dried under nitrogen, authentic standards located, and 10 mm slices counted for radioactivity. Arrow indicates solvent front.
(c) Reversed Phase Thin Layer Chromatography

Retinol and retinoic acid (all trans isomers) standards 10 μg each) in ethanol were applied with micropipette tips under nitrogen, in the dark at 4°C to Whatman KC 18 reversed phase plates (20 x 5 cm) (Maidstone, Kent, U.K.). Reconstituted extracted radioactivity was applied as described above, but at more than one position to prevent volume overloading.

The chromatotank was pre-equilibrated in the cold room with 50 ml of an 80%(v/v) ethanol solvent system. The reversed phase plate was developed at 4°C, in the dark for about 4 hours. At the end of this time, the plate was dried under nitrogen and the positions of standards located under u/v light. Slices of 10 mm were then scraped into counting vials and radioactivity determined.

(d) Removal of Unlabelled All Trans Retinol and Retinoic Acid From Solution With Charcoal Pellets

Working solutions

retinol (all trans) in ethanol - 100 μg/ml
retinoic acid (all trans) in ethanol - 20 μg/ml

Method

Charcoal pellets in test tubes were formed from 0.5, 1.0, 2.0, 4.0, 8.0 and 10.0 volumes (1 volume = 150 μl) of DCC suspension by centrifugation at 100 x gAV for 5 minutes. The supernates were discarded.

All subsequent procedures were undertaken in subdued lighting. Aliquots (150 ml) of the working solutions were dispensed into tubes containing charcoal pellets. The tubes were mixed and incubated for 10 minutes at 4°C. All tubes were agitated after the first five minutes of incubation. After the 10 minute period, 1.85 ml of Tris/ED buffer was added and tubes centrifuged at 800 x gAV (5 minutes) to remove the charcoal.
Spectrophotometric Readings

A Pye Unicam (SP6 550 uv/vis) was set to 325 nm (peak absorbance for retinol) or 350 nm (retinoic acid peak absorbance) and zeroed with 7.5% ethanol in Tris/ED buffer. The contents of each tube were read for absorbance at the appropriate wavelength.

It is evident from Table 1 that pellets prepared from 8 and 4 volumes of DCC suspension will remove over 90% of unlabelled retinol and retinoic acid respectively from solution. In fact, further studies with radiolabelled vitamin A alcohol and acid, indicated that this method was capable of removing over 99% of either compound from buffer containing $10^{-7}$M[^3]H retinoid. (table 1b)
TABLE 1a  Charcoal Removal of All Trans Retinol and Retinoic Acid from Solution

All trans retinol and retinoic acid solutions (100 and 20 μg/ml respectively) were treated with charcoal pellets prepared from increasing volumes of DCC suspension. After a period of 10 minutes, charcoal was removed at 800 x g_{AV} and retinol and retinoic acid absorbance read at 325 and 350 nm respectively.

TABLE 1b

[3H] Retinol and [3H] retinoic acid (100 nM) in Tris/ED buffer were treated with charcoal pellets formed from 10 and 8 volumes of DCC suspension as described in the legend to table 1a. The charcoal was removed and the buffer counted for radioactivity.
### TABLE 1

<table>
<thead>
<tr>
<th>CHARCOAL PELLETS (VOLUME RATIO OF SUSPENSION)</th>
<th>0</th>
<th>0.5</th>
<th>1.0</th>
<th>2.0</th>
<th>4.0</th>
<th>8.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRANS RETINOL ABSORBANCE AT 325 nm</td>
<td>1.535</td>
<td>(-)</td>
<td>0.845</td>
<td>0.289</td>
<td>0.168</td>
<td>0.110</td>
<td>0.136</td>
</tr>
<tr>
<td>TRANS RETINOIC ACID ABSORBANCE AT 350 nm</td>
<td>0.361</td>
<td>0.155</td>
<td>0.061</td>
<td>0.021</td>
<td>0.021</td>
<td>(-)</td>
<td>(-)</td>
</tr>
</tbody>
</table>

### TABLE 1(b)

<table>
<thead>
<tr>
<th>$^3$H Retinoid</th>
<th>Total Radio-activity incubated (dpm)</th>
<th>Radioactivity remaining after charcoal treatment (dpm)</th>
<th>Efficiency of charcoal procedure (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RETINOL</td>
<td>1.78x10^6</td>
<td>5.86x10^3</td>
<td>99.7</td>
</tr>
<tr>
<td>RETINOIC ACID</td>
<td>1.55x10^6</td>
<td>7.24x10^3</td>
<td>99.5</td>
</tr>
</tbody>
</table>
2.4 Prostate Obtainment

General Procedures

Human prostate clinically and histologically diagnosed as hyperplastic or malignant was removed by electroresection or open prostatectomy and transported to the laboratory in ice-cold saline [0.9% (W/v)]. The tissue was washed three times with Tris/ED buffer and blotted dry with tissue paper. Prostate tissue removed by open prostatectomy was cut into 2 gm blocks and then divided 2 - 3 blocks per glass Universal. All tissues were snap-frozen in liquid nitrogen and stored at - 70°C until required.

Selection of Malignant Material for Biochemical Work

Tissues confirmed with the malignant disease were only included in comparative studies if the patients had not undergone any previous treatment (X - irradiation, endocrine manipulation, chemotherapy).

Prior to electroresection, those patients with cancer were bimanually clinically staged to determine the extent of disease (Harmer 1978). Incidental tumours (T₀) and those staged as T₁ were excluded from biochemical studies on the grounds that the portion of gland assessed by histopathology may be unrepresentative of that retained for scientific purpose. All other malignancies were assigned a Gleason histological score (Gleason 1966) by a pathologist. Briefly, this scoring system describes the overall differentiation of the tumours on a scale from 2 - 10; well differentiated cancers are assigned a value of 2 - 4; poorly differentiated, values of 6 - 10.

2.5. General Methods

(a) Subcellular Fractionation

All procedures were undertaken in the cold room.

Hyperplastic prostate tissue (2 - 5 gm unless otherwise stated) was finely minced with scissors and pulverised for 20s in a precooled
Teflon vial (-20°C) using a Mikrodisembrator II (B Braun Melsungen AG, FRG) at maximum setting. Subcellular fractionation was essentially that described by Chamberlain et al (1966).

**Cytosol**

Pulverised tissue was transferred to a "Quick Fit" test tube (10 ml) and homogenised in 1 - 5 volumes of Tris/EDG buffer with a Ystral homogeniser (speed 7, (3 x) 15 s bursts with 1 minute intermittent cooling). The homogenate was transferred to plastic low spin centrifuge tubes and spun at 800 x g<sub>AV</sub> for 15 minutes in a Chilspin (MSE, Sussex, U.K.) centrifuge to remove tissue debris and nuclei. The supernatant was harvested with a Pasteur pipette, care being taken to avoid floating lipid substance. The supernatant was dispensed into 10 ml capacity polycarbonate ultracentrifuge tubes (Sorvall, Herts, U.K.) which were loaded in an angled T 865.1 rotor (Du Pont Instruments, Herts, U.K.). Ultracentrifugation was carried out at 100,000 x g<sub>AV</sub> for 1 hour. The resulting clear supernate was adjusted to a protein concentration of 1 - 5 mg/ml and retained for subsequent experiments.

**Mitochondrial and Microsomal Pellets**

Homogenisation of tissue and low spin centrifugation were identical to "Cytosol Preparation" but in this case Tris/EDG was replaced with Tris/ED buffer.

A mitochondrial pellet was prepared by ultracentrifugation of the 800 x g<sub>AV</sub> supernatant at 15,000 x g<sub>AV</sub> (15 minutes). The supernatant was stored and the pellet scraped from the bottom of the tube with a spatula. Tris/ED buffer (2 ml) was added to each polycarbonate tube and pellet in buffer transferred to a glass homogeniser (Jencoons, Herts, U.K.). The mitochondrial pellet was dispersed with 6 strokes of the teflon pestle and the resulting suspension retained for further experiments.
Microsomal pellets were prepared by ultracentrifugation of the 15,000 x g_{AV} supernate at 100,000 x g_{AV} for 1 hour. The cytosol was decanted and microsomes resuspended in 2 ml of Tris/ED buffer in an identical manner to that for mitochondria.

**Nuclei**

Pulverised prostate (500 - 2000 mg) was homogenised in 5 - 10 volumes of Tris/MgCl$_2$/TX100 under identical conditions to other subcellular fractions. The homogenate was filtered through one layer of nylon gauze (John Stainer & Co, Manchester, U.K.) to remove tissue clumps and spun at 20 x g_{AV} for 15 minutes to sediment cellular debris. The supernatant was transferred by Pasteur pipette to another low spin centrifuge tube and a nuclear pellet prepared by centrifugation at 800 x g_{AV} for 15 minutes. At the end of the spin, the supernate was discarded and nuclei resuspended in 2 - 3 ml of Tris/MgCl$_2$/TX100 buffer with the aid of a Pasteur pipette. Nuclei were pelleted at 800 x g_{AV} for 10 minutes and 2 further washes undertaken.

Washed nuclei (30 - 60 mg DNA) were resuspended in the appropriate buffer and retained for further experiments. Examination of nuclei by light microscopy (Giemsa stain) established the purity of this fraction (plate).

**Preparation of an Untreated Nuclear Salt Extract**

Nuclei resuspended in Tris/MgCl$_2$/KCl were extracted, with gentle stirring, at 4°C for 2 hours. At the end of this time, insoluble material was removed at 15,000 x g_{AV} (15 minutes) and the supernatant retained.

(b) **Retinol Binding Assay**

The assay for retinol binding in various subcellular fractions was essentially that of Bashor et al (1973) with minor modifications.
Prostate Nuclei From Human Hyperplastic Gland

Nuclei were prepared as described in section 2.5(a) and an aliquot smeared on a microscope slide. The preparation was dried on a Tecam\textsuperscript{R} Dri Block set at 30\textdegree C and subsequently fixed with absolute ethanol for 10 minutes. Nuclei were stained with Giemsa.
Retinol binding was assessed in triplicate (unless otherwise stated) at a single point concentration ($10^{-7}$ M) of radiolabel. Parallel experiments were carried out in the presence of a 50/100 fold excess of unlabelled trans retinol to determine non specific binding. The level of ethanol carrier was either 5.0 or 7.5% (V/v).

All procedures were carried out in subdued lighting. Unlabelled retinol was dispensed with a Hamilton syringe. Both $[^3H]$ ligand solution and subcellular fractions were added by Gilson pipette.

Incubations were performed in the dark at 4°C for 18 hours, (unless stated otherwise). After this time, ligand binding was determined by the DCC method (section 2.5(d)).

(c) Retinoic Acid Binding Assay

Retinoic acid binding was assayed by the method of Brandes (1981) with minor modifications.

Ligand binding was determined at a single concentration ($10^{-7}$ M) of $[^3H]$ retinoic acid with or without unlabelled trans retinoic acid in a 50/100 fold excess. Each assay was performed in triplicate, (unless stated otherwise). The level of ethanol carrier was 5% or in some cases 7.5%.

All procedures were undertaken in subdued lighting. Ligand solution was dispensed with the aid of a fixed micropipette. Subcellular fraction was added using a variable Gilson micropipette.

The tubes were agitated and incubated either at 4°C overnight for cytosol, mitochondria and microsomes or at 37°C for 30 minutes (unless otherwise stated) for nuclear assays. At the end of these times, $[^3H]$ retinoic acid binding was determined by the DCC method (section 2.5(d)).

(d) DCC Method

Charcoal suspension (0.8 or 1.0 ml for retinoic acid and retinol binding assays respectively) was dispensed into a set of test
tubes. Charcoal pellets were formed by centrifugation (Chilspin MSE) at \(100 \times g_{AV}\) for 5 minutes and the buffer discarded.

At the end of incubation with radioligand, aliquots of subcellular fraction were transferred to the tubes containing charcoal. The mixture was agitated and incubated at \(4^\circ C\) for 10 minutes. After this time, charcoal was sedimented at \(800 \times g_{AV}\) for 5 minutes. Aliquots of the treated subcellular fraction were counted for radioactivity or, if stated, subjected to sucrose gradient analysis.

Specific ligand association was determined by subtracting non specific binding assessed in the presence of unlabelled compound, from total binding observed in the absence of competitor.

(e) Metabolism Studies

Cytosol, nuclear salt extract or buffer was incubated in glass test tubes (protected from light) with \(^{3}H\) retinol or \(^{3}H\) retinoic acid (\(10^{-7} \text{ M}\)) under identical conditions to the binding assays (sections 2.5(b)(c)). In some experiments, nuclear salt extract was incubated with \(10^{-5} \text{ M} \) \(^{3}H\) retinoic acid (specific activity = 390 mCi/mmol).

At the end of incubation 1 ml of ice-cold diethylether was added to each tube and mixed well. The aqueous phase was allowed to settle and the organic phase aspirated with a Pasteur pipette. The solvent extract was collected in 2 ml glass bottles wrapped with aluminium foil. The subcellular fraction was extracted for a second time, in a similar way, and solvent extracts combined. The glass bottles were placed in a light-shielded vacuum oven and the ether evaporated without heat. The residual extracted material was reconstituted in 50 \(\mu\)l of ice-cold ethanol. Reversed phase chromatography has been described in section 2.3(c).
Sucrose Gradient Centrifugation (Rate Zonal)

Sucrose Solutions

A 20% (w/v) sucrose solution was made in Tris/ED buffer. Working solutions (7.5, 10.0, 12.5, 15.0, 17.5 and 20%) were prepared by dilution of the 20% solution with appropriate volumes of Tris/ED. Working solutions were stored at 4°C in counting vials for no longer than 1 week.

Sucrose Gradients

Sucrose gradients (7.5 - 20%) were set up in the following way. A polyallomer tube (Du Pont Instruments) was mounted vertically in a bench clamp. A syringe needle bent at right angles was attached to a 1 ml barrel. The syringe was loaded with 0.71 ml of the 20% solution which was then delivered to the bottom of the tube. The barrel was re-loaded with the 17.5% sucrose, and this solution layered onto the 20% sucrose, care being taken to minimise mixing. The remaining solutions were delivered in a similar fashion with the least dense (7.5%) solution occupying the top of the tube. A period of 8 - 12 hours at ambient temperature was allowed for establishment of a continuous gradient by diffusion.

Sample Loading

Prior to ultracentrifugation, sucrose gradients were loaded in a pre-cooled vertical rotor (TV 865 Du Pont Instruments). The rotor was then placed in the refrigerated cabinet of the ultracentrifuge (OTD 65 - Du Pont Instruments) to allow cooling of the gradients to 4°C.

To load samples, the rotor was retrieved from the cabinet and fixed in a nearby bench clamp. Aliquots (150 μl) of samples to be centrifuged were carefully overlayed the gradients with a Gilson pipette. The tubes were secured with a plug and screw cap.
Centrifugation

The OTD 65 ultracentrifuge was programmed for slow acceleration and deceleration to allow minimal disturbance of the gradients during the orientation and re-orientation phases. Gradients were centrifuged at 400,000 x g<sub>AV</sub> for 2 hours at 4°C (excluding acceleration times).

Fraction Collection

Fractions (20 x 200 µl) were collected from the top of the tube using a Gilson pipette. Initially, fractions were harvested by upward displacement with a Gradient Unloader (Nyegaard U.K.). This method was not found to be superior to that of collection by pipette. Rather, an imperfect seal between tube and Unloader in many cases, resulted in unsuccessful harvesting of some gradients.

External Markers

Myoglobin (2S) and BSA (4.6S) were dissolved in Tris/EDG buffer to give protein strengths of 1 - 4 mg/ml. Aliquots (150 µl) of each marker were loaded on separate gradients. Gradient centrifugation and fraction collection have been described. Each fraction was collected in plastic test tubes and 2 ml of Tris/ED buffer added. The contents of each tube were mixed and absorbance read at 280 nm.

(g) Ultrogel AcA 34 Gel Filtration

An Amicon glass column (Amicon, Gloucestershire, U.K.) of dimensions 60 x 1.0 cm was used in these experiments. Ultrogel AcA 34 in sodium citrate buffer was provided by LKB, Orsay, France.

Packing of the column

All procedures were carried out at 4°C. The column was mounted in a retort stand at 45° to the vertical. The Ultrogel AcA 34 was decanted into a beaker containing Tris/MgCl₂/KCl and mixed with a glass rod to give a thin slurry. The gel was poured into the column.
Figure 6. Sucrose Gradient Centrifugation of Myoglobin (2S) and BSA (4.6S)

Aliquots (150 µl) of myoglobin and BSA in buffer were centrifuged at 400,000 x $g_{av}$ for 2 hours on sucrose gradients (7.5 - 20%). Fractions (20 x 200 µl) were harvested and myoglobin (▲) and BSA (●) absorbance read at 280 nm.
MYOGLOBIN
which was then reorientated to the vertical position. The column was packed under a force of 15 cm of water. To ensure that the packing was uniform, further additions of the Ultrogel slurry was preceded by vigorous disturbance of the top portion of the gel column. The final dimensions of the gel column were 55 × 1.0 cm with a bed volume of 43 ml.

**Equilibration of the Column**

The column was equilibrated by running through 3 bed volumes of the appropriate buffer containing 5%(V/v) ethanol. For this purpose, a LKB (Croydon, Surrey, U.K.) peristaltic pump was used and programmed to deliver 5 ml of buffer per hour.

**Determination of the Void Volume**

Dextran Blue 2000 was dissolved in Tris/MgCl₂ buffer to give a 1 mg/ml solution. An aliquot (1 ml) of the solution was applied to the top of the gel, eluant collection started and the Dextran Blue allowed to drain into the gel. The column outflow was then stopped and the gel "topped" up with a head of buffer. This procedure minimised gel disturbance in subsequent buffer inflow. Eluant collection was then continued and monitored for absorbance at 620 nm. The void volume was defined as the volume of buffer required to elute the marker.

**Sample Application and Gel Filtration**

Sample application and gel filtration were essentially as described for Dextran Blue 2000 with the exception that these experiments were conducted in the dark. All elution buffers contained 5%(V/v) ethanol.

**Fraction Collection**

A LKB Ultrorac II was used for this purpose and programmed to collect 1 ml fractions.
2.6. Other Methods

(a) Protein Assay

Protein determination was by the method of Bradford (1976).

Bradford Reagent

Coomassie Brilliant Blue G (100 mg) was dissolved in 50 ml of a 95%(V/v) ethanolic solution. To this, 100 ml of phosphoric acid (85%(V/v)) was added and mixed. The solution was made up to 1 l with distilled water and finally passed through Whatman filter paper to remove insoluble material.

Bovine Serum Albumin Solution

A Pye Unican (model SP6 - 550 uv/vis) spectrophotometer was set at 280 nm and zeroed with distilled water in a quartz cuvette.

A solution of BSA in distilled water (approximately 1.0 mg/ml) was made and its absorbance read at 280 nm. The concentration of the solution was adjusted to give an absorbance reading of 0.650 units which corresponds to a protein strength of 1.0 mg/ml. This solution was stored at -20°C until required for the assay.

Assay

Varying amounts of the BSA solution (0 - 80 μg) were dispensed into standard tubes and their volumes brought to 80 μl with buffer.

Assay tubes contained 20 μl of subcellular fraction and 60 μl of buffer. Bradford Reagent (5 ml) was added to each tube and the contents vortexed.

The spectrophotometer was zeroed with the blank (0 μg BSA) and the contents of the standard and assay tubes read for absorbance at 595 nm.

A calibration curve was constructed from the standards. The amount of protein in the assay tubes was determined by use of the standard curve.

The assay was linear over a protein range of 0 - 80 μg BSA. In excess of 80 μg BSA, absorbance at 595 nm was found to level off. Also worthy of comment is that buffer components had minimal effects on the assay.
Figure 7. Protein Calibration Curve.

Increasing amounts (10 - 80 μg) of a BSA solution (1.0 mg/ml) were mixed with Bradford Reagent and read for absorbance at 595 nm.
(b) **DNA Assay**

DNA was assayed by the method of Giles and Myers (1965).

**Solutions**

- perchloric acid - 10% (V/V)
- diphenylamine in glacial acetic acid - 4% (w/v) (freshly made)
- calf thymus DNA in 5 mM sodium hydroxide - 1.0 mg/ml
- aqueous acetaldehyde - 1.6 mg/ml

**Method**

Perchloric acid (1.5 ml) was added to a set of tubes containing either standard DNA solution (0 - 80 µg) in a final volume of 80 µl or to nuclear pellets. The tubes were heated to 70°C for 30 minutes, after which centrifugation at 800 x $g_{av}$ (10 minutes) was undertaken to remove insoluble material. The supernates were decanted into a separate set of tubes and 2 ml of the diphenylamine reagent added. Following the addition of 100 µl of aqueous acetaldehyde, all tubes were mixed and incubated at 37°C overnight. After this time, the contents of all tubes were read at 600 nm. A DNA calibration curve was constructed and used to determine the amount of DNA present in each assay tube.

Figure 8 shows a typical calibration curve. It is evident that absorbance at 600 nm is linear over a DNA range of 0 - 80 µg.

Worthy of comment is that nuclear pellets prepared in the presence and absence of sodium molybdate gave similar DNA values. Smith et al (1983a) had cautioned that the presence of sodium molybdate in nuclear salt extracts resulted in an overestimation of DNA. These conflicting data may be resolved by the fact that in these experiments, DNA assays were carried out not on nuclear salt extracts but on nuclear pellets.
Figure 8. DNA Calibration Curve.

Perchloric acid extracts of increasing amounts of DNA (0 – 80 µg) were mixed with diphenylamine reagent and aqueous acetaldehyde. The preparations were incubated at 37°C overnight, after which samples were read for absorbance at 600 nm. A calibration curve was constructed accordingly.
(c) **Immunoprecipitation**

Anti-human anti albumin (Sigma Chemicals) was reconstituted in 2 ml of distilled water by rotating gently until the powder dissolved in solution. Working aliquots (200 µl) were stored at -20°C until needed. When required, frozen aliquots were thawed and serial dilutions (1/4, 1/8, 1/16, 1/32) made in distilled water.

Cytosol (900 µl) was reacted with 100 µl of the diluted anti albumin solution at 4°C or 20°C for 16 hours. At the end of this time, the visible precipitate was removed by ultracentrifugation at 15,000 x g_{AV} (15 minutes) and the treated cytosol retained for the retinoic acid binding assay (2.5(c)).

(d) **Ultrafiltration**

An Amicon Stirred Cell and Diaflo XM 50 membrane were used in these experiments. The membrane rejection rate for myoglobin and BSA, as quoted by the manufacturers were 20 and > 90% respectively.

The XM 50 membrane was soaked in Tris/EDG buffer for 30 minutes prior to its use. Cytosol (5 - 10 ml) was introduced into the cell and the nitrogen source adjusted to supply a pressure head of 40 pounds per square inch. To minimise membrane clogging, the cytosol was kept in constant motion with a magnetic stirrer at low setting. The filtrate was collected at 4°C and retained for further analysis.

(e) **Blue Sepharose CL - 6B Dye Affinity Chromatography**

Blue Sepharose CL - 6B (Pharmacia Fine Chemicals) was supplied preswollen in distilled water by a fellow associate working locally (Protein Fractionation Centre, Edinburgh). All procedures were carried out at 4°C. An Amicon glass column (5 x 0.9 cm) was packed under gravity with Blue Sepharose CL - 6B to a height of 4 cm (bed volume = 2.5 ml). The column was equilibrated with 3 bed volumes of Tris/EDG buffer.
Cytosol (1 ml, 2 - 5 mg protein/ml) was run into the column under the force of gravity and eluted with 1 bed volume of Tris/EDG buffer. Fractions (4 x 1 ml) were collected manually after sample application. The elution of cytosol from the column was monitored by assaying for protein (Bradford 1976). In a separate experiment, cytosol was adjusted to 0.2 M with KCl and chromatographed as described above. In this case, the eluting buffer, Tris/EDG contained 0.2 M KCl.

(f) DEAE Ion Exchange Chromatography

All procedures were carried out in the cold room. DE 52 anion exchanger (Whatman) supplied preswollen was resuspended in ice-cold Tris/ED buffer to give a thin slurry. With the aid of a glass rod, the slurry was poured into a vertically mounted Amicon column (15 x 1.6 cm). An exchange column of 5 cm height (bed volume = 10 ml) was formed under a constant pressure of 10 cm of water. The column was equilibrated with 3 bed volumes of Tris/ED buffer supplied by a LKB peristaltic pump set at 15 ml/hour.

Cytosol (4 ml, 1 - 5 mg protein/ml) was applied to the top of the column and allowed to drain in. The column was washed through with one bed volume of Tris/ED buffer and the eluant retained for further experiment. Step wise elution of the column with KCl (0 - 0.4 M in Tris/ED; 10 ml per step) was then undertaken. Fractions (5 ml) were collected with a LKB Ultrorac II.

(g) Dialysis

Visking 8/32 tubing (London) was used in these experiments. All procedures were carried out at 4°C.

Untreated nuclear salt extract (2 ml) was dialysed for 16 hours against 2 l of Tris/MgCl$_2$ or Tris/CaCl$_2$ buffer. Over the course of the experiment, dialysing buffer was kept in constant motion with a magnetic stirrer.
Experiments With Human Plasma

Preparation of Human Plasma

Blood drawn from patients diagnosed with benign prostate hyperplasia was collected in containers with anti-coagulant and delivered to the laboratories on ice.

The blood was transferred to low spin centrifuge tubes and cells removed at 800 x g_{AV} (15 minutes). The clear supernatant (plasma) was aspirated and divided into 1 ml aliquots for storage at -20°C.

Incubations With [{H}[^3]] Retinoids

Plasma was thawed and diluted with Tris/EDG to give a final protein concentration of 1 - 5 mg/ml.

Diluted plasma was incubated with [{H}[^3]] retinol or [{H}[^3]] retinoic acid (10^{-7} M) in the presence and absence of unlabelled like competitors in a 50 fold excess under conditions specified for the cytosol assays (2.5(b)(c)).

At the end of incubation, free ligand was removed by the DCC method (2.5(d)) and aliquots (150 μl) of the radiolabelled plasma subjected to sucrose gradient analysis (2.5(f)).

2.7. Validation of the Assay for cRBP

The retinol binding assay (section 2.5(b)) was validated using dog liver, a tissue known to contain cRBP (Bashor et al 1973).

Liver was obtained from a male dog, at the time of autopsy, and transported to the laboratory on ice. The tissue was washed with Tris/EDG buffer and used immediately.

Preparation of liver cytosol was as described for the equivalent prostate fraction (section 2.5(a)). The soluble fraction was diluted with Tris/EDG buffer to give a protein concentration of 1 - 5 mg/ml.

The subcellular fraction was assessed in duplicate at 4°C for
6 hours for \(^{3}\text{H}\) retinol binding (section 2.5(b)). After incubation, the mixture was briefly treated with charcoal pellets as described in the DCC method (section 2.5(d)) and the cytosol subjected to sucrose gradient centrifugation (section 2.5(f)).

2.8. Characterisation of Vitamin A Alcohol and Acid Binding in Prostate Cytosol

All characterisation studies were carried out using benign hyperplastic prostate.

(a) Specificity Studies

\(^{3}\text{H}\) Retinol and retinoic acid binding in the presence of various competitors was assessed by the assays described elsewhere (2.5(b)(c)) but with minor modifications.

Briefly, subcellular fraction (185 \(\mu\)l) was incubated in duplicate at 4\(^{\circ}\)C for 18 hours with 15 \(\mu\)l of ligand solution containing radioactive compound (10\(^{-7}\) M) in the presence and absence of competitors in a 50 fold excess. After this time, free ligand was removed with charcoal (section 2.5(d)) and the cytosol analysed by sucrose gradient centrifugation (section 2.5(f)).

(b) Heat/Protease Treatments

Prostate cytosol (750 \(\mu\)l) was incubated at 37\(^{\circ}\)C for 90 minutes with 250 \(\mu\)l of trypsin (0.05\%/\(v/v\) final concentration) in Tris/ED or buffer alone. In parallel experiments, 1 ml of the subcellular fraction was heated to 60\(^{\circ}\)C for 10 minutes.

The treated preparations were incubated with \(^{3}\text{H}\) retinol or \(^{3}\text{H}\) retinoic acid (10\(^{-7}\) M) in the presence and absence of like competitors in a 50 fold excess under conditions (18 hour incubation) specified in the assays (2.5(b)(c)). At the end of incubations, excess ligand was removed by the DCC method (2.5(d)) and cytosol (150 \(\mu\)l) analysed on 7.5 - 20\% sucrose gradients (2.5(f)).
(c) The Effect of Charcoal Pre-treatments on $[^3H]$ Ligand Binding

Charcoal pellets were prepared by centrifuging (Chilspin, MSE) 8 ml of DCC suspension at 100 $x$ g$_{AV}$ for 15 minutes. The buffer supernates were discarded.

Cytosol (2 ml) was divided 2 ways and one half incubated with the charcoal pellets at 4°C for 30 minutes. The suspension was agitated after the first 15 minutes of incubation. At the end of the 30 minutes the charcoal was removed by centrifugation at 800 $x$ g$_{AV}$ for 10 minutes.

Treated and untreated cytosol preparations were radiolabelled with $[^3H]$ retinol and $[^3H]$ retinoic acid ($10^{-7}$ M) under the conditions specified in the assays (2.5(b)(c)) and after a brief treatment with charcoal (2.5(d)) analysed on sucrose gradients (2.5(f)).

(d) Binding of $[^3H]$ Retinol at 4°C - A Time Study

Cytosol (185 µl) was incubated in triplicate with 15 µl of ligand solution ($[^3H]$ retinol final concentration $10^{-7}$ M with or without unlabelled retinol in 100 fold excess) at 4°C for varying times.

Incubations were terminated at the appropriate times by removal of excess ligand with charcoal pellets (2.5(d)). Aliquots (150 µl) of the treated cytosol were counted for radioactivity. Non specific binding was corrected for by subtracting the binding observed in the presence of competitor from that determined in its absence.

(e) Scatchard Analysis of Retinol Binding

Aliquots (185 µl) of subcellular fraction (0.1 - 0.5 mg protein/ml) were incubated in triplicate at 4°C for 18 hours with increasing concentrations (10 - 100 nM) of $[^3H]$ retinol. Non specific binding was assessed in parallel experiments conducted in the presence of unlabelled retinol in a 100 fold excess.
At the end of incubations, free ligand was removed with charcoal (2.5(d)) and aliquots (150 µl) of cytosol counted for radioactivity.

(f) Effects of PMSF, Aprotinin and Sodium Molybdate on Cytosol Retinol Binding

Prostate tissue (1.0 - 2.0 gm) was finely minced with scissors and pulverised in a Mikrodisembrator (2.5(a)). The homogeneous paste was divided by weight into equal amounts and further homogenised in 10 volumes of Tris/EDG buffer with or without enzyme inhibitor (PMSF 1mM, aprotinin 100 mg/l, sodium molybdate 10 mM; Rao et al 1979, Smith et al 1983a,b). Cytosol was prepared from each homogenate and the protein concentrations adjusted to 2 - 5 mg/ml. The presence of enzyme inhibitors was found to have minimal effects on the Bradford protein assay (1976). The subcellular fraction prepared with or without enzyme inhibitor was compared for [³H] retinol binding (2.5(b)(d)).

2.9. Other Experiments With Prostate Cytosol

The specificity studies and gel filtration experiments described in this section were carried out to allow a comparison of the cytosol and nuclear binding data.

(a) Specificity Studies

In these experiments, cytosol was prepared in Tris/MgCl₂ and adjusted to 0.6 M with KCl. The cytosol was assayed for [³H] retinol binding (2.5(b)(d)) in the absence and presence of unlabelled retinol, retinal and retinoic acid (all trans isomers) in a 100 fold excess.

(b) Gel Filtration

Cytosol (925 µl) prepared in Tris/MgCl₂ buffer was incubated at 4°C for 16 hours with 75 µl of ligand solution containing [³H]
retinol (10-7 M final concentration) with or without unlabelled retinol in 100 fold excess. At the end of this time, the incubation mixture was treated to a charcoal pellet prepared from 2 ml of DCC suspension, and finally subjected to gel filtration (2.5(g)). The elution position of myoglobin standard was determined in a separate experiment.

(c) Removal of the Non-Specific Retinoic Acid Binding Component

Cytosol preparations subjected to (a) no treatment, (b) immunoprecipitation (2.6(c)), (c) dye affinity chromatography (2.6(e), (d) ultrafiltration (2.6(d)) and (e) DEAE ion exchange chromatography (2.6(f)), were radiolabelled with [3H] retinoic acid under conditions specified in the assay (2.5(c)) and analysed on sucrose gradients (2.5(f)).

(d) Metabolism Studies

The soluble fraction incubated with either [3H] retinol or [3H] retinoic acid (10-7 M) under identical conditions to the binding assays (2.5(b)(c)) was ether extracted. The ether was evaporated (2.5(e)), the extracted radioactivity reconstituted in ethanol and chromatographed with authentic standards on reversed phase T.L.C. plates (2.5(e)).

2.10. Studies With Intact or Sonicated Nuclear Pellets

All studies were carried out using benign hyperplastic tissue.

(a) Experiments With Ligand Only

Nuclear pellets were prepared (30 - 60 µg DNA) (2.5(a)). The subcellular fraction was resuspended in Tris/MgCl2 with six strokes of a Jencons glass homogeniser (Herts., U.K.) and one half of the preparation sonicated for 10 minutes on ice (Ultrasonicator MSE, Crawley, Sussex, U.K.).

Aliquots of the untreated or sonicated nuclear suspension (1 - 5 µg DNA) were incubated in triplicate in the dark at 4°C for 18 hours.
with either $[^3]H$ retinol or $[^3]H$ retinoic acid ($10^{-7}$ M final concentration). Parallel incubations were conducted in the presence of unlabelled like competitor in a 100 fold excess to assess non specific binding. At the end of this time, 1 ml of Tris/MgCl$_2$ buffer containing 5% ethanol (v/v) was added to each tube. The tubes were vortexed, centrifuged at 800 x g$_{AV}$ for 5 minutes and supernates discarded. A second wash was undertaken in a similar manner to remove free ligand. The washed nuclear pellets were extracted with 500 µl of absolute ethanol. Nuclei were re-pelleted at 800 x g$_{AV}$ and 200 µl of the ethanol extract counted for radioactivity. Non specific ligand association was defined as the radioactivity extracted from nuclei incubated in the presence of unlabelled excess competitor.

(b) Incubation of Nuclei With $[^3]H$ Retinol Labelled Cytosol

The cytosol fraction was incubated in 3 parallel experiments with $[^3]H$ retinol in the presence or absence of unlabelled retinol in 100 fold excess (2.5(b)).

A suspension of untreated or sonicated nuclei (2.10(a)) containing 30 - 60 µg DNA was prepared in Tris/MgCl$_2$/TX100 (2.5(a)). The nuclear suspensions were dispensed into separate test tubes and centrifuged at 800 x g$_{AV}$ for 10 minutes. The buffer was discarded. At the end of the 6 hour incubation period, cytosol was either assayed for $[^3]H$ retinol binding (2.5(d)) or decanted into tubes containing untreated or sonicated nuclei. Nuclear-cytosol mixtures were vortexed and incubated for a further 4 hours at 4°C. After this time, 1 ml of ice-cold Tris/MgCl$_2$ buffer containing 5% ethanol was added to each tube, the mixture vortexed and nuclei sedimented at 800 x g$_{AV}$ (5 minutes). The buffer was discarded and a second cycle performed in a similar fashion. Tubes were then extracted with 500 µl of absolute ethanol, nuclei removed at 800 x g$_{AV}$ and aliquots (200 µl)
counted for radioactivity. Non specific ligand interaction was defined as the radioactivity in extracts from nuclei incubated with radiolabelled cytosol and unlabelled competitor.

2.11 (a) (I). Optimisation of the $[^3\text{H}]$ Retinoic Acid Binding in the Nuclear Salt Extract

Assaying for $[^3\text{H}]$ retinoic acid binding in the untreated nuclear salt extract (section 2.5(a)(c)) resulted in an average 1.0 fMOL\text{E bound per µg DNA} (Figure 9).

Rennie et al (1983) had reported that sonication of nuclei had improved the extractability of the androgen receptor. In addition, the ability of charcoal to enhance ligand binding in radioreceptor assays had been documented for the prolactin binding site (Leake et al 1983). Both procedures were therefore investigated for their effects on $[^3\text{H}]$ retinoic acid binding in the nuclear salt extract.

Intact nuclei (30 - 60 µg DNA) were prepared (section 2.5(a)) and resuspended in 6 ml of Tris/MgCl$_2$/KCl buffer. The suspension was divided 3 ways. One portion was retained (untreated nuclear salt extract - control), the other 2 sonicated on ice (Ultrasonicator MSE) for 10 minutes at maximum amperage. Following ultrasound, all 3 preparations were extracted with gentle stirring at 4°C for a period of 2 hours. At the end of this time, nuclei were removed at 15,000 x $g_{AV}$ (15 minutes). One of the 2 sonicated nuclear salt extracts was treated at 4°C for 1 hour with a pellet of charcoal formed from 4 volumes (8 ml) of DCC suspension. Charcoal was kept in suspension by agitation of the mixture every 15 minutes. After the 1 hour, charcoal was sedimented for 10 minutes at 800 x $g_{AV}$ and the nuclear salt extract retained for analysis. All three preparations (control, sonicated, sonicated and charcoal treated) were then compared for $[^3\text{H}]$ retinoic acid binding by the assay described elsewhere (section 2.5(c)). It is evident from Figure 9 that sonication results in a five-fold increase in the amount
of $[^3\text{H}]$ retinoic acid specifically bound. However, when sonication of nuclei was combined with charcoal pretreatment, an impressive 34.2 fMOLES $[^3\text{H}]$ vitamin A acid was bound specifically in the nuclear salt extract (Figure 9).

To optimise the effect of charcoal, a time course study was undertaken. The preparation and extraction of sonicated nuclei was as described above. The extractable fraction was incubated with a charcoal pellet, and at appropriate times, 1.5 ml of the mixture removed. Charcoal was sedimented and the supernates assayed for $[^3\text{H}]$ retinoic acid binding (2.5(c)). It is evident from Figure 10 that the effect of charcoal pretreatment on $[^3\text{H}]$ retinoic acid binding is time dependent, with exposure times of less than 1 hour being sub-optimal. Maximal binding was observed at 1 hour with no decrease up to 2 hours of incubation.

Salt extraction periods were also compared for their effect on $[^3\text{H}]$ retinoic acid binding. Briefly, sonicated nuclear suspensions (30 - 60 µg DNA) were prepared, divided 2 ways and extracted for either 2 or 16 hours (Smith et al 1983b). After these times, insoluble material was removed at 15,000 x $g_{AV}$ (15 minutes) and both extracts treated with charcoal pellets for 1 hour. Assaying for $[^3\text{H}]$ retinoic acid in the charcoal treated preparations revealed the 2 hour extraction period to be superior to that of 16 hours (Table 2) inasmuch as 40% less retinoic acid was bound with the latter extraction time.

2.11 (a) (II). Optimisation of $[^3\text{H}]$ Retinol Binding in the Nuclear Salt Extract

Due to the restricted availability of $[^3\text{H}]$ retinol, it was not possible to carry out the extensive optimisation studies undertaken for $[^3\text{H}]$ retinoic acid binding in the nuclear salt extract. Thus, experiments with $[^3\text{H}]$ retinol were limited to:-

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Suspension of nuclei (30 - 60 µg DNA) in Tris/MgCl₂/KCl were either untreated (control), or sonicated for 10 minutes. The preparations were extracted at 4°C for 2 hours, after which nuclei were removed at 15,000 x g<sub>AV</sub>. The salt extract of sonicated nuclei were divided 2 ways and one portion treated with a charcoal pellet for 1 hour. Charcoal was sedimented at 800 x g<sub>AV</sub> and the nuclear salt extract retained. The 3 preparations (control, sonicated, sonicated and charcoal treated were compared for [³H] retinoic acid binding by the assay described in section 2.5(c). Data are expressed as mean values ± S.D. The experiments were repeated with 4 separate sets of tissues.
[³H] Retinoic Acid Bound Specifically
(f moles/µg DNA)
Figure 10. $[^3\text{H}]$ Retinoic Acid Binding in Nuclear Salt Extract - Optimisation of the Charcoal Pretreatment

The salt extract of sonicated nuclei (30 – 60 μg DNA) was incubated with a charcoal pellet at 4°C for varying periods. At appropriate times aliquots of the mixture were removed and centrifuged at 800 x g$_{av}$ (10 minutes). The treated nuclear salt extracts were compared for $[^3\text{H}]$ retinoic acid binding by the assay detailed in section 2.5(c). Data are expressed as mean values ± S.D. The number of experiments are indicated in parenthesis.
SPECIFIC [3H] RETINOIC ACID BINDING
(\% MAXIMUM)
TABLE 2. A Comparison of 2 and 16 hour KCl Extraction Periods on $[^3H]$ Retinoic Acid Binding

Suspensions of nuclei in Tris/MgCl$_2$/KCl were sonicated and extracted at 4°C for 2 or 16 hours. After these times, nuclei were removed at 15,000 x g$_{AV}$ and the extracts pretreated with charcoal for 60 minutes. The charcoal was then pelleted at 800 x g$_{AV}$ and the supernates assayed for $[^3H]$ retinoic acid binding. Data are expressed as the mean values of 3 experiments $\pm$ S.D.
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<th>Extraction Period (h)</th>
<th>[ ^{3}\text{H} ] Retinoic Acid Bound Specifically (%)</th>
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<td>2</td>
<td>100</td>
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<tr>
<td>16</td>
<td>62 ± 3.5</td>
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**Table 2**
(a) A time course study of charcoal pretreatment

The preparation and extraction of sonicated nuclei (30 - 60 μg DNA) were as described in the previous section. The extractable fraction was incubated with a charcoal pellet, and at appropriate times, 1.5 ml of the mixture removed. The charcoal was sedimented and the supernate assessed for [3H] retinol binding at 4°C for 18 hours (2.5(b)). It is apparent from Figure 11 that a charcoal exposure period of 60 minutes is required for maximal binding of [3H] retinol. Extension of this period up to 90 minutes resulted in no decline of [3H] retinol binding.

(b) Comparison of 2 and 16 hour extraction periods

Sonicated nuclear suspensions were prepared, divided into equal volumes and extracted for 2 or 16 hours. At the end of these times, insoluble material was sedimented at 15,000 × g_{AV} and both extracts pretreated with charcoal for 1 hour. The nuclear salt extracts were then compared for [3H] retinol binding (2.5(b)). Table 3 illustrates that the 2 hour period is superior to 16 hours inasmuch as approximately 30% less [3H] retinol was specifically bound using the latter time.

In all subsequent experiments, the protocol adopted for the preparation of nuclear salt extract was as described in this section, utilising 10 minutes of sonication, a KCl extraction period of 2 hours and a charcoal exposure time of 60 minutes.
Figure 11. [\textsuperscript{3}H] Retinol Binding in Nuclear Salt Extracts
Optimisation of the Charcoal Pretreatment

The nuclear salt extract of sonicated nuclei was incubated with a charcoal pellet at 4\textdegree{}C for varying times. At appropriate times, aliquots of the mixture were removed and centrifuged at 800 x \textit{g}_{AV}. The treated nuclear extracts were compared for [\textsuperscript{3}H] retinol binding (2.5(b)). These experiments were repeated 4 times. Results are expressed as mean values \pm S.D.
$[^3H] \text{Retinol bound specifically (\% maximum)}$
TABLE 3.  A Comparison of 2 and 16 hour KCl Extraction Periods on $[^{3}\text{H}]$ Retinol Binding

Suspensions of nuclei in Tris/MgCl$_2$/KCl were sonicated and extracted at 4°C for 2 or 16 hours. After these times, nuclei were removed at 15,000 $\times$ g$_{AV}$ and the extracts pretreated with charcoal for 60 minutes. The charcoal was pelleted at 800 $\times$ g$_{AV}$ and the supernates assayed for $[^{3}\text{H}]$ retinol binding (2.5(b)). Data are expressed as mean % values of 3 experiments $\pm$ S.D.
### Table 3

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<td>[3H] Retinol Bound Specifically (%)</td>
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<td></td>
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2.11 (b). Characterisation of Retinoic Acid and Retinol Binding in the Nuclear Salt Extract

All studies in this section were carried out using benign hyperplastic tissue.

(i) Specificity studies

Competition experiments were essentially those described in section 2.8(a). Briefly, nuclear salt extract was incubated at 4°C for 16 hours with \([^3\text{H}]\) retinoic acid or \([^3\text{H}]\) retinol (10\(^{-7}\) M) in the presence and absence of various competitors. After incubation, unbound ligand was removed by the DCC method (2.5(d)) and nuclear salt extract counted for radioactivity. The competition observed with unlabelled ligand competitor was defined as 100%.

(ii) Pronase digestion experiments

Prior to assaying for \([^3\text{H}]\) retinoic acid or \([^3\text{H}]\) retinol binding (2.5(b)(c)), nuclear salt extract was incubated at 37°C for 2 hours with "Pronase" (3 IU/ml - final concentration) or buffer (Tris/MgCl\(_2\)).

(iii) Temperature/time studies

Nuclear salt extract was incubated with \([^3\text{H}]\) retinol at 4°C or with \([^3\text{H}]\) retinoic acid (10\(^{-7}\) M) at 4°C, 25°C or 37°C for varying periods (section 2.8(d)). Parallel experiments were conducted in the presence of unlabelled like competitor in a 100 fold excess. At appropriate times, incubations were terminated by the DCC method (2.5(d)) and the specific binding of either ligand determined.

(iv) Scatchard analysis of retinol and retinoic acid binding

Aliquots of nuclear salt extract were incubated (2.8(e)) in triplicate with \([^3\text{H}]\) retinol at 4°C for 18 hours or with \([^3\text{H}]\) retinoic acid at 37°C for 30 minutes (unless stated otherwise) over a
range (5 - 60 nM) of ligand concentrations.

Non specific binding was determined in parallel experiments conducted in the presence of unlabelled like competitor in 100 fold excess. At the end of incubations free ligand was removed by the DCC method (2.5(d)) and the nuclear salt extract counted for radioactivity. The data was analysed by the Scatchard method (1949).

(v) Gel filtration studies

Nuclear salt extract was radiolabelled with $10^{-7}$ M $[^3H]$ retinol as described in section 2.9(b) and gel filtered on an Ultrogel AcA 34 column (2.5(g)).

In parallel studies, the nuclear salt extract (950 µl) was incubated at 37°C for 30 minutes or at 4°C for 16 hours with 50 µl of ligand solution containing $[^3H]$ retinoic acid ($10^{-7}$ M final concentration) and unlabelled competitor in 100 fold excess. After these times, the mixture was treated with a charcoal pellet and finally gel filtered on the Ultrogel AcA 34 column (2.5(g)). In one experiment, the effect of sodium molybdate (10 mM) on the elution profile was investigated. Nuclear salt extract prepared in the presence of sodium molybdate, was radiolabelled with retinoic acid and gel filtered on the Ultrogel AcA 34 column equilibrated with buffer containing 10 mM sodium molybdate.

In another experiment, the effect of desalting on the elution profile of retinoic acid was tested. Nuclei were sonicated for 10 minutes and extracted with Tris/MgCl$_2$/KCl (2.11(a)). The extract was dialysed against Tris/MgCl$_2$ (2.6(g)), pretreated with charcoal for 1 hour and finally radiolabelled with retinoic acid for gel filtration.

The effect of micrococcal nuclease was also investigated. Digestion experiments were essentially those of Rennie et al (1983).
A nuclear pellet (30 - 60 μg DNA) was prepared (section 2.5(a)) and extracted for 2 hours with Tris/CaCl₂ containing 0.6M KCl. Insoluble material was removed at 15,000 x g_{AV} and the supernatant dialysed extensively against Tris/CaCl₂. The subcellular fraction was charcoal treated and subjected to micrococcal nuclease (5 IU/ml, 37°C for 1 hour). The preparation was radiolabelled with retinoic acid and gel filtered.

(vi) The effect of enzyme inhibitors

Nuclear salt extracts were prepared in the presence of various enzyme inhibitors, phenylmethylsulphonyl fluoride (PMSF) 1 mM, aprotinin 100 mg/l, sodium molybdate 10 mM and compared for [³H] retinoic acid binding (2.5(c)).

To optimise the effect of sodium molybdate, nuclear salt extracts prepared in the absence and presence of increasing (0.1 - 50 mM) concentration of inhibitor were compared for [³H] retinoic acid binding.

2.11 (c). Metabolism Studies

Incubations of nuclear salt extract with [³H] retinol (10⁻⁷ M) or [³H] retinoic acid (10⁻⁷, 10⁻⁵ M) were performed under identical conditions to the nuclear binding assays (2.5(b),(c)).

Extraction and reversed phase chromatography of standards and radioactive material has been described elsewhere (2.3(c), 2.5(e)).

2.12. Comparison of Retinol Binding in Hyperplastic and Malignant Prostate

(a) Retinol Binding Assay

The assay was essentially that described elsewhere (2.5(b)) but with minor modifications. Cytosol was prepared from resected hyperplastic and malignant gland and the protein concentration adjusted to 0.5 mg/ml. The subcellular fraction was incubated with [³H] retinol (10⁻⁷ M) in the presence and absence of competitor at 4°C for 4 hours after which unbound ligand was removed by the DCC method (2.5(d)).
Data Analysis

The differences in specific binding of $[^3]H$ retinol between hyperplastic and malignant prostates were tested for statistical significance by the Mann-Whitney U-Test.

(b) Validation of the Assay

The assay was validated using hyperplastic prostate (unless stated otherwise).

(i) Optimisation of the DCC method

To optimise charcoal post-treatments, cytosol incubated with $[^3]H$ retinol in the presence and absence of unlabelled ligand, was treated with charcoal pellets for periods of 5, 10 and 15 minutes. At appropriate times, charcoal was removed at $800 \times g_{av}$ and the treated subcellular fractions compared for $[^3]H$ retinol binding.

(ii) Linearity of the assay with protein concentration

Serial dilutions of cytosol were made in Tris/EDG buffer to give a range (0.1 - 2.5 mg/ml) of protein concentrations. The original and diluted preparations were compared for $[^3]H$ retinol binding by the assay described in this section.

(iii) Effects of snap-freezing and storage of tissue at $-70^\circ C$

A homogeneous tissue paste was prepared by pulverisation of finely minced prostate on a Mikrodismembrator (B Braun) and divided 6 ways (500 mg portions). Five portions were snap frozen in liquid nitrogen and stored at $-70^\circ C$ for future assays. After allowing a 30 minute period for equilibration of tissue with the freezer temperature, one portion was retrieved, thawed in the cold room, and assayed for retinol binding in parallel with the prostate not frozen. Aliquots of prostate (500 mg) were removed from the freezer after 1, 2, 4 and 8 weeks of storage and retinol binding determined in each case.
(iv) **Saturation analysis of retinol binding in malignant gland**

Saturation and Scatchard (1949) analysis of the retinol binding protein in malignant prostate cytosol was identical to that detailed in 2.8(e) with the exception that the incubation period was reduced to 4 hours.

(v) **Metabolism studies with malignant prostate cytosol**

The soluble fraction of malignant gland was incubated with $[^3H]$ retinol under conditions specified in the assay (2.12(a)). Extraction of radioactivity and reversed phase chromatography have been described elsewhere (2.5(e), 2.3(c)).

(vi) **Inter and intra assay variation**

Finely minced prostate was pulverised on a Mikrodismembrator and the resulting homogeneous paste divided by weight into 5 x 500 mg portions. The cytosol fraction was prepared from each portion and assayed separately for retinol binding.

Inter-assay variation was expressed as the % change in retinol binding between assays; intra assay variation described the difference between triplicate values of the same assay.
CHAPTER 3. (RESULTS)
CHAPTER 3. RESULTS

In this chapter, results will be presented as follows:

(1) characterisation of the cytosolic binding for
   (a) retinol
   (b) retinoic acid

(2) other experiments with prostate cytosol

(3) mitochondrial and microsomal binding of vitamin A alcohol and acid

(4) (a) binding of retinol/retinoic acid in nuclear pellets
    (b) characterisation of the nuclear extractable component for retinoic acid and retinol

(5) comparison of retinol binding in prostate hyperplasia and malignancy.
3.1. Cytosol Binding of Retinoids

(a) Retinol

Validation of the binding assay

Cytosol from dog liver, a tissue known to possess cRBP (Bashor et al 1973) was incubated with \(^{3}\text{H}\) retinol (10\(^{-7}\)M) at 4°C for 6 hours and analysed on sucrose gradients (Figure 12). The characteristics of ligand binding in these studies (saturability, specificity and a sedimentation coefficient of 2S) are identical to those observed by Bashor's group (1973) and suggest that \(^{3}\text{H}\) retinol is binding to the classical cRBP macromolecule.

Characterisation of retinol binding in human hyperplastic prostate

(i) Specificity studies

Incubation of prostate cytosol (2 - 5 mg protein/ml) with \(^{3}\text{H}\) retinol (10\(^{-7}\)M) at 4°C for 18 hours followed by sucrose gradient analysis revealed a peak of radioactivity coincident with the 2S external marker (Figures 13, 14, 15). The binding of radioligand was abolished with a 50 fold excess of unlabelled trans retinol but not by trans retinoic acid (Figure 13), the synthetic analogues RO 10-1670, RO 13-7410 (Figure 14), or steroid hormones (Figure 15). All trans retinal (Figure 13) and 13 cis retinoic acid (Figure 14) showed some affinity for the peak as indicated by the partial displacement of radioligand. This saturable peak is unlikely to be a blood contaminant of prostate cytosol since experiments with \(^{3}\text{H}\) retinol radiolabelled human plasma revealed the absence of ligand binding in the 2S region of sucrose gradients (Figure 16).

(ii) Inactivation of retinol binding by protease and heat treatments

Protease experiments were undertaken to determine the nature of the retinol binding macromolecule. It is evident from
Figure 12. Sucrose Gradient Analysis of Retinol Radiolabelled Dog Liver Cytosol

Dog liver cytosol was radiolabelled with $[^3$H$]$ retinol $(10^{-7} \text{M})$ at $4^\circ\text{C}$ for 6 hours in the absence (▲) or presence of unlabelled trans retinol (●) or trans retinoic acid (○) in a 50 fold excess. After incubation, preparations were briefly treated with charcoal and finally centrifuged on 7.5 - 20% sucrose gradients at 400,000 x $g_{AV}$ for 2 hours. The experiment was repeated 3 times.
Figure 13. Specificity of [³H] Retinol Binding in Cytosol From Benign Human Prostate - Sucrose Gradient Analysis

Aliquots of prostate cytosol were incubated at 4°C for 18 hours with [³H] retinol (10⁻⁷M) alone (▲) or in the presence of unlabelled retinol (●), retinal (●), retinoic acid (○) (all trans isomers) in a 50 fold excess. After incubation, unbound ligand was removed by the DCC method and cytosol analysed on sucrose gradients. The experiments were repeated 3 times.
Figure 14. Specificity of [3H] Retinol Binding in
Hyperplastic Prostate Cytosol

Aliquots of prostate cytosol were incubated at 4°C for
18 hours with [3H] retinol alone (10^-7M) (▲) or in the
presence of unlabelled RO 10-1670 (■), RO 13-7410 (○)
13 cis - retinoic acid (●) in a 50 fold excess. After
free ligand was removed by the DCC method, [3H] retinol
binding was assessed by sucrose gradient centrifugation.
The experiment was repeated with 3 separate tissues.
Figure 15. $[^{3}H]$ Retinol Binding in Hyperplastic Prostate Cytosol - Specificity Studies

Cytosol was incubated with $[^{3}H]$ retinol (10$^{-7}$M) at 4°C for 18 hours, alone (▲) or in the presence of dihydrotestosterone (■), testosterone (●), oestradiol - 17β (○) in a 50 fold excess. The preparations were treated with charcoal, and cytosol subjected to sucrose gradient centrifugation. The results shown are representative of 3 experiments.
Human plasma was diluted in Tris/EDG buffer to give a protein concentration of 1 - 5 mg/ml. The preparation was incubated with $[^3H]$ retinol ($10^{-7} M$) at 4°C for 18 hours, after which free ligand was removed with charcoal and the sample analysed on 7.5 - 20% sucrose gradients. The data is representative of 2 experiments with plasma from different patients.
Figure 17 that the binding of radio-active retinol in the 2S region of the gradient is sensitive to trypsin (0.05%(W/v)) and heat (60°C, 10 minutes) pre-treatments. These data would suggest that the 2S binding of \[^{3}H\] retinol is to a prostate protein.

(iii) Effect of charcoal pretreatment

To test whether endogenous ligand was interfering with the assay, thereby resulting in an underestimation of cytosol retinol binding sites, the subcellular fraction (2 - 5 mg protein/ml) was exposed to a charcoal pellet for 30 minutes and then assayed for \[^{3}H\] retinol binding (section 2.5(b)). The specific binding data was found to be unaltered by charcoal pre-treatment (data not shown).

(iv) \[^{3}H\] Retinol binding at 4°C - a time study

In view of the instability of \[^{3}H\] retinol, the optimal time for radioligand exchange was determined at 4°C only. The subcellular fraction was incubated with \[^{3}H\] retinol (10\(^{-7}\)M) at 4°C for varying periods. At appropriate times, the reaction was terminated by removal of free ligand with charcoal and the cytosol counted for radioactivity. It is evident from Figure 18 that the cytosol binding of ligand at this temperature, is time dependent. Equilibrium of binding was established after 4 hours of incubation and maintained for up to 24 hours.

(v) Scatchard analysis

Under optimal conditions for the retinol binding assay (an 18 hour incubation at 4°C) saturation analysis was undertaken over a range of ligand concentrations (10 - 100 nM). Manipulation of the resulting saturation data (Figure 19a) by the method of Scatchard (1949) revealed the binding of \[^{3}H\] retinol to be of high affinity with a \(K_D\) of 33±6 nM (Figure 19b).
Figure 17. Inactivation of [3H] Retinol Binding by Heat and Trypsin Treatments - Sucrose Gradient Analysis

The soluble fraction of human, benign prostate was treated with trypsin (0.05% w/v) or briefly heated to 60°C. Untreated cytosol was incubated with [3H] retinol (10^-7M) in the absence (▲) and presence (●) of unlabelled trans retinol in a 50 fold excess; trypsin (●) and heat (○) treated preparations were radiolabelled with retinol in the absence of competitor. Samples were overlayed sucrose gradients (7.5 - 20%) after charcoal treatment and centrifuged at 400,000 x g_{AV} for 2 hours. All studies were performed in triplicate.
Figure 18. Retinol Binding in Prostate Cytosol -
A Time Course Study

Cytosol was incubated at 4°C for varying times with
[3H] retinol (10⁻⁷M) in the presence or absence of
unlabelled compound in 100 fold excess. At appropriate
times, unbound ligand was removed by the DCC method
(section 2.5(d)) and aliquots of cytosol counted for
radioactivity. Results are expressed as mean values of
4 experiments ± S.D.
**[3H]** Retinol bound specifically (% maximum)

**Incubation time (h)**

- 0.5
- 1
- 2
- 3
- 4
- 18
- 24
Binding of [³H] retinol was assessed over a range (10 - 100 nM) of ligand concentrations with or without unlabelled compound in 100 fold excess. Incubations were carried out at 4°C for 18 hours, after which specific binding was determined by the DCC method. The specific binding data (a) was plotted by the method of Scatchard (b) to yield a value for the dissociation constant.

The experiments were repeated 5 times. (▲) specific binding of retinol; (●) non specific ligand binding.
(vi) The effect of various enzyme inhibitors on \[^{3}\text{H}]\ retinol binding

The well documented observation of high levels of endogenous proteases in the prostate (Gotterer et al 1956, Isaacs and Coffey 1984) plus a recent report that sodium molybdate improved androgen receptor assays (Smith et al 1983a) prompted an investigation of the protease inhibitors PMSF (1.0 mM), aprotinin (100 mg/l) and sodium molybdate (10 mM) a phosphatase inhibitor. Under the conditions of the assay (section 2.5(b)) retinol binding at 4°C was not enhanced by the presence of these enzyme blockers (Table 4). These data would suggest either of 2 possibilities. Firstly, enzymes involved in the degradation of the prostate retinol binding protein may be inactive at 4°C. Alternatively, this vitamin A binding protein may be insensitive to those enzymes blocked by the above-mentioned inhibitors.

(b) Retinoic Acid Binding

Although Brandes (1981) had detected a saturable retinoic acid binding component in human hyperplastic prostate cytosol, this worker had failed to characterise the binding site in terms of specificity, ligand affinity and heat/protease sensitivity. The results in this section are an extension to these earlier findings.

(i) Specificity studies

Sucrose gradient analysis of prostate cytosol (2 - 5 mg protein/ml) radiolabelled at 4°C for 18 hours with \[^{3}\text{H}]\ retinoic acid (10^{-7}M) revealed a peak of radioactivity in the 2S region (Figures 20, 21, 22) which was abolished in the presence of all trans retinoic acid (Figure 20), the synthetic analogues RO 10-1670, RO 13-7410 and 13 cis-retinoic acid (Figure 21). Steroid hormones were poor competitors for the binding of \[^{3}\text{H}]\ retinoic acid (Figure 22) but somewhat surprising both the alcohol and aldehyde (Figure 20) forms of vitamin A displayed
TABLE 4. The Effect of Various Enzyme Inhibitors on Retinol Binding in Prostate Cytosol

Cytosol prepared in the absence (control) and presence of PMSF (1 mM), aprotinin (100 mg/l) and sodium molybdate (10 mM) were compared for retinol binding. Subcellular fraction was incubated at 4°C for 18 hours with \([^3H]\) retinol (10\(^{-7}\)M). Non specific binding was determined by parallel incubations with unlabelled retinol in 100 fold excess. After incubation, unbound assay ligand was removed by the DCC method, and aliquots of cytosol counted for radioactivity. Each experiment was repeated 4 times.
<table>
<thead>
<tr>
<th>Enzyme Inhibitor</th>
<th>[^{3}H] Retinol Specifically Bound (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent PMSF (1 mM)</td>
<td>94 ± 14</td>
</tr>
<tr>
<td>Aprotinin (100 mg/l)</td>
<td>88 ± 15</td>
</tr>
<tr>
<td>Sodium Molybdate (10 mM)</td>
<td>95 ± 10</td>
</tr>
</tbody>
</table>
Figure 20. Specificity of $[^3H]$ Retinoic Acid Binding in Cytosol From Human Hyperplastic Prostate - Sucrose Gradient Analysis

Aliquots of prostate cytosol were incubated at $4^\circ$C for 18 hours with $[^3H]$ retinoic acid ($10^{-7}$M) alone (Δ) or in the presence of unlabelled competitor ($5 \times 10^{-6}$M); retinoic acid (○), retinal (●), retinol (■) (all trans isomers); After incubation, samples were treated with charcoal and subjected to sucrose gradient (7.5 - 20%) analysis. The experiments were repeated 3 times.
[\textsuperscript{3}H] Retinoic acid bound d.p.m. ($\times 10^{-3}$)
Prostate cytosol was incubated at 4°C for 18 hours with $[^3]H$ retinoic acid (10$^{-7}$M) alone (△) or in the presence of unlabelled RO 10-1670 (■), RO 13-7410 (○), 13 cis-retinoic acid (●) in a 50 fold excess. After this time, unbound ligand was removed with charcoal and the cytosol overlayed 7.5 - 20% sucrose gradients. The data are representative of 3 separate experiments.
$[^3H]$ Retinoic acid bound d.p.m. ($\times 10^{-3}$)

Fraction number
Figure 22. Specificity of $[^3H]$ Retinoic Acid Binding in Cytosol From Hyperplastic Prostate - Sucrose Gradient Centrifugation

Cytosol was incubated with $[^3H]$ retinoic acid ($10^{-7}$M) at $4^\circ$C for 18 hours, alone ($\Delta$) or in the presence of excess unlabelled testosterone ($\bullet$), dihydrotestosterone ($\blacksquare$), all trans retinoic acid ($\circ$). At the end of this time, free ligand was removed by the DCC method, and the subcellular fraction analysed on 7.5 - 20% sucrose gradients. The experiments were repeated with 3 separate sets of tissues.
some affinity for the 2S component. A second peak of radioactivity coincident with the 4.6S external marker was unaffected by any competitor in a 50 fold excess and therefore regarded as non specific binding.

The prostatic origin of the 2S retinoic acid binding component was suggested by experiments with human plasma. These studies clearly showed the absence of a 2S binding site on sucrose gradients (Figure 23).

(ii) Protease and heat inactivation

To establish the nature of the prostate retinoic acid binding component, cytosol was pre-incubated with trypsin (0.05% \( \text{W/v} \)) or briefly heated to 60°C. The preparations were radiolabelled with retinoic acid \((10^{-7}\text{M})\) at 4°C for 18 hours and analysed on sucrose gradients. It is clear from Figure 24 that these treatments inactivate the cytosol binding of \(^3\text{H}\) vitamin A acid. These observations are consistent with the contention that ligand association is with a protein macromolecule.

(iii) Effect of charcoal pre-treatment

The possibility that endogenous ligand was interfering with the assay for \(^3\text{H}\) retinoic acid binding was tested indirectly by pre-exposure of cytosol to charcoal for a 30 minute period. Treated and untreated subcellular fractions were then radiolabelled with \(^3\text{H}\) retinoic acid \((10^{-7}\text{M})\) and compared for specific binding on sucrose gradients. Data not shown indicated that charcoal pre-treatment was without effect on the binding of ligand.
Figure 23. \(^{3}H\) Retinoic Acid Binding in Human Plasma

Human plasma diluted in Tris/EDG buffer to give a protein concentration of 1 - 5 mg/ml was incubated with \(^{3}H\) retinoic acid \((10^{-7}M)\) at 4°C for 18 hours. After this time, free ligand was removed with charcoal and the preparation overlayed 7.5 - 20% sucrose gradients. The results shown are typical of separate experiments with plasma from different patients.
$[^3\text{H}]$ Retinoic acid bound (dpm x 10$^{-3}$)
Figure 24. Inactivation of $[^{3}\text{H}]$ Retinoic Acid Binding by Heat and Trypsin Treatments - Sucrose Gradient Analysis

The soluble fraction was treated with trypsin (0.05%W/v) or briefly heated to 60°C. Untreated cytosol was incubated with $[^{3}\text{H}]$ retinoic acid $(10^{-7}\text{M})$ in the absence ($\Delta$) and presence ($\blacksquare$) of unlabelled trans-retinoic acid in 50 fold excess; trypsin ($\bullet$) and heat treated ($\circ$) preparations were radiolabelled with retinoic acid in the absence of competitor. At the end of incubation, samples were treated with charcoal, and cytosol analysed by sucrose gradient (7.5 - 20%) centrifugation. The experiments were repeated with 3 sets of tissues.
3.2. Other Experiments with Prostate Cytosol

(a) Metabolism Studies

The ability (albeit limited) of all trans retinol to compete for $^{3}$H retinoic acid binding (Figure 20) was surprising. To test whether this observation was a consequence of in vitro ligand conversion metabolism studies were undertaken. Cytosol incubated with $^{3}$H retinol or $^{3}$H retinoic acid (10$^{-7}$M) under identical conditions to the binding assays (2.5(b)(c)) were extracted and the radioactive material chromatographed on reversed phase plates. It is apparent from Figures 25 and 26 that in vitro ligand conversion is not manifested under the assay conditions used. In fact the chromatograms were identical to those in which radioligands were extracted from buffer alone.

(b) Removal of the 4.6S Non Specific Binding Component for $^{3}$H Retinoic Acid

Sucrose gradient analysis is suitable for obtaining semi-quantitative data as required in specificity studies or protease sensitivity experiments. However, obtainment of quantitative data by this method is extremely difficult and a prerequisite to further characterisation of the prostate retinoic acid binding protein. As a preliminary to the establishment of a quantitative assay, the non-specific binding component as evident in the 4.6S region of sucrose gradients would have to be substantially reduced. The results in this section document the abilities of various methods to remove the interfering component. All of the methods assessed (immunoprecipitation, dye affinity chromatography, ultrafiltration, and DEAE ion exchange) were successful insofar as $^{3}$H ligand binding in the 4.6S region was reduced (the results of dye affinity chromatography and ultrafiltration are shown in Figure 27). Unfortunately, concomitant with this reduction was a loss of specific vitamin A acid binding (Figure 27).
Cytosol incubated with \([^3H]\) retinol (10\(^{-7}\)M) under conditions identical to the binding assays was extracted with diethylether. The extracts were evaporated of solvent under vacuum and reconstituted in 50 \(\mu\)l of absolute ethanol. Reconstituted material and authentic standards were applied to reversed phase plates and developed at 4\(^\circ\)C in an ethanol: water (8:2) solvent system. Plates were dried under \(N_2\), standards located, and 5 or 10 mm slices counted for radioactivity. The experiments were repeated with 4 separate tissue preparations.
Retinol standard

RADIOACTIVITY (dpm x 10^-3)

Retinoic acid standard

Solvent front

Slice number
Figure 26. Metabolism of [\(^3\)H] Retinoic Acid by Hyperplastic Prostate Cytosol

Cytosol was incubated at 4°C for 18 hours with 10^{-7}M \(^3\)H retinoic acid. After this time, the mixture was extracted, twice, with diethylether. The ether solvent was evaporated under vacuum, the residual radioactive material reconstituted in ethanol and applied, together with authentic standards, to reversed phase T.L.C. plates. The plates were developed at 4°C for 4 hours in an ethanol:water (8 : 2) solvent system. Plates were finally dried under nitrogen, authentic standards located and 5 or 10 mm slices counted for radioactivity. The data are representative of 5 separate experiments.
Figure 27. The Effect of Ultrafiltration and Dye Affinity Chromatography on Cytosol Binding of $[^3\text{H}]$ Retinoic Acid

Cytosol subjected to no treatment (▲), dye affinity chromatography (●) or ultrafiltration (○) was incubated with $[^3\text{H}]$ retinoic acid ($10^{-7} \text{M}$) at 4°C for 18 hours. After this period, excess assay ligand was removed with charcoal and the preparations analysed by sucrose gradient (7.5 - 20%) centrifugation. The experiments were repeated twice.
(c) **Gel Filtration**

Prostate cytosol (2 - 5 mg protein/ml) was radiolabelled with $[^3H]$ retinol ($10^{-7}$M) at 4°C for 16 hours and the preparation gel filtered on an Ultrogel AcA34 column. As evident in Figure 28, a saturable retinol binding component co-eluted with the myoglobin standard in fraction 37. In addition, a second peak of bound radioactivity was observed in the void volume of the column and probably represents non specific ligand association since unlabelled retinol in excess was without effect on its height.
Figure 28. Gel Filtration of Cytosol Radiolabelled with Retinol

Cytosol prepared in Tris/MgCl₂ buffer was reacted with [³H] retinol (10⁻⁷M) at 4°C for 16 hours in the presence (●) or absence (▲) of unlabelled all trans retinol. After a brief treatment with charcoal, cytosol was gel filtered on an Ultrogel AcA34 column. The column was equilibrated and eluted with Tris/MgCl₂ buffer containing 5% (v/v) ethanol. The experiment was repeated twice.
3.3. Retinoid Binding in Prostate Microsomes and Mitochondria

Suspensions of microsomes and mitochondria in Tris/ED buffer were incubated with \(^{3}H\) retinol or \(^{3}H\) retinoic acid (10\(^{-7}\)M) at 4\(^\circ\)C overnight. At the end of this time, free ligand was removed with charcoal pellets and radioactivity determined in the microsomes and mitochondria. In all tissues examined, no specific binding of either ligand could be detected in the microsomal or mitochondrial fractions.
3.4. Nuclear Studies

Nuclear binding studies were carried out on:

(a) nuclear pellets
(b) nuclear salt extracts.

The preparation of nuclear pellets and salt extracts were as described in sections 2.5(a) and 2.11(a).

(a) Studies With Nuclear Pellets

Specific binding of retinol and retinoic acid could not be detected in intact or sonicated nuclei incubated with \[^3H\] ligand at 4°C for 18 hours. Similarly, experiments in which nuclei were incubated with \[^3H\] retinol labelled cytosol (25,000 dpm bound specifically) also revealed the absence of specific ligand association at this subcellular site.

(b) Characterisation of Retinoic Acid and Retinol Binding in Nuclear Salt Extracts

(i) Specificity studies

The nuclear salt extract was incubated with \[^3H\] retinoic acid or \[^3H\] retinol at 4°C for 16 hours in the presence and absence of various unlabelled competitors. At the end of this time, free ligand was removed with charcoal and the nuclear salt extract counted for radioactivity.

Table 5 illustrates the specificity of \[^3H\] retinoic acid binding. \[^3H\] ligand was displaced by all trans retinoic acid (100%), all trans retinol (95%) and one of the synthetic analogues, RO 10-1670 (91%). All trans retinal and RO 13-7410 showed lower affinities for the component (60% competition). In contrast, testosterone, dihydrotestosterone, oestradiol-17β and progesterone were without effect.

In a more limited study, all trans retinoic acid (Table 6) but not the steroid dihydrotestosterone appeared just as effective as all
trans retinol in competing for the specific binding of $[^3\text{H}]$retinol. In contrast, the ability of retinal (trans) to compete for $[^3\text{H}]$ retinol binding was intermediate (Table 6, 56%). This value in fact is identical to that observed in studies with $[^3\text{H}]$ retinoic acid as radioligand (Table 5, 60%) and retinal as competitor.

(ii) Protease sensitivity

The nature of the nuclear derived vitamin A alcohol and acid binding component (S) was investigated by digestion experiments. Incubation of the nuclear salt extract at $37^\circ\text{C}$ for 2 hours with "Pronase" (3 IU/ml) totally abolished the specific binding of $[^3\text{H}]$retinol and $[^3\text{H}]$ retinoic acid (Table 7). These data are in accordance with the view that the binding of vitamin A alcohol and acid in nuclear salt extracts is to a protein macromolecule (S).

(iii) Temperature/time studies

The stability of retinoic acid allowed the investigation of ligand binding in nuclear salt extracts over a range of temperatures. This was not the case for $[^3\text{H}]$ retinol where some degradation of the compound can be expected at elevated temperatures. Nuclear salt extract was incubated with $[^3\text{H}]$retinol at $4^\circ\text{C}$ or with $[^3\text{H}]$ retinoic acid ($10^{-7}\text{M}$) at $4^\circ$, $25^\circ$ or $37^\circ\text{C}$ for varying periods after which free ligand was removed with charcoal and extract counted for radioactivity.

It is evident from Figure 29 that the binding of $[^3\text{H}]$ retinoic acid is time dependent. At $37^\circ\text{C}$ ligand binding rapidly came into equilibrium, decreasing after a 30 minute incubation period. In contrast, at the lower temperatures of $4^\circ\text{C}$ and $25^\circ\text{C}$, equilibrium was slower to establish requiring 4 and 2 hours respectively; at these temperatures, there was no decline in retinoic acid binding for up to 16 hours of incubation.
TABLE 5. Specificity of [3H] Retinoic Acid Binding in Nuclear Extracts

Nuclear salt extract was incubated at 4°C for 16 hours with [3H] retinoic acid (10^-7 M) in the presence and absence of various competitors in 100 fold excess. At the end of incubation, free ligand was removed with charcoal treatment and aliquots of extract counted for radioactivity. Competition by a 100 fold excess of all trans retinoic acid was defined as 100%. The ability of other compounds to compete was expressed relative to this value.
<table>
<thead>
<tr>
<th>COMPETITOR IN 100 FOLD EXCESS</th>
<th>% COMPETITION (MEAN ± S.D.)</th>
<th>NUMBER OF EXPERIMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>RETINOIC ACID (TRANS)</td>
<td>100</td>
<td>7</td>
</tr>
<tr>
<td>RETINOL (TRANS)</td>
<td>95 ± 12</td>
<td>14</td>
</tr>
<tr>
<td>RO 10 1670</td>
<td>91 ± 7</td>
<td>3</td>
</tr>
<tr>
<td>RO 13 7410</td>
<td>60 ± 2</td>
<td>3</td>
</tr>
<tr>
<td>RETINAL</td>
<td>60 ± 4</td>
<td>3</td>
</tr>
<tr>
<td>DIHYDROTESTOSTERONE</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>TESTOSTERONE</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>OESTRADIOL - 17β</td>
<td>9 ± 9</td>
<td>3</td>
</tr>
<tr>
<td>PROGESTERONE</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>
TABLE 6. \[^{3}\text{H}]\text{ Retinol Binding in Nuclear Extracts - Competition Studies}\n
Nuclear extract was incubated at 4°C for 16 hours with \[^{3}\text{H}]\text{ retinol (10}^{-7}\text{M)}\) in the presence and absence of unlabelled trans competitor in 100 fold excess. After this time, excess ligand was removed with charcoal pellets and aliquots of extract counted for radioactivity. The competition observed with all trans retinol was defined as 100%; the ability of various agents to compete for binding was expressed relative to this value.
<table>
<thead>
<tr>
<th>COMPETITOR IN 100 FOLD EXCESS</th>
<th>% COMPETITION (MEAN ± S.D.)</th>
<th>NUMBER OF EXPERIMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>RETINOL (TRANS)</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td>RETINAL (TRANS)</td>
<td>56 ± 6</td>
<td>3</td>
</tr>
<tr>
<td>RETINOIC ACID (TRANS)</td>
<td>91 ± 22</td>
<td>3</td>
</tr>
<tr>
<td>DIHYDROTESTOSTERONE</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>
TABLE 7. The Effect of "Pronase" Treatment on $[^3\text{H}]$ Retinoic Acid and $[^3\text{H}]$ Retinol Binding in Nuclear Salt Extracts

Nuclear salt extract was incubated at 37°C for 2 hours with buffer or "Pronase" (3 IU/ml). After this time, binding of $[^3\text{H}]$ retinol and $[^3\text{H}]$ retinoic acid was assayed in protease treated and untreated samples by the methods described in sections 2.5(b)(c).
<table>
<thead>
<tr>
<th>Patient</th>
<th>Pronase</th>
<th>3h Retinol Bound</th>
<th>3h Retinal Bound</th>
<th>3h Retinoic Acid</th>
<th>DNA Mols/ug</th>
<th>3h Retinoic Acid Bound</th>
<th>DNA Mols/ug</th>
<th>3h Retinol Bound</th>
<th>3h Retinal Bound</th>
<th>3h Retinoic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Present</td>
<td>27.8</td>
<td>27.8</td>
<td>27.8</td>
<td>0.0</td>
<td>3</td>
<td>0.0</td>
<td>30.6</td>
<td>0.0</td>
<td>30.6</td>
</tr>
<tr>
<td>2</td>
<td>Present</td>
<td>10.4</td>
<td>11.8</td>
<td>11.8</td>
<td>0.0</td>
<td>2</td>
<td>0.0</td>
<td>11.8</td>
<td>0.0</td>
<td>11.8</td>
</tr>
<tr>
<td>3</td>
<td>Present</td>
<td>3.4</td>
<td>3.4</td>
<td>3.4</td>
<td>0.0</td>
<td>1</td>
<td>0.0</td>
<td>25.0</td>
<td>0.0</td>
<td>25.0</td>
</tr>
</tbody>
</table>

**TABLE 7**
Figure 29. $[^3H]$ Retinoic Acid Binding in Nuclear Extract: A Time/Temperature Study

Nuclear salt extract was incubated with $[^3H]$ retinoic acid (10$^{-7}$M) at 4$^\circ$C (●), 25$^\circ$C (▲) and 37$^\circ$C (○) for various periods. Non specific binding was determined in parallel experiments with unlabelled trans retinoic acid in 100 fold excess. At appropriate times, the binding reaction was terminated by charcoal treatment (DCC method) and aliquots of extract counted for radioactivity. Data are expressed as a % of the binding at 37$^\circ$C (30 minutes). Figures in parentheses refer to the number of experiments.
$[^3]H$ RETINOIC ACID SPECIFICALLY BOUND
(% MAXIMUM AT 37°C)
Studies with $[^3]$H retinol revealed that the binding of this radioligand was also dependent on time requiring a period of 3 hours for equilibration (Figure 30). No decrease in the binding of $[^3]$H retinol was observed with incubation times up to 18 hours.

(iv) **Scatchard analysis**

Saturation of the retinol and retinoic acid binding components was carried out over a range (5 - 60 nM) of ligand concentrations. Incubations were performed at $4^\circ$C (16 hours) for retinol and at $37^\circ$C (30 minutes) for retinoic acid. Typical binding curves are shown in Figures 31a and 32a. Analysis of the data by the Scatchard method (Figures 31b, 32b) gave $K_D$ values of 12.5±1.0 nM (binding capacity 50±13 fMOLES/μg DNA) for retinol and 15.0±2.0 nM (binding capacity 48.2±18 fMOLES/μg DNA) for retinoic acid. Also worthy of comment is that these retinoic acid binding data did not differ if the incubation temperature and time was changed to $4^\circ$C for 16 hours (data not shown).

(v) **The effects of enzyme inhibitors**

The elevated temperature of $37^\circ$C used for the nuclear assays could have activated enzymes involved in the regulation of the vitamin A acid binding protein. The abilities of PMSF (1.0 mM), aprotinin (100 mg/l) and sodium molybdate (10 mM) to protect retinoic acid binding in nuclear salt extracts was therefore investigated. In this respect, PMSF and aprotinin were ineffective (Table 8). In fact, the binding of $[^3]$H retinoic acid was less in the presence than in the absence of inhibitor. However, sodium molybdate (10 mM) increased specific ligand binding at $37^\circ$C by 18%. Molybdate concentrations lower than or in excess of 10 mM were either without effect or inhibitory.

(vi) **Gel filtration**

In light of reports from Mehta et al (1982a) and
Figure 30. Binding of \([^3H]\) Retinol in Nuclear Salt Extracts - A Time Course Study

Nuclear salt extract was incubated at 4°C for varying times with \([^3H]\) retinol (10\(^{-7}\)M). Parallel experiments were conducted in the presence of unlabelled all trans retinol in 100 fold excess. At appropriate times, the reactions were terminated by removal of unbound ligand with charcoal and nuclear salt extract counted for radioactivity. The experiment was repeated 3 times. Data are expressed as mean values (% of maximal binding) ± S.D.
[\textsuperscript{3}H] Retinol Bound specifically in nuclear salt extracts (% maximum)

Incubation time (h)
Figure 31. Scatchard Analysis of Retinoic Acid Binding in Nuclear Salt Extracts

Nuclear extract was incubated with a range (5 - 60 nM) of \[^3H\] retinoic acid concentrations at 37°C for 30 minutes. Non specific binding was determined in each case by parallel incubations with unlabelled like-competitor in 100 fold excess. At the end of incubation, unbound assay ligand was removed with charcoal, and nuclear extract counted for radioactivity. After correction for non specific binding (●) the specific binding (▲) data (a) was plotted by the method of Scatchard (b) to yield a value for the dissociation constant. The experiments were repeated at least 5 times.
- [3H] Retinoic Acid Bound (dpm x 10^-3)

- Retinoic Acid Free Concentration (nM)

- Retinoic Acid Bound Specifically (fmol/µg DNA)

- Bound/Free
Figure 32. Scatchard Analysis of Retinol Binding in Nuclear Salt Extracts

Nuclear preparation was incubated at 4°C for 16 hours with increasing concentration of \([^3\text{H}]\) retinol (5 - 60 nM). Non specific binding was assessed in parallel incubations in the presence of unlabelled retinol in 100 fold excess. Unbound ligand was then removed by the DCC method and nuclear extract counted for radioactivity. After correction for non specific ligand association (●) the specific data (▲) in (a) was analysed by the method of Scatchard (b) and a value for the dissociation constant calculated. The experiments were repeated with 3 sets of tissues.
TABLE 8. The Effect of PMSF and Aprotinin on Retinoic Acid Binding in Nuclear Salt Extracts

Nuclear salt extract prepared in the absence (control) and presence of PMSF (1 mM) or aprotinin (100 mg/l) were compared for [3H] retinoic acid binding. Briefly, nuclear extract was incubated at 37°C for 30 minutes with [3H] retinoic acid (10^-7M). Non specific binding was determined by parallel experiments with unlabelled retinoic acid in 100 fold excess. After incubation, excess assay ligand was removed by the DCC method and aliquots of extract counted for radioactivity. Results are expressed as mean values of 4 experiments ± S.D.
<table>
<thead>
<tr>
<th>ENZYME INHIBITOR</th>
<th>CONTROL (%)</th>
<th>SPECIFICALLY BOUND [3H] RETINOIC ACID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aprotinin (100 μM/l)</td>
<td>69 ± 19</td>
<td>100</td>
</tr>
<tr>
<td>PSMP (1 mM)</td>
<td>ABSENT</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 8
Figure 33. The Effect of Sodium Molybdate on the Nuclear Binding of [\(^3\)H] Retinoic Acid

Nuclear salt extract prepared in the absence (control) or presence of various levels of sodium molybdate (0.1 - 50 mM) were assayed for retinoic acid binding as described in section 2.5(c). Specifically bound retinoic acid in extracts without sodium molybdate was defined as 100%. Figures in parentheses refer to the number of experiments.
$[^{3}H]$ RETINOIC ACID SPECIFICALLY BOUND
($\%$ of control)

SODIUM MOLYBDATE CONCENTRATION (mM)

0.0  0.1  1.0  10.0  20.0  50.0

CONTROL

$\%$
Sani and Donovan (1979) on the nuclear location of cRABP (extractability of a 2S protein which showed the same ligand specificity as the cytosolic sites), the idea that the prostate nuclear and cytosolic sites were identical, was initially entertained. However, when gradient centrifugation of nuclear preparation was undertaken the radioactivity appeared at the bottom of the tube (data not shown). As an alternative, the nuclear salt extract was radiolabelled with either retinoic acid or retinol (10^{-7}M) and gel filtered on an Ultrogel AcA 34 column. In both cases (Figures 34, 35), radioactivity eluted in the void volume (exclusion limit 600,000 daltons) and at position 41 (ml) of the column. Reduction of the first but not second peak by unlabelled ligand indicated a saturable component in the void volume (Figures 34, 35). Radioactivity in the second peak (41 ml) probably represents free ligand, since gel filtered [^3H] retinol or [^3H] retinoic acid in buffer eluted at this position.

The saturable retinoic acid binding peak was not shifted in position by a number of procedures including (1) digestion with micrococcal nuclease (5 Iu/ml) (Figure 34b), (2) dialysis (Figure 34c), (3) inclusion of 10 mM sodium molybdate (Figure 34d). Also no change in the elution profile of [^3H] retinoic acid could be found if the incubation conditions were altered from 37°C for 30 minutes to 4°C for 16 hours (data not shown).
Figure 34. Gel Filtration of Nuclear Salt Extract Radiolabelled with Retinoic Acid

Nuclear extract untreated (a), treated with micrococcal nuclease (b), desalted by dialysis (c), prepared in the presence of 10 mM sodium molybdate (d) was incubated at 37°C for 30 minutes with [³H] retinoic acid (10⁻⁷M) in the presence (●) or absence (▲) of unlabelled trans retinoic acid in a 100 fold excess. After incubation, unbound assay ligand was removed with charcoal and nuclear extract gel filtered on an Ultrogel AcA 34 column of bed volume 43 ml. The column was eluted with appropriate buffer. Arrow indicates void volume.
Figure 35. Gel Filtration of Nuclear Salt Extract Radiolabelled with Trans-Retinol

Nuclear extract and $[^3H]$ retinol were incubated at 4°C for 16 hours with (●) or without (▲) unlabelled trans retinol. After a brief charcoal treatment, nuclear extract was gel filtered on an Ultrogel AcA 34 column (43 ml = bed volume). The column was eluted with Tris/MgCl$_2$/KCl containing 5% ethanol and 50 x 1 ml fractions collected. A representative profile of 3 separate experiments is shown. Arrow indicates void volume.
3.4 (c) Metabolism Studies

The abilities of all trans retinol and all trans retinoic acid to displace each other from the nuclear extracted protein, could have been a consequence of in vitro ligand conversion. To determine whether this was the case, metabolism studies using both vitamin A alcohol and vitamin A acid were undertaken. Nuclear salt extract incubated with $[^{3}\text{H}]$ retinol ($10^{-7}$M) or $[^{3}\text{H}]$ retinoic acid ($10^{-7}$M, $10^{-5}$M) under identical conditions to the nuclear assays were extracted with ether and radioactive material subjected to reversed phase thin layer chromatography.

$[^{3}\text{H}]$ Retinoic Acid

The majority of radioactive material extracted from nuclear preparations or buffer, co-chromatographed with authentic all trans retinoic acid (Figure 36 a,b). However, studies with nuclear salt extract revealed a minor peak of radioactivity (Figure 36b) more polar than retinoic acid. The peak accounted for less than 5% of the chromatographed material. Quantitation of the unidentified peak after incubation with $10^{-5}$M substrate resulted in again, less than 5% conversion.

$[^{3}\text{H}]$ Retinol

The chromatograms of radioactive material extracted from buffer or nuclear salt extract were identical (data not shown).
Figure 36. Metabolism Studies with Nuclear Salt Extract - Reversed Phase Chromatography

Nuclear salt extract (36b) or buffer (36a) was incubated with $[^{3}\text{H}]$ retinoic acid ($10^{-7}\text{M}$) under conditions identical to the binding assay. After this period, incubation mixtures were extracted with diethyl ether. The ether was evaporated under vacuum and extracted material reconstituted in 50 $\mu$l of ethanol. Reconstituted material and authentic standards were applied under nitrogen to reversed phase thin layer chromatography plates. The plates were developed at 4°C in an ethanol : water (8 : 2) solvent system. After evaporation of the solvent system under nitrogen and location of authentic standards, 10 mm slices were scraped into counting vials and radioactivity counted. The experiment was repeated 5 times.
3.5. Comparison of Cytosol Retinol Binding in Hyperplastic and Malignant Prostate

Previous studies have documented altered $[^{3}H]$ retinol binding in various malignancies including lung and ovaries (Palan and Romney 1980). More important, (Brandes 1984) had found an increased expression of prostate cRABP when progressing from normal to benign to malignant states. To the knowledge of this worker no study has, as of yet, been reported on $[^{3}H]$ retinol binding in prostate malignancy. This investigation was therefore undertaken to compare the levels of retinol binding protein in prostate hyperplasia and malignancy. Such information might infer a certain requirement by the cell for vitamin A alcohol.

(a) Validation of the Assay

(i) Optimisation of the DCC method

Cytosol (0.5 mg protein/ml) was incubated with $10^{-7}$M $[^{3}H]$ retinol at $4^\circ$C for 4 hours. After this time, the subcellular fraction was treated with charcoal pellets for 5, 10 or 15 minutes and counted for radioactivity. It is apparent from the results in Table 9 that a 10 minute charcoal post-treatment period is optimal for assessment of retinol binding. Exposure times of 5 and 15 minutes resulted in 15% less binding.

(ii) Effect of protein concentration on $[^{3}H]$ retinol binding

Serial dilutions ($1/2$, $1/4$, $1/8$) of cytosol (1.0 mg protein/ml) were made in Tris/EDG buffer and the preparations assayed for $[^{3}H]$ retinol binding. Figure 37 illustrates the linear relationship between retinol binding and protein concentration. Retinol binding was found to level off with protein concentrations in excess of 1.0 mg/ml (data not shown).
TABLE 9. The Effect of the DCC Method on the Cytosol Retinol Binding Assay

Cytosol (0.5 mg protein/ml) was incubated with 
$[^3H]$ retinol ($10^{-7}$M) in the presence and absence of unlabelled retinol in 100 fold excess under conditions specified in the assay. At the end of incubation, the radioactive cytosol was treated with charcoal pellets for 5, 10 or 15 minutes. At appropriate times, the charcoal was removed at 800 x $g_{AV}$ and aliquots of cytosol counted for radioactivity. The results are expressed as mean values of 4 experiments ± S.D.
<table>
<thead>
<tr>
<th>[³H] Retinol Bound Specifically (fMoles/mg)</th>
<th>Charcoal Treatment (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>$3306 \pm 365$</td>
</tr>
</tbody>
</table>
Prostate cytosol protein concentration was adjusted to 1.0 mg/ml and serial dilutions made in Tris/EDG buffer. The 1 mg/ml fraction and serial dilutions were assayed for retinol binding as described in section 2.12(a). Non specific ligand association was determined in parallel incubations with unlabelled retinol in 100 fold excess. The experiment was performed with 3 separate tissues.
$[^3]H$ RETINOL BOUND NON SPECIFICALLY (p moles)

PROTEIN CONCENTRATION (mg/ml)

$[^3]H$ RETINOL BOUND SPECIFICALLY (p moles)
(iii) Freezing and storage of prostate at \(-70^\circ\text{C}\)

Portions of homogeneous prostate mince frozen at \(-70^\circ\text{C}\) were retrieved at appropriate times and assayed for \(^{3}\text{H}\) retinol binding under conditions specified in section 2.12(a). The effect of storage of prostatic tissue on retinol binding is illustrated in Figure 38. It is evident from these data that neither freezing nor storage of prostate at \(-70^\circ\text{C}\) for periods of up to 8 weeks has adverse effects on the assay.

(iv) **Scatchard analysis of retinol binding in malignant gland**

Malignant prostate cytosol (0.5 mg protein/ml) was analysed for ligand binding at \(4^\circ\text{C}\) over a range of \(^{3}\text{H}\) retinol concentrations (10 - 100 nM). A typical saturation curve is illustrated in Figure 39a. Manipulation of the data to yield a Scatchard plot (Figure 39b) revealed a ligand dissociation constant of \(31 \pm 15\) nM, a value almost identical to the \(33 \pm 6\) nM quoted for hyperplastic tissue (Figure 19).

(v) **Metabolism of \(^{3}\text{H}\) retinol by malignant prostate cytosol**

To determine whether, under the conditions of the assay, malignant prostate cytosol was capable of metabolising \(^{3}\text{H}\) retinol, subcellular fraction was incubated at \(4^\circ\text{C}\) for 4 hours with radioligand. After this time, the incubation mixture was extracted with ether and the extracted radioactivity analysed by reversed phase T.L.C. (2.5(e), 2.3(c). It is evident from Figure 40 that the soluble fraction of malignant prostate lacks the capacity to convert \(^{3}\text{H}\) retinol in vitro. The absence of retinol metabolism by the malignant tissue fraction is consistent with the results obtained with hyperplastic gland (Figure 25).

(vi) **Inter / intra assay variations**

Inter- and intra- assay variations were \(6\pm 4\%\) and \(10\pm 7\%\) respectively.
Figure 38. The Effect of Snap Freezing and Storage of Prostate Tissue at ~70°C on the Cytosol Retinol Binding Assay

Prostate tissue pulverised into a homogeneous paste was divided by weight into equal portions and either assayed for cytosol retinol binding or snap frozen in liquid nitrogen and stored at ~70°C for varying periods. At appropriate times, prostate was retrieved, a cytosol prepared and specific retinol binding determined. These data represent average values (± S.D.) of 4 different prostates.
[3H] RETINOL BOUND SPECIFICALLY (%)
Prostate cytosol was incubated at 4°C for 4 hours with a range of [3H] retinol concentrations (10 - 100 nM). Non specific binding was determined in parallel incubations in the presence of unlabelled compound in 100 fold excess. After incubation, free ligand was removed with charcoal and aliquots of cytosol counted for radioactivity. The specific binding data (a) corrected for non specific ligand association was plotted by the method of Scatchard (b) to yield a value for the dissociation constant. The experiment was repeated 3 times.
Specifically (μ moles)

\[ K_0 = 31.0 \pm 15.0 \text{μM} \]

RETINOL BOUND

[\text{[^3]H RETINOL]}

BOUND SPECIFICALLY (fmols)

\[ [\text{[^3]H RETINOL}] \times 10^{-3} \]

FREE RETINOL

CONCENTRATION (μM)
Figure 40. Metabolism of $[^3\text{H}]$Retinol by the Cytosol of Malignant Prostate

Cytosol from malignant gland was incubated with $[^3\text{H}]$ retinol ($10^{-7}\text{M}$) at $4^\circ\text{C}$ for 4 hours after which radioactivity was extracted with diethyl ether. Extracts were evaporated of solvent under vacuum, reconstituted in $50\ \mu\text{l}$ of ethanol and together with authentic standards subjected to reversed phase thin layer chromatography as described in the text (2.3(c)). After development, plates were dried under $\text{N}_2$, standards located and $10\text{ mm}$ slices counted for radioactivity. The experiment was repeated with 3 separate sets of tissues.
3.5(b)

(i) Comparison of Retinol Binding in Hyperplastic and Malignant Prostate Cytosol

Cytosol from benign hyperplastic prostate bound an average of $4.0 \pm 1.6$ pMOLES $[^3H]$ retinol per mg of protein (Figure 41a) (range 1.9 - 8.4 pMOLES/mg). In contrast, less retinol ($1.7 \pm 1.6$ pMOLES/mg) was specifically bound by the soluble fraction of malignant gland. This difference was found to be statistically significant by the Mann Whitney U-Test ($p < 0.01$). Examination of the data revealed an approximate 40\% overlap of both groups. Although all benign hyperplastic prostates assayed for retinol binding were found to be positive, this was not the case in malignancy where 3/15 glands were devoid of binding, or at least below the detection limits of the assay (100 fMOLES/mg).

(ii) Retinol Binding and Histological Differentiation

The reduced binding of $[^3H]$ retinol by malignant gland prompted the author to investigate whether this observation was further amplified in un-differentiated tumours. For this reason, the specific binding of retinol was compared with the Gleason score (Figure 41b) (sum of primary and secondary histological grades) which is an assessment of the overall histological pattern. It is apparent from Figure 41b that no relationship exists between $[^3H]$ retinol binding and tumour differentiation. In fact, anaplastic (Gleason score 7 - 8), and well-differentiated tumours (Gleason score (3 - 4)) bound similar amounts of retinol. Furthermore, data not shown, indicated the absence of a correlation between $[^3H]$ retinol binding and the primary Gleason score.
Malignant or hyperplastic prostate cytosol was prepared and adjusted to 0.5 mg protein/ml. Aliquots of subcellular fraction were incubated at 4°C for 4 hours with \([^{3}\text{H}]\) retinol \((10^{-7}\text{M})\). Non specific binding was assessed by parallel incubations with unlabelled retinol in 100 fold excess. After incubation, excess assay ligand was removed with charcoal treatment (10 minutes) and aliquots of cytosol counted for radioactivity.
[\textsuperscript{3}H] Retinol bound specifically (p moles/mg)

Benign

Malignant

[\textsuperscript{3}H] Retinol bound specifically (p moles/mg)

3-4

5-6

7-8

Gleason Score
CHAPTER 4. (DISCUSSION)
High affinity binding sites for all trans retinol and retinoic acid have been detected in the subcellular fractions of human hyperplastic prostate. The cytosolic sites recognised vitamin A alcohol and acid preferentially but not steroid hormones. Although specific components for either ligand could not be identified in suspensions of microsomes or mitochondria, a soluble protein showing different characteristics to the cytosolic sites was extracted from prostate nuclei.

4.1 Characterisation of Retinoid Binding in Hyperplastic Gland

The features of retinol and retinoic acid binding in prostate cytosol are similar to those reported for other vitamin A target tissues. In this regard, both prostate binding sites bound their respective ligands preferentially (Figures 13, 20) co-sedimented with the 2S external marker on sucrose gradients and showed little affinity for steroid hormones (Figures 15, 22). In addition, the dissociation constant \( K_d \) for prostate retinol binding (Figure 19) was calculated to be \( 33 \pm 6 \) nM, a value not inconsistent with the 16 nM published for purified rat liver protein (Ong and Chytil 1978). The observations suggest, at least, that the prostate cytosol retinol binding site is identical to the cRBP characterised in other tissues including rat testes, liver and lung (Bashor et al 1973, Ong and Chytil 1975).

However, in spite of some similarities between the prostate retinoic acid binding component and cRABP (sedimentation coefficient = 2.0S, displacement of radioactive vitamin A acid by all trans retinoic acid and synthetic analogues (Figures 20, 21)), these sites may very well be separate. Evidence for this stems from one particular feature of \([^3H]\) retinoic acid binding; that is the observed competition
by excess unlabelled all trans retinol and retinal (Figure 20). Although these agents were less active than all trans retinoic acid in displacing $[^3]H$ retinoic acid, other reports using rat testes cytosol for example (Ong and Chytil 1975) have clearly demonstrated the retinoid specificity of binding to cRABP. It would appear that the end function of the vitamin A molecule is an important discriminant of ligand association with either cRBP or cRABP. Certainly these characteristics are of relevance to the prostate cytosol retinol binding component where unlabelled trans retinoic acid in excess proved ineffective in displacing $[^3]H$ retinol from the saturable 2S peak (Figure 13). The competition for $[^3]H$ retinoic acid binding by excess retinol and retinal could alternatively have been explained by in vitro ligand conversion. However, this worker was unable to detect the in vitro conversion of either $[^3]H$ retinol (Figure 25) or $[^3]H$ retinoic acid (Figure 26) and for this reason it would appear that the competition for $[^3]H$ retinoic acid binding by vitamin A alcohol is genuine and reflects the particular characteristics of the prostate protein. Although in a previous study (Brandes 1980) binding of $[^3]H$ retinoic acid in the soluble fraction of human prostate had been reported, no attempt was made to characterise the binding in terms of ligand specificity, affinity and protease/heat sensitivity. It was clear that further studies were required to confirm and extend these earlier findings.

In addition to the specific binding of $[^3]H$ retinoic acid observed on the sucrose gradient, a second peak representing non specific ligand association was apparent in the 4.6S region (Figures 20, 21, 22). The appearance of radioactive retinoic acid at this position is not novel, and in fact a common observation with various tissue cytosols (Sani et al 1978, Brandes 1981). Sani et al (1978) have
proposed that this component is contaminating serum albumin. In agreement with this contention, experiments with chick embryo skin extracts treated with an anti albumin immunoglobulin (Sani et al. 1977) revealed the efficacy of the antibody in reducing the size of the non specific peak on sucrose gradients.

In an attempt to establish a quantitative assay for prostate retinoic acid binding protein, various methods were assessed for their abilities to separate specific and non specific components. In this regard, a number of methods including (i) immunoprecipitation, (ii) dye affinity chromatography (Figure 27), (iii) ultrafiltration (Figure 27), (iv) DEAE ion exchange, proved capable of removing the non specific component. Unfortunately, as evident in Figure 27, these techniques failed to discriminate between specific and non specific components or possibly interfered with \(^{3}\text{H}\) ligand binding. Noteworthy, was the inefficacy of immunoprecipitation in separating specific and non specific retinoic acid binding. As previously mentioned, this method had proved successful in experiments with chick embryo skin extracts (Sani 1977). The inability of this procedure to selectively remove the 4.6S component in human prostate cytosol may, in fact, be a result of a less specific antibody. Alternatively, one may speculate that the 4.6S binding component is not actually contaminating serum albumin but a low affinity high capacity prostate derived storage site for retinoic acid. No attempts were made to resolve both components by gel filtration since dilution of the fractionated protein would require a "reconcentration" step such as ultrafiltration. In view of these difficulties, further characterisation of the prostate cytosol vitamin A acid binding was not possible.

The presence of prostate cytosol binding sites for retinol and retinoic acid argues for a role of vitamin A in the function of the
gland. Certainly, with regard to mouse prostate in culture, the ability of retinol and retinoic acid to reverse squamous metaplasia and benign hyperplasia has been demonstrated by others (Laznitski 1963, Chopra and Wilkoff 1976). Furthermore, in response to various retinoids, malignant rat prostate cells were contact inhibited at lower cell densities than untreated controls; a feature of in vitro differentiation (Reese et al 1983).

Although the mechanism by which retinoids govern these cellular events is unclear, it may depend on the interaction of these agents with prostate microsomes/mitochondria or nuclei. However, in view of the absence of specific ligand association with the microsomes and mitochondria, a finding endorsed by other workers (Sani and Donovan 1979) using the equivalent subcellular fractions of chick embryo skin, it seems unlikely that retinoids modulate cell function at this level.

Alternatively, and as the evidence suggests, at least for other tissues, vitamin A is capable of influencing nuclear events. In this regard, a number of workers have reported the ability of vitamin A to alter the synthesis of mRNA in rat liver, testes and hamster trachea (Tryfiates and Krause 1971, Omori and Chytil 1982, Kaufman et al 1972). Although these findings have not, as yet, been reproduced for prostate tissue, the vitamin A induced morphological changes in cultured gland (Laznitski 1963, Chopra and Wilkoff 1976) would suggest a control of the genome.

Genomic expression could be regulated at the nuclear level by the vitamin A molecule or a complex of ligand and its binding site. In accordance with this view, a number of laboratories have identified cRABP in nuclear extracts of Lewis lung carcinoma and rat mammary adenocarcinoma (Sani and Donovan 1979, Mehta et al 1982). Preliminary
experiments in this laboratory revealed the absence of specific ligand binding in nuclei or nuclei incubated with retinol radio-labeled cytosol. However, in further studies, it was found that if nuclei were subjected to shear forces (sonication) and the high salt extract pretreated with charcoal, substantial binding of retinol (Figure 32) and retinoic acid (Figure 31) was observed (50 ± 13; 48 ± 18 f MOLES/μg DNA respectively). This effect of charcoal may have been due to the removal of an endogenous inhibitor. Indeed, the ability of charcoal to remove interfering substances, thereby enhancing $^{125}$I-prolactin binding in radioreceptor assays, has been reported previously (Leake et al 1983). At the present time, the identity of the inhibitor of $^{3}$H retinol and $^{3}$H retinoic acid binding in nuclear salt extracts is not known. Clearly, further studies, possibly involving fluorimetry at 350 nm to determine the level of endogenous retinoid in nuclear salt extracts (Ong and Chytil 1978) will be required to establish the nature of this binding inhibitor.

In addition to the enhancing effects of shear and charcoal treatments on $^{3}$H retinoic acid binding in nuclear salt extracts (Figure 9), this worker found a small (18%) but reproducible increase in specific binding of this ligand in the presence of 10 mM sodium molybdate (Figure 33). Such a phenomenon has not been reported previously for retinoic acid binding assays. Recent years have seen wide use of this ion in steroid receptor assays (Smith et al 1983a, Trachtenberg et al 1981); the protection of steroid binding sites has been ascribed to the inhibition of endogenous phosphatases (Paigen 1958, Roberts and Bazer 1976) by the molybdate ion. If this is in fact the mechanism of action of molybdate, the absence of an effect on cytosol retinol binding is surprising. Smith et al (1983a) had reported a
50% increase in the levels of assayable cytosolic androgen receptor which would indicate the presence of active phosphatase in this fraction. One possible explanation for the absence of an effect on cytosol retinol binding may be that this site unlike the nuclear extracted protein is insensitive to the effects of phosphatase.

Another interesting observation and worthy of comment at this stage was that charcoal treatment did not augment the cytosolic binding of either retinol or retinoic acid. These data could be explained in either of two ways. Firstly, under the conditions used for the cytosol assays, ligand exchange may be complete. Alternatively, on the basis of the "translocation theory" for steroid hormones, it could be argued that binding sites residing in the soluble compartment are vacant and available for radiolabelling. The weight of evidence, however, would suggest that the former postulate is correct. In this regard, it is well documented that cytosolic sites, namely cRBP and cRABP from other vitamin A target tissues are occupied at a 40 - 100% saturation level with their respective endogenous ligands (Ong and Chytil 1974, Saari et al 1982).

Since the presence of cRABP has been demonstrated in nuclear salt extract from other vitamin A target organs, it was initially felt that a similar situation may exist for human prostate. However, a number of observations were inconsistent with such a hypothesis. Saturation analysis of the nuclear extracted protein (S) indicated one class of sites capable of binding [³H] retinol (Figure 32) and [³H] retinoic acid (Figure 31) with equal affinity (K_D = 12.5 + 1.0; 15.0 + 2.0 nM) and capacity (50 + 13; 48 + 12 f MOLES/µg DNA respectively). More important, competition studies revealed vitamin A alcohol (Table 5) and acid (Table 6) to be equally active in displacing either radioligand.
These observations could not be accounted for by in vitro ligand conversion since under the conditions of the nuclear assays little metabolism of retinol and retinoic acid (Figure 36) could be detected. In addition, control experiments (2.9(a)) indicated that the different competition profiles for nuclear and cytosolic proteins was not a result of the buffer components (data not shown). Put together, these data would suggest that the nuclear extracted retinol and retinoic acid binding sites are identical.

To determine whether, in fact, the cytosolic and nuclear proteins were separate, experiments were undertaken to estimate the size of the nuclear extracted component. Preliminary experiments had indicated sucrose gradient analysis to be unsuitable for this purpose since protein bound radioactivity was found to sediment to the bottom of the tube. Further studies were therefore performed on an Ultrogel AcA 34 column of large pore size. At this stage, it is appropriate to point out that for comparative purpose, gel filtration of cytosol and nuclear salt extract was performed on [3H] retinol labelled samples. This worker was unable to detect cytosolic retinoic acid binding sites on the Ultrogel column; these results might be explained by the long gel filtration period (10 hours) in which ligand dissociation could have occurred. Gel filtration of cytosol and nuclear salt extract resulted in very separate profiles. Elution of these preparations from the column revealed saturable peaks of bound radioactivity in the void volumes for the nuclear salt extract (Figure 35) and fraction 37 for the cytosol (Figure 28). The latter fraction corresponds to the elution position of myoglobin standard (molecular weight 17,000) and more than likely this peak of bound radioactivity represents the prostate retinol binding protein.
characterised on sucrose gradients (Figures 13, 14, 15). The voiding of nuclear protein labelled with either \(^{3}\text{H}\) retinol (Figure 35) or \(^{3}\text{H}\) retinoic acid (Figure 34) could very well have been due to aggregation of binding sites. However, in light of the absence of such a phenomenon for the cytosolic retinol binding component (Figure 28) it is unlikely that the voided bound radioactivity constitutes aggregated cytosolic sites.

Alternatively, chromatin bound "receptor" may have been extracted as a consequence of nuclear sonication (Liau et al 1981). To check this possibility, nuclear salt extract was treated with micrococcal nuclease, radiolabelled with retinoic acid and the preparation gel filtered (Figure 34(b)). The inability of the nuclease to alter the elution profile, does not lend credence to the contention that nuclear extracted protein is chromatin associated.

At this point it is appropriate to comment on the inability of 10 mM sodium molybdate to alter the elution profile of the nuclear extracted component (Figure 34(d)). Rowley's group (1984) had reported changes in the sedimentation properties (as analysed on sucrose gradients) of the androgen receptor in the presence of this ion. These data would suggest that the physical properties of the nuclear retinoid binding protein, unlike the androgen receptor, cannot be modified by the molybdate ion.

The physiological significance of the nuclear derived retinoid binding protein is not clear and requires further attention. It does seem somewhat strange that a nuclear protein should recognise both vitamin A alcohol and acid while cytosolic components are able to discriminate between either ligand.

Perhaps relevant to these studies are the recent findings by
Liau et al (1981) and Cope et al (1984). Both groups detected binding sites for vitamin A alcohol and acid in nuclei from liver and testicular cells which were quite separate from cRBP or cRABP. These observations led Liau to propose the existence of nuclear acceptor sites. Although evidence is lacking at this stage, the possibility exists that the prostate nuclear binding sites are identical to the acceptor sites postulated by Liau (1981) and Cope (1984). If indeed this hypothesis is correct, a radiolabelling of the putative prostate acceptor sites might be anticipated in experiments where retinol radiolabelled cytosol was interacted with intact nuclei. The absence of specific ligand transfer from cytosolic to nuclear components was therefore surprising. However, these data may reflect the in vitro conditions rather than anything else. Certainly, as is the case for steroid receptors (Jensen and De Sombre 1972) the temperature activation of cytosolic receptors is a prerequisite to the nuclear interaction of these proteins. Although Mehta and coworkers (1982a) could not find any effect of temperature activation on the nuclear association of cellular retinoic acid binding protein, such an event could be of relevance to the retinol binding system. Unfortunately, studies of this phenomenon in regard to retinol nuclear interactions are limited by the relative instability of radioligand.
4.2 Comparison of Retinol Binding in Hyperplastic and Malignant Prostate Cytosol

Data presented in this thesis suggest that malignant prostate cytosol has a decreased ability to bind retinol when compared with the hyperplastic gland. Suppressed retinol binding in a number of human malignancies including lung, ovaries and breast has been reported by others (Palan and Romney 1980) and interpreted to mean fewer copies of cRBP per cell. To the knowledge of this worker, no evidence was presented to support such a contention. Decreased ligand binding may reflect other factors, e.g. (1) a reduced affinity of cRBP for ligand, (2) the presence of interfering endogenous retinol. To exclude these possibilities for the prostate data, saturation analysis was performed on the cytosol of malignant gland. The calculated $K_D$ for malignant prostate cytosol was $31 \pm 15$ nM (Figure 39), a value close to the $33 \pm 6$ nM determined for hyperplastic tissue (Figure 19). In addition metabolism studies clearly demonstrated the inability of malignant prostate cytosol to convert $[^3H]$ retinol (Figure 40) under the conditions of the binding assay; these observations were in fact consistent with results obtained with hyperplastic gland. These data do not therefore support the contention that the above-mentioned factors can account for the suppressed binding of retinol in the cancerous tissue fraction. More than likely, the suppressed binding of $[^3H]$ retinol in cancerous tissue reflects a reduced expression of the prostate binding protein. Such a concept is not novel; a number of proteins in a variety of cancers are either re-expressed (carcino-embryonic antigen - Hall et al 1973) from foetal development, or masked, as cRABP is in anaplastic breast cancer (Mehta et al 1982b).

The lack of correlation between the binding of retinol and histological grading (Figure 41(b)) requires some attention. The absence
of such a trend may very well have been a consequence of using the Gleason sum which includes the secondary histological pattern. However, data not shown indicated that this was not the case and in fact omission of the secondary grade resulted in little alteration of the retinol binding profile illustrated in Figure 41(b).

Alternatively, the similar retinol binding observed in well and poorly differentiated specimens would suggest that the suppression of the binding protein in malignancy cannot be further amplified by the undifferentiation process.

The aetiological significance of these findings with regard to prostate malignancy can only be speculated on. Perhaps noteworthy is that such changes do not necessarily infer a particular requirement by the malignant cell for vitamin A (Abelev 1971).

On the other hand, the possibility exists that these data may be of prognostic significance. In this regard, the observation that a number of patients with benign prostate hyperplasia possessed relatively low retinol binding in the cytosol of this gland (Figure 41(a)) led this worker to speculate on whether these individuals might represent a "high risk" group predisposed to the malignant disease. With this in mind, these patients with benign hyperplasia and low retinol binding are being followed in an attempt to assess the potential of this parameter as a marker of undifferentiation.
The results presented in this thesis support the contention that the human hyperplastic and malignant prostate is a target organ for vitamin A and its natural metabolites. However, at this stage a number of questions remain unanswered.

Firstly, the endogenous levels of vitamin A and its metabolites in the normal and diseased human prostate are, at present, not known. Epidemiology studies (Kark et al. 1982) have demonstrated an inverse relationship between serum levels of vitamin A and the incidence of prostate cancer. Low serum vitamin A could hypothetically result in deficient tissue levels of this endogenous agent. Such a situation might cause the disruption of the vitamin A biological system required in the normal functioning of the gland.

Secondly, it remains to be seen whether the retinoid binding proteins are distributed equally between prostate stroma and epithelium or, alternatively, localised to one cell type. Such information would not only be of academic interest since it may indicate from a biological standpoint, at least, which prostate disease could be managed with retinoids; i.e. the hyperproliferation of stromal cells in benign hyperplasia or the control of aberrant epithelial cells in malignancy.

Thirdly, in view of earlier findings demonstrating the effects of retinoids on the differentiation and proliferation of cultured mouse and rat prostate (Laznitski 1963, Chopra and Wilkoff 1976) it would be worth investigating whether similar responses might be achieved with human tissue in vitro.
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A comparison of retinol binding in human hyperplastic and malignant prostate

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Vitamin A is an important regulator of cell differentiation and function. In its absence, a number of cells in vivo undergo squamous metaplasia (Wolbach & Howe, 1925) which in the case of mouse prostate in vitro may be reversed by vitamin A (Laznitski, 1963). This type of cell transformation is thought to indicate neoplastic potential, a situation clearly evident in the vitamin A deficient rat where a higher rate of spontaneous carcinomas is detected (Fujimaki, 1926).

There is evidence to suggest that vitamin A (retinol) may mediate its biological actions through cellular retinol binding protein (cRBP) which binds the ligand specifically and with high affinity (Ong & Chytiril, 1975, 1978). cRBP may serve to deliver retinol from the cytoplasm to nuclear receptor sites where it affects genetic read out (Liau et al., 1981).

The presence of cRBP in human (benign) hyperplastic prostate (BPH) has been reported by this laboratory (Boyd et al., 1984). The protein binds retinol with high affinity (35 nM) and shows similar characteristics to the component analysed in other tissues (Bashor et al., 1973).

Reports from several laboratories suggest that the expression of cRBP in malignancy may be altered. In this respect, examination of epidermoid carcinomas of the oral cavity and oropharynx revealed cRBP levels to be higher in the malignancy than in normal adjacent tissue (Ong et al., 1982). In another study (Palan & Romney, 1980) reduced levels of cRBP were reported in a number of human tumours including lungs, ovaries and endometrium. However, to the knowledge of these workers, no study has yet been undertaken to compare cRBP levels in human hyperplastic and malignant prostate (CaP).

In this investigation, we have compared retinol binding in human, benign and malignant prostates, the results of which are presented herein.

11,12 (n)-[3H] vitamin A free alcohol (all-trans-retinol) (sp. act. = 43 Ci mmol−1) was purchased from Amersham, Bucks, UK. Radioactive retinol was checked for purity every two weeks as described previously (Boyd et al., 1984). All trans-retinol (unlabelled) was obtained from Sigma Chemicals, Poole, Dorset, UK. All retinoids were stored in ethanol solution, under nitrogen, in the dark at −20°C. Other chemicals were of analytical grade and obtained from Sigma, BDH, Poole, Dorset, UK or Fisons, Leicester, UK. The following buffers were used in these studies; TEDG containing Tris 10 mM, EDTA 1.5 mM, dithiothreitol 1.0 mM, glycerol 10% (v/v) pH 7.4; DCC containing dextran 0.025%, gelatin 0.1%, activated charcoal 0.25% (w/v) in TED pH 7.4 buffer.

The patients entered in the present study were in the age range 50−70 years. None of the cancer patients had received any therapy (endocrine, radiation etc.) prior to entry into this study. Prior to the prostatectomy malignant tumours were clinically staged by digital palpation using the TNM system (Harmer, 1978). Incidental tumours (T0) and those clinically staged as T1 were excluded from this study on the grounds that the portion of gland assessed as malignant may be unrepresentative of that selected for scientific work.

Prostate tissues were removed by trans-urethral resection. Large uncharred prostatic chippings were selected for our biochemical studies and transported to the laboratory in ice-cold saline (0.9% w/v). A portion of each chipping was retained for histological analysis and assigned a Gleason Score (Gleason, 1966) by the pathologist. Specimens used for biochemical studies were snap frozen in liquid nitrogen and stored at −70°C until required for further analysis. In this study, a total of 16 hyperplastic and 15 malignant glands were assessed for retinol binding.

The following procedures were carried out at 4°C. Prostate tissue (0.5−2.0 g) was finely minced with scissors and pulverised for 20 sec in a Teflon vial pre-cooled at −20°C using a Mikro-dismembrator II (B. Braun, Melsungen AG, FRG). The tissue was homogenised in 5−10 vol of TEDG buffer as described previously (Boyd et al., 1984) and ultracentrifuged at 100,000 g for 1 h to obtain the cytosol fraction.

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The retinol binding assay for cRBP was as described previously (Boyd et al., 1984) but with minor modifications. Briefly cytosol (protein concentration 0.5 mg ml⁻¹) was incubated in triplicate at 4°C for 4 h with [³H]retinol (10⁻⁷ M) in the presence and absence of unlabelled competitor in 100-fold excess. Preliminary studies had established the reproducibility of the assay; inter and intra assay variations were <15%. In addition, the specific binding of [³H]retinol was found to be linear over a protein concentration range of 0.1–1.0 mg ml⁻¹ and unaffected by either freezing or storage of tissue at −70°C for up to 8 weeks.

After incubation with radio-ligand, cytosol was treated with charcoal pellets (Boyd et al., 1984) to remove unbound ligand, and finally counted for radioactivity in 6 ml of scintillation cocktail.

Non specific binding was corrected for, by subtracting the radioactivity bound in the presence of unlabelled retinol from that observed in the absence of competitor. Control experiments had shown that charcoal treatment removed over 99.5% of free [³H]retinol from buffer and that remaining radioactivity in no way interfered with the measurement of prostate cRBP.

Cytosol protein was assayed by the method of Bradford (1976) using BSA as standard. The difference in cRBP levels between benign and malignant prostates was tested for statistical significance by the Mann–Whitney U-test.

Hyperplastic prostate cytosol bound an average of 4.0 ± 1.6 pmol of [³H]retinol per mg of protein (Figure 1) (range: 1.9–8.4 pmol ml⁻¹ protein). In contrast less retinol (1.7 ± 1.6 pmol mg⁻¹; range: 0–6.5 pmol mg⁻¹ protein) was specifically bound by the malignant gland. In spite of an overlap between the two groups there was a statistically significant difference between the levels of cRBP in BPH and CaP (P < 0.01). Although all benign prostates assayed for cRBP were found to be positive, this was not the case in malignancy where 3/15 glands were devoid of retinol binding, or at least below the detection limits of the assay (100 fmol mg⁻¹).

In contrast to the differences observed between BPH and CaP, these workers could find no relationship between the levels of cRBP and histological differentiation. In fact, similar amounts of [³H]retinol were bound in well (Gleason sum 3–4; 1.0 ± 0.3 pmol mg⁻¹) and poorly differentiated (Gleason sum 7–8; 1.1 ± 1.5 pmol mg⁻¹) tumours.

Data presented in this report suggest that malignant prostate has a decreased ability to bind retinol when compared with the benign, hyperplastic gland. Suppressed retinol binding was also found in a number of other human malignancies including lung, ovarian and breast as reported by Palan & Romney (1980). These data were interpreted to mean fewer copies of cRBP per cell although no evidence was presented to support such a contention. The possibility exists that decreased binding of radiolabelled ligand may reflect other factors e.g.: (1) alteration of the cRBP molecule with a reduced binding affinity for ligand. (2) the presence of interfering endogenous retinol. (3) enzymatic inactivation of cRBP. In this study we found no alteration in the dissociation constant (Kd) for retinol binding in prostate cancer (Kd = 31 ± 15 nM – data not shown). Thus, it would seem unlikely that endogenous ligand and/or alteration in the cRBP molecule can account for our observations of suppressed binding in malignancy. Also, since various enzyme inhibitors including aprotinin, phenylmethyl-sulphonyl-fluoride and sodium molybdate did not augment radioligand binding in he prostate subcellular fraction (data not shown), it is unlikely that the difference in cRBP values between both sets of tissues is a consequence of enhanced inactivation of the macromolecule in prostate cancer. For these reasons the possibility that prostate cRBP is being expressed in reduced amounts in cancerous tissue must be entertained. Such a concept is not novel; a number of proteins in a variety of cancers are either re-expressed from foetal development e.g. carcinoembryonic antigen (Hall et al., 1973) or masked as cellular retinoic acid binding protein is in anaplastic breast cancer.
In view of the reduced levels of cRBP in malignant tissue compared to BPH, it was surprising not to find any correlation between the levels of these binding sites and the histological differentiation of the tumour. Similar observations were made by Mehta et al. (1982) when investigating retinoic acid binding in human breast cancer.

The aetiological significance of these findings with regard to prostate malignancy can only be speculated on. Perhaps noteworthy is that such changes do not necessarily infer a particular requirement by the malignant cell for vitamin A (Abelev, 1971). On the other hand, the possibility exists that these data may be of prognostic significance. In this regard low retinol binding in patients with BPH could be used to identify those, who, in time would progress to malignancy.

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Nuclear retinoic acid binding protein in human prostate adenomas

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ABSTRACT

A retinoic acid binding protein has been detected in salt extracts of nuclei obtained from human prostate adenoma. The binding was characterized by competition experiments, temperature/time studies and saturation analysis.

Substantial binding was only observed after sonication of nuclei and charcoal-pretreatment of a salt extract. The binding of radiolabelled all-trans-retinoic acid was displaced by all-trans-retinoic acid, retinol and to a lesser extent retinal and two synthetic retinoids, RO 10-1670 and RO 13-7410. Testosterone and dihydrotestosterone, at a 100-fold excess, had little effect on the binding.

The association between retinoic acid and nuclear protein was both temperature and time dependent. At 37 °C, equilibrium was rapidly reached (30 min) whereas at 4 and 25 °C, ligand binding occurred at a slower rate. Saturation analysis performed under steady-state conditions yielded a dissociation constant of 15 ± 2 nmol/l.

Metabolism studies failed to show conversion of either radiolabelled all-trans-retinol or [3H]retinoic acid in vitro; these data suggest that both acid and alcohol forms of vitamin A are recognized by the extracted nuclear protein.

The effect of three enzyme inhibitors on [3H]retinoic acid binding was studied. Binding was unaltered in the presence of aprotinin and phenylmethylsulphonyl fluoride but sodium molybdate (10 mmol/l) increased binding by 18%.

The presence of a specific retinoid binding protein in prostate nuclei suggests that retinoids may play some role in the function of the gland.


INTRODUCTION

The importance of vitamin A in maintaining cell integrity and function has been well documented. There is now ample evidence to suggest that retinoids are also vital for the normal growth and function of male secondary sex organs and in some early studies Lasnitzki (1963) was able to prevent benign transformation of mouse prostate in culture by the addition of vitamin A. More recently, Reese, Gordon, Gratzner et al. (1983) found that rat adenocarcinoma cells in culture responded to vitamin A by a 40% reduction of cell saturation density, a feature of in-vitro differentiation.

The mechanism(s) by which vitamin A maintains cell integrity and directs differentiation are presently unknown. However, binding proteins for retinol (vitamin A alcohol) and retinoic acid (vitamin A acid) have been detected in the cytosols of a number of normal and pathological tissues (Sani & Corbett, 1977; Palan & Romney, 1980), including human prostate adenoma (Boyd, Beynon, Chisholm & Habib, 1984).

The possibility that these sites may be mediating the actions of vitamin A through interactions with nuclear components has been put forward by a number of workers. In support of this concept, Sani & Donovan (1979) were able to extract a retinoic acid binding protein from nuclei of Lewis lung carcinoma which showed the same ligand specificity and sedimentation value as the cytosol retinoic acid binding protein. Similar results have been achieved by Mehta, Cerny & Moon (1982) with rat mammary adenocarcinoma, a tissue reported to possess cytosolic binding sites.

In view of these findings and in an attempt to further our understanding of the biological actions of vitamin A in the human prostate, we have investigated ligand binding in the nuclear fraction, the results of which are reported herein.
MATERIALS AND METHODS

Radiochemicals

[11,12(n)-3H]Vitamin A, free alcohol (retinol; specific activity 60 Ci/mmole) was purchased from Amersham International plc, Bucks. [11,12-3H]Vitamin A acid (trans-retinoic acid; specific activity 23 Ci/mmole) was a generous gift from Hoffmann La Roche, Nutley, NJ, U.S.A. Both radiolabelled compounds were stored in the dark at -20°C under a nitrogen atmosphere. The purity of each compound was assessed at regular intervals, as described previously (Boyd et al. 1984).

Other chemicals

Unlabelled retinol, retinal and retinoic acid (all-trans-isomers) and steroid hormones were obtained from Sigma Chemicals, Poole, Dorset. The synthetic retinoid analogues, RO 10-1670 (all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-non-tetraenoic acid) and RO 13-7410 (P-(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl benzoic acid), were generous gifts from Roche, Welwyn Garden City, Herts. Unlabelled compounds were stored in solid form in the dark at -20°C. All other reagents were of analytical grade and obtained from Sigma Chemicals.

Buffers

The following buffers were used in this investigation: buffer A (Tris (10 mmole/l), MgCl₂ (5 mmole/l), 1% (v/v) Triton X-100, pH 7-4); buffer B (Tris (10 mmole/l), MgCl₂ (5 mmole/l), KCl (0-6 mol/l), pH 7-4); dextran-coated charcoal (DCC; dextran (0-025%, w/v), gelatin (0-1%, w/v), activated charcoal (0-25%, w/v), Tris (10 mmole/l), EDTA (1-5 mmole/l), pH 7-4).

Preparation of nuclear pellets

The preparation, transport and storage of prostate tissue removed by transurethral resection have been described elsewhere (Boyd et al. 1984). Fragments of prostate tissue were retained for histological analysis. Only those with confirmed benign disease were used in these studies.

Prostate tissue was minced finely with scissors and a microdismembrator (Braun, Melsungen, F.R.G.) was used to pulverize the tissue for 20 s in a Teflon vial precooled to -20°C. All subsequent procedures were carried out at 4°C unless stated. The tissue was then homogenized in 5-10 vol. buffer A using 3 × 15 bursts (speed 7) of an Ystral homogenizer with 1 min intermittent cooling. The homogenate was passed through one layer of nylon gauze (Nybolt 9-150; John Stainer & Co., Manchester) and centrifuged at 20g for 20 min to remove unhomogenized tissue. A nuclear pellet was prepared by centrifugation of the supernate at 800g for 10 min (Chilspin MSE). The pellet was washed three times in the same buffer and finally resuspended in the required volume of buffer B. Examination of nuclear preparation by light microscopy (Giemsa stained) revealed the presence of intact nuclei uncontaminated by unhomogenized tissue.

Nuclear extraction

Nuclear suspensions (20-50 μg DNA) in buffer B were stirred gently for 2 h at 4°C. At the end of this period, insoluble material was removed by centrifugation at 15 000g (Sorvall T865-1 rotor) and the resulting supernate retained for subsequent experiments.

Exchange assay

Unless stated otherwise, nuclear extract equivalent to 1-5 μg DNA was incubated at 37°C for 30 min with [3H]retinoic acid (100 nmol/l) in the presence and absence of unlabelled competitor at 10-fold excess. Free ligand was removed by the DCC method (Boyd et al. 1984) and aliquots of extract were counted in 6 ml scintillation cocktail (Vickers Labs, Burley-in-Wharfdale, West Yorkshire). Non-specific binding was assessed using retinoic acid as competing ligand at 100-fold excess.

Sonication

A report by Rennie, Bruchovsky & Cheng (1983) that sonication increased the extractability of androgen receptor from prostate nuclei prompted us to investigate this procedure. Suspensions of nuclei were divided into equal volumes. One portion was sonicated (Ultra-sonicator; MSE Scientific Instruments, Crawley, Sussex) for 10 min at maximum amperage, the other half retained as control. In all other experiments, nuclei were routinely sonicated.

Charcoal pretreatment of KCl extract

Post-sonication nuclear extracts were incubated for 60 min at 4°C with DCC pellets formed from 4 vol. of charcoal suspension. Charcoal was removed by centrifugation and extracts assessed for retinoic acid binding as described above.

In a time-course study undertaken to optimize the charcoal pretreatment, aliquots of extract/charcoal mixture were removed at various times, the charcoal was sedimented and the level of retinoic acid binding determined by the 'exchange assay' method.
Other experiments
The DNA content of the nuclear pellet was assayed by the method of Giles & Myers (1965) using calf thymus as standard.

Characterization of binding
Protease treatment
Nuclear extracts were incubated at 37 °C for 2 h with 0.05% pronase (Sigma Chemicals) in buffer B. Extracts were then assayed for [3H]retinoic acid binding as described previously.

Competition studies
Salt extracts were incubated with [3H]retinoic acid at 37 °C for 30 min in the presence and absence of unlabelled competitor at 100-fold excess. Free ligand was removed with DCC and aliquots of extracts were counted directly in 6 ml scintillation cocktail. The displacement of binding by all-trans-retinoic acid at 100-fold excess was defined as 100% competition; the binding of all other agents was expressed relative to this value.

Temperature/time studies
Binding of [3H]retinoic acid was compared at 4, 25 and 37 °C. Briefly, nuclear extract was incubated with [3H]retinoic acid (100 nmol/l) for varying times; non-specific binding was corrected for as described above. Specific binding at each temperature/time was expressed as a per cent of that observed after 30 min at 37 °C.

Saturation analysis
Saturation analysis was performed over a range between 5 and 60 nmol/l for [3H]retinoic acid and [3H]retinol concentrations. Non-specific binding at each concentration of radiolabel was determined in the presence of appropriate unlabelled competitor at 100-fold excess. Binding of retinoic acid was assessed under equilibrium conditions, i.e. 37 °C for 30 min and 16 h at 4 °C. Saturation analysis of the retinol binding was carried out at 4 °C for 16 h. The saturation data were corrected for non-specific binding and the specific binding data analysed by the method of Scatchard (1949) to yield dissociation constants (Kd) for both retinol and retinoic acid binding.

Enzyme inhibition
The possibility of underestimating [3H]retinoic acid binding in nuclear KCl extracts due to endogenous inactivation of binding sites prompted assessment of the effects of various enzyme inhibitors. Aprotinin (0.1 g/l), phenylmethyl sulphonyl fluoride (PMSF; 1 mmol/l) and sodium molybdate (10 mmol/l) were selected and included in both homogenizing and extracting buffers. Prostate mince was divided by weight into equal amounts and homogenized in buffer A in the presence or absence of inhibitor. Likewise, extraction of nuclei with buffer B was performed with and without inhibitor. The levels of retinoic acid binding in extracts were determined by 'exchange assay'. Specific binding in the absence of inhibitors was regarded as 100%.

Metabolism studies
Nuclear extract was incubated under conditions identical to the exchange assay with 100 nmol/l of either [3H]retinoic acid or [3H]retinol. Extraction of radiolabelled compounds and separation of metabolites by reversed-phase thin-layer chromatography have been described elsewhere (Boyd et al. 1984).

RESULTS
Effects of sonication and charcoal pretreatment on [3H]retinoic acid binding
Untreated nuclear KCl extracts bound little if any [3H]retinoic acid. However, when nuclei were sonicated before extraction an average of 5 ± 1 fmol [3H]retinoic acid was bound per µg DNA. If charcoal and sonication treatments were combined, substantial binding (34 ± 3 fmol/µg DNA) of retinoic acid was observed.

The inactivation of specific binding by pronase treatment confirmed that the [3H]retinoic acid was associating with a protein component.

A time-course study undertaken to optimize the charcoal pretreatment of extract revealed that maximal binding of [3H]retinoic acid was achieved after 60 min of incubation with no decline up to 120 min.

Specificity studies
[3H]Retinoic acid binding
Testosterone and dihydrotestosterone at a 100-fold excess showed no affinity for the retinoic acid binding protein. In contrast, all-trans-retinol (95 ± 12% (s.d.)), all-trans-retinoic acid (100%), RO 10-1670 (91 ± 7%), and to a lesser extent retinal (60 ± 4%) and RO 13-7410 (60 ± 2%) were effective competitors for the binding of [3H]retinoic acid.

Temperature/time studies
At the raised temperature of 37 °C, [3H]retinoic acid binding was transient (Fig. 1), decreasing after a 30-min incubation period. However, at the lower temperatures of 4 and 25 °C, the rate of ligand association was slower, reaching equilibrium at 4 and 2 h respectively. In addition, at both of these temperatures, binding was maintained for up to 16 h.
D. boyd and others • Retinoid binding in human prostate

Retinoid binding in human prostate (4)

Incubation time (h)

**Figure 1.** [3H]Retinoic acid binding in nuclear extracts: a temperature/time study. Nuclear extract was incubated with 100 nmol [3H]retinoic acid/l at 4°C (●), 25°C (▲) and 37°C (O) for varying times. Non-specific binding was assessed by running parallel experiments in the presence of unlabelled retinoic acid at a 100-fold excess. At the end of each incubation, unbound ligand was removed with dextran-coated charcoal suspension and aliquots of extract were counted in 6 ml scintillation cocktail. Each point represents an average value ± s.d.; the numbers of experiments are in parentheses.

**Saturation analysis**

Incubation of the nuclear extract with increasing concentrations of [3H]retinoic acid (5–60 nmol/l) revealed a saturation of the binding protein at 60 nmol/l (Fig. 2). Scatchard analysis yielded a Kd value of 15 ± 2 (s.d.) nmol/l. Parallel experiments using [3H]retinol as a ligand gave a similar value (12.5 ± 1.0 nmol/l) for the Kd (Fig. 3).

**Metabolism studies**

Reversed-phase chromatography of radioactive material extracted after incubation of nuclear preparation with [3H]retinoic acid resulted in a major peak co-migrating with the all-trans-retinoic acid standard. A minor peak (5% of the chromatographed radioactivity) more polar than the retinoic acid and of unknown identity was also detected. Incubation of KCl extract with [3H]retinol followed by extraction and chromatography resulted in an unchanged chromatograph compared with [3H]retinol incubated with buffer alone.

**Enzyme inhibition**

The levels of [3H]retinoic acid binding in KCl extracts were unaltered by the presence of aprotinin and PMSF. However, inclusion of sodium molybdate (10 mmol/l) increased the binding of [3H]retinoic acid by 18%. The effect of various concentrations of sodium molybdate on [3H]retinoic acid binding was then studied; sodium molybdate concentrations below or in excess of 10 mmol/l were either without effect or inhibited [3H]retinoic acid binding.

**Figure 2.** Saturation and Scatchard analysis of [3H]retinoic acid binding. Nuclear extracts were incubated at 37°C for 30 min and 4°C for 16 h with a range (5–60 nmol/l) of [3H]retinoic acid concentrations in the presence and absence of unlabelled retinoic acid at a 100-fold excess. Free ligand was removed with dextran-coated charcoal suspension and the specific binding (▲) determined after correction for non-specific ligand association (●). The experiment was repeated five times. Kd, dissociation constant.
DISCUSSION

A binding protein which binds all-trans-retinoic acid with high affinity was detected in salt extracts of human prostate adenoma. The protein did not bind testosterone and dihydrottestosterone and in this regard it appears unlikely that the nuclear-extracted component is the androgen receptor reported by others (Smith, Sutherland, Chisholm & Habib, 1983).

Although experiments using other target tissues did not reveal binding of [3H]retinoic acid in nuclear extracts (Wiggert, Russel, Lewis & Chader, 1977; Mehta et al. 1982), exposure of rat mammary adenocarcinoma nuclei to cytosol radiolabelled with retinoic acid resulted in a retention of radioactivity (Mehta et al. 1982). Other studies (Sani & Donovan, 1979) have revealed similar findings and support the contention that the biological actions of retinoic acid may be mediated through nuclear interactions.

Our preliminary binding experiments indicated no binding of retinoic acid with either intact nuclei or nuclei treated with ligand-labelled cytosol. However, sonication of nuclei followed by charcoal treatment of extract resulted in substantial binding. The ability of charcoal to remove endogenous inhibitors of binding in exchange-assay systems has been reported before (Jitendra & Witorsch, 1982). Clearly further studies will be required to establish the identity of the retinoic acid binding inhibitor present in prostate nuclear extracts.

One interesting feature of the competition studies was the finding that retinol effectively competed for [3H]retinoic acid binding, an observation at variance with those of Sani & Donovan (1979) for nuclear-derived cellular retinoic acid binding protein. Our initial fear that this might be due to ligand conversion in vitro was not substantiated by metabolism studies using either retinol or retinoic acid as substrates. Although some conversion of the [3H]retinoic acid could be detected, this amounted to less than 5% of the chromatographed material and is unlikely to be of any significance to these studies. Furthermore, as no conversion of [3H]retinol occurred in vitro, it would appear that the nuclear-derived protein is able to bind both alcohol and acid forms of vitamin A. In support of this contention, unpublished data (D. Boyd, G. D. Chisholm & F. K. Habib) revealed retinoic acid to be just as active as retinol in displacing [3H]retinol binding in salt extracts. In addition, Scatchard analysis indicated that the retinol binding was indeed of high

FIGURE 3. Saturation and Scatchard analysis of [3H]retinol binding. Salt extracts were incubated at 4°C overnight with a range (5-60 nmol/l) of [3H]retinol concentrations with or without unlabelled retinol at a 100-fold excess. Unbound ligand was removed with dextran-coated charcoal suspension and the specific binding (A) of [3H]retinol calculated after correction for non-specific ligand association (●). The experiment was repeated three times. Kd, dissociation constant.
affinity \(K_d = 12.5 \pm 1.0 \text{ nmol/l}\) and exhibited a \(K_d\) value similar to that for retinoic acid binding \(K_d = 15.0 \pm 2.0 \text{ nmol/l}\).

While the evidence at this stage is scant, it is possible that the cytosol binding proteins characterized previously (Boyd et al. 1984) are separate from the nuclear binding proteins. In support of such a view, fractionation experiments indicated that the nuclear and cytosolic retinoid binding components were indeed different (D. Boyd, G. D. Chisholm & F. K. Habib, unpublished data). In addition, the ability of cytosol but not nuclear binding proteins to discriminate between alcohol and acid forms of vitamin A is in accordance with such a hypothesis (Boyd et al. 1984). Perhaps relevant to the present studies are the findings by Liau, Ong & Chytil (1981) and Cope, Knox & Hall (1984) of acceptor sites for retinol and retinoic acid in the nuclei of liver and testicular cells. Ligand-exchange studies and sodium dodecyl sulphate gel electrophoresis confirmed that these sites were different to the cytosol binding proteins.

Clearly further studies will be required to establish the true identity and physiological relevance of these prostate nuclear sites.

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Characterization of the Retinol and Retinoic Acid Binding Proteins in the Human Prostate

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ABSTRACT

A retinol-binding protein has been detected in the cytosol of human prostates with benign hyperplasia. The binding was of high affinity and specific for retinol (Kd = 35 nM), with other retinoids such as trans-retinoic acid, retinal, and the synthetic analogues, all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid and p-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl]benzoic acid, showing little or no competition. The retinoid binding, which sedimented as a 2S component on sucrose density gradients, was also unaffected by the addition of excess unlabeled steroid hormones. Furthermore, pretreatment of the cytosol proteins with heat and/or trypsin totally abolished the retinol binding.

Parallel experiments with trans-retinoic acid suggest that the hyperplastic prostate possesses a second retinoid-binding site which is specific for retinoic acid and distinct from the retinol-binding component. Experiments with serum from patients with benign prostate hyperplasia revealed no binding at the 2S sedimentation position; this suggests that the retinoid-binding proteins were exclusively associated with prostatic tissue and were not therefore derived from serum.

INTRODUCTION

Vitamin A is an important regulator of epithelial differentiation. In 1925, Wolbach and Howe (23) demonstrated widespread epithelial lesions in rats fed vitamin A-deficient diets. A year later in 1926, Fujimaki (9) confirmed these findings interpreting this pathological state to be a prerequisite for neoplastic transformation.

In contrast to a vitamin A deficiency, hypervitaminosis induced by synthetic analogues and natural metabolites of vitamin A appeared protective against the development of papillomas in mice (3). Furthermore, carcinogen-induced benign and malignant states in a number of in vitro and in vivo systems were prevented by vitamin A analogues (8, 13, 21); this was particularly evident in the male secondary sex organs. In this regard, Laznitski (12) reported that chemically induced squamous metaplasia in cultured mouse prostate was reversed by the addition of vitamin A to the culture medium. Recently, Reese et al. (16) have found that the sensitivity of rat prostate adenocarcinoma cell lines to retinoids manifests as a 40% decrease in cell saturation density.

The molecular mechanisms by which vitamin A directs differentiation and mediates its chemopreventive actions are poorly understood. Specific binding proteins for 2 natural forms of vitamin A (retinol and retinoic acid) have been detected in the soluble fraction of a number of normal and pathological tissues (2, 10, 15), and these may play a role in the actions of vitamin A. This concept is supported by the finding that the abilities of a number of retinoids to promote growth in epidermal cell cultures correlated with their binding efficacy to the retinoic acid-binding protein (7).

In view of the findings that vitamin A is transported in the blood as the alcohol (11) and that cellular uptake is an active process, an investigation into the binding of retinol by human hyperplastic prostate seemed a logical approach. Furthermore, we have also described some of our findings on the characteristics of the retinoic acid-binding properties of the human hyperplastic prostate. Although in an earlier investigation Brandes (5) had detected a saturable retinoic acid-binding component in the soluble fraction of the benign and malignant prostate, no characterization of the specific retinoid acid binding protein has so far been undertaken.

MATERIALS AND METHODS

Radiochemicals

All-trans-11,12-[	extsuperscript{3}H]retinoic acid (32 Ci/mmol) was a generous gift from Hoffmann-LaRoche, Basel, Switzerland. All-trans-11,12-[	extsuperscript{3}H]retinol (60 Ci/mmol) was purchased from Amersham International, Inc., High Wycombe, Buckinghamshire, United Kingdom. Radiochemicals were stored under nitrogen at −20°C and the purity checked every 2 weeks by thin-layer chromatography using methylene chloride:ethanol (97:3, v/v) for the retinol (1) and benzene:chloroform:methanol (4:1:1, v/v/v) for retinoic acid (17).

Other Chemicals

The synthetic retinoids RO 10-1670 and RO 13-7410 and 13-cis-retinoic acid were gifts from Hoffman LaRoche, Welwyn Garden City, Hertfordshire, United Kingdom. Retinol, retinal, retinoic acid (all-trans isomers), and various steroids were purchased from Sigma Chemicals, Poole, Dorset, United Kingdom. Solutions of natural and synthetic retinoids were stored in ethanol under nitrogen at −20°C. All other chemical were of analytical grade and purchased from either Sigma or BDH Chemicals, Ltd., Poole, Dorset, United Kingdom.

Tissue Preparation

Freshly obtained transurethral resected or retroperitoneal human hyperplastic prostates were transported to the laboratory in ice-cold 0.9% NaCl solution (saline). The tissue was either used fresh or snap-frozen in liquid nitrogen and stored at −20°C until analysis. Fragments of every prostatic specimen included in the study were examined histologically.

The abbreviations used are: RO 10-1670, all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid; RO 13-7410, p-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl]benzoic acid; TEDG buffer, 10 mM Tris:1.5 mM EDTA:1.0 mM dithiothreitol:glycerol, 10% (v/v), pH 7.4.

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and in each case benign hyperplasia was confirmed. Unless specified, all subsequent procedures were carried out at 4°C. The prostate tissue was washed in TEDG2 buffer, blotted dry, and minced finely with scissors. The tissue was pulverized for 20 sec on a Mikrodisseminator II (El. Braun Melsungen, A. G., Federal Republic of Germany) in a precooled Teflon container and homogenized in 2 to 4 volumes of TEDG buffer with a Ystral GM homogenizer (three 15-sec bursts; speed 7 with 1-min intermittent cooling). The homogenate was filtered through one layer of nylon gauze (Nybott 9-150; John Stainer & Co., Manchester, United Kingdom) and centrifuged to obtain the cytosol fraction (105,000 Xg supernatant) as described previously (6).

Blood obtained from patients with the benign disease was fractionated to give the serum component. The serum was diluted with an appropriate volume of TEDG buffer to give a protein concentration comparable with prostate cytosol.

Retinol Binding Assay

Retinol binding in the cytosol fraction was assessed as described previously (18). Briefly, aliquots of subcellular preparation were incubated at 4°C in the dark for 18 hr with [3H]retinol (100 nM) in the presence and absence of unlabeled ligand in a 100-fold excess. Preliminary studies indicated that pellets formed from dextran-coated charcoal suspension (activated charcoal (0.25%), dextran (0.025%), and gelatin (0.1%) in 10 mM Tris, 1.5 mM EDTA, and 1.0 mM dithiothreitol buffer, pH 7.4) removed greater than 90% of retinol and retinoic acid from solution.

After incubation of cytosol with [3H]retinol, aliquots were transferred to tubes containing preformed charcoal pellets and mixed. After a 10-min incubation period, charcoal was sedimented at 1500 Xg in a refrigerated Chlispin centrifuge (MSE). The supematant was then either overlaid in sucrose gradients (7.5 to 20%) or counted in 6 ml of scintillation cocktail (Triton X-100 based; Vickers Laboratories, Ltd., Burley-in-Wharfdale, West Yorkshire, United Kingdom).

To establish what the binding of [3H]retinol had reached equilibrium, cytosol was incubated with the radiolabeled compound at 4°C for varying lengths of time in the presence and absence of a 100-fold excess of unlabeled retinol. Free ligand was removed with charcoal, and the treated soluble fraction was counted in 6 ml of scintillation cocktail.

Saturation analysis was carried out by incubating the subcellular fraction with an increasing concentration (10 to 100 nM) of [3H]retinol at 4°C for 18 hr in the presence and absence of a 100-fold excess of unlabeled retinol. Unbound retinol was removed with charcoal and the treated supematant counted for radioactivity in a Packard liquid scintillation spectrometer with a counting efficiency of 40%. The binding data were analyzed by the method of Scatchard (20).

Sucrose Density Gradients

Linear sucrose gradients (7.5 to 20%) were centrifuged at 400,000 Xg for 2 hr in a Sorvall TV 865 vertical rotor (DuPont Instruments). Myoglobin (2S) and bovine serum albumin (4.6S) (Sigma Chemicals) were run on separate gradients as external standards. Fractions (200 ml X 20) were harvested by upward displacement using 40% sucrose as displacing agent.

Other Experiments

[3H]Retinoic Acid Binding Assay. The binding of trans-retinoic acid was assessed by the method of Brandes (5); cytosol was incubated with [3H]retinoic acid (100 nM) in the dark at 4°C for 18 hr in the presence and absence of a 25-fold excess of unlabeled ligand. Unbound ligand was removed by charcoal as described above and the treated subcellular preparation analyzed by sucrose density gradient centrifugation.

Serum Binding of Retinol and Retinoic Acid. Serum diluted in TEDG buffer was incubated with [3H]retinol or [3H]retinoic acid (100 nM) in the dark at 4°C overnight. Excess ligand was removed with charcoal and the treated preparation analyzed on sucrose gradients.

Protein Measurement. Cytosol protein concentration was measured by the method of Bradford (4) using bovine serum albumin as standard.

Effect of Heat and Trypsin. Prior to incubation with [3H]retinol or retinoic acid, cytosol was heated to 60°C for 10 min or treated with trypsin (0.05% bovine pancreas, type 1; Sigma Chemicals) at 37°C for 90 min. The binding of [3H]retinol and [3H]retinoic acid were assessed by sucrose gradient analysis after incubation with 100 nM of the radiolabeled compounds as described above.

Metabolism Studies. In order to ensure that retinol or retinoic acid were not metabolized under our receptor assay conditions, the cytosol fraction was incubated with 100 nM of either [3H]retinol or [3H]retinoic acid at 4°C for 18 hr. The cytosol was then extracted with 2 X 1 ml of diethyl ether to remove the radioactivity from the incubation medium. Following the drying down of the diethyl ether, the residue was reconstituted in 50 U of ethanol and subjected to reversed-phase chromatography using Whatman KC 18 thin-layer chromatographic plates (Whatman, Maidstone, Kent, United Kingdom). The plates were run at 4°C in subdued lighting using an ethanol-water (8:2, v/v) solvent system. At the end of the run, the plates were dried down, markers were located, and the positions of the [3H]-labeled retinoids were identified in relation to the reference standards. The appropriate zones containing the various retinoids were cut from the plates and inserted directly into the counting vials, and the radioactivity was measured.

RESULTS

Characterization of Retinol Binding

Specificity. Sucrose gradient analysis of cytosol labeled with [3H]retinol revealed a peak of activity coincident with the 2S external marker (Chart 1). The peak was abolished with a 25-fold excess of retinol (Chart 1a) but was unaffected by trans-retinoic acid (Chart 1a), the synthetic retinoids RO 10-1670 and RO 13-7410 (Chart 1b), testosterone, dihydrotestosterone, and 17β-estradiol (Chart 1c). In contrast, Chart 1, a and b, illustrates that retinol and cis-retinoic acids both have some affinity for the binding component.

Effects of Heat and Trypsin. It is apparent from Chart 2 that protease and heat pretreatment inactivate the 2S binding of [3H]retinol. These data suggest that [3H]retinol is bound by a protein macromolecule derived from the soluble fraction of human prostate.

Serum Binding of [3H]Retinol and [3H]Retinoic Acid. Chart 3 illustrates the results of sucrose gradient analysis of serum labeled with [3H]retinol. A peak of activity was observed in the 4.6S but not in the 2S region of the gradient. These findings confirm those of other workers (2) who were unable to detect any retinol binding in the 2S region of the sucrose gradient for serum specimens treated with radiolabeled retinol.

Affinity and Binding Capacity. Prior to saturation analysis of the soluble component, it was important to establish that the binding of [3H]retinol was in equilibrium under conditions of time and temperature. It is apparent from Chart 4 that the specific binding of [3H]retinol is at equilibrium after 4 hr and extends to 24 hr of incubation at 4°C.

Scatchard analysis of the saturation data determined under equilibrium conditions of exchange revealed a dissociation constant of 35 nM with a binding capacity of 3.5 pmol/mg cytosol protein (Chart 4).

Metabolism Studies. Chart 5, a and b, illustrates the absence of [3H]retinol and [3H]retinoic acid metabolism in vitro. The chro-
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Chart 1. Specificity of [3H]retinol binding in cytosol from benign human prostate: sucrose gradient analysis. Aliquots of prostate cytosol were incubated for 18 hr at 4°C with either 100 nM [3H]retinol alone (A) or in the presence of 25-fold excess of unlabeled (a) retinol (B), retinal (C), trans-retinol-acid (O), RO 10-1670 (B), RO 13-7410 (C), cis-retinol-acid (B), (c) dihydrotestosterone (B), testosterone (B), 17β-estradiol (B). The binding of [3H]retinol was assessed by sucrose gradient centrifugation as described in "Materials and Methods." Each experiment was repeated 3 times.

matographic profiles obtained were identical to those for [3H]retinol and [3H]retinoic acid extracted from buffer alone (data not shown).

Chart 2. Inactivation of [3H]retinol binding by heat and trypsin treatments: sucrose gradient analysis. The soluble fraction of human prostate was treated with either 0.05% trypsin or briefly heated to 60°C. Untreated (A), heated (O), trypsin (■), and 100-fold excess unlabeled retinol (■) treated cytosols were then incubated with 100 nM [3H]retinol and overlayed on sucrose gradients. All studies were performed in triplicate.

Chart 3. Binding of [3H]retinol and [3H]retinoic acid in serum from patients with benign prostate hypertrophy. Serum from patients with benign prostate hyperplasia was diluted with TEDG buffer to give protein concentrations of 5 to 10 mg/ml. The diluted serum preparations were incubated with either 100 nM [3H]retinol (A) or [3H]retinoic acid (■) at 4°C for 18 hr. The binding of radiolabeled ligands was analyzed by sucrose gradient centrifugation as described in "Materials and Methods." These measurements were repeated at least 3 times.

Radiolabeled Retinoic Acid Binding

Sucrose gradient centrifugation of cytosol labeled with [3H]retinoic acid revealed peaks of activity in the 2S and 4.6S regions (Chart 6). While the 2S peak was abolished by a 25-fold excess of retinoic acid (all trans- and cis-isomers; Chart 6, a and b), RO 10-1670 and RO 13-7410 (Chart 6c), dihydrotestosterone, and testosterone were with little effect (Chart 6c). However, it is apparent from Chart 6b that both retinol and retinal possess some affinity for the 2S binding component. In contrast to the binding profile of the 2S peak, the secondary peak of activity in the 4.6S region was unaffected by any competitor present in excess, suggesting a nonspecific association.

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It is also evident from Chart 7 that the retinoic acid binding component is degraded by both protease and heat treatment, indicating that the component is a heat-labile protein.

**DISCUSSION**

A binding component for retinol has been detected in benign human hyperplastic prostate. The component exhibits similar characteristics to the cellular retinol binding protein present in a number of other Vitamin A target organs (18, 22). In common with cellular retinoic acid binding protein (Chart 6), the prostate binding component has a sedimentation coefficient of 2S (Chart 1) and was inactivated by protease and heat treatments (Chart 2). Although this binding site failed to recognize trans-retinoic acid (Chart 1a), the profile illustrated in Chart 1b indicates some competition by the cis-isomer; this may reflect the different stereochemistries of the 2 isomers. Furthermore, this site binds retinol avidly with a dissociation constant of 35 nM (Chart 4); this value is not inconsistent with the 16 nM reported for purified cellular retinol binding protein (14).

The present results also suggest that human hyperplastic prostate is able to recognize the acid form of Vitamin A as a distinct entity. Although the binding sites for retinol and retinoic acid are similar in that they are both heat-labile proteins with sedimentation coefficients of 2S, it is unlikely that they are identical. In support of this, trans-retinoic acid was not recognized by the retinol-labeled peak (Chart 1a). Conversely, retinol displayed little affinity for the retinoic acid-binding site (Chart 6).

Furthermore, while the retinoic acid-binding site bound the synthetic analogues RO 10-1670 and RO 1-7410 (Chat 6), these compounds did not associate with the retinol recognizing structures (Chart 1). The data on the retinoic acid binding confirm and extend the findings of Brandes (5) who detected the 2S saturable component for retinoic acid in the cytosol of benign and malignant human prostate; this author, however, did not attempt to check the specificity of his binding component. Furthermore, our findings that retinol showed some affinity for [H]retinoic acid binding are at variance with other reports on the specificity of retinoic acid.
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Chart 6. Specificity of [%H]retinol acid binding in cytosol from benign human prostate sucrose gradient analysis. Aliquots of prostate cytosol were incubated for 18 hr at 4°C with either 100 nM [%H]retinol acid alone (A) or in the presence of 2.5 μM unlabeled (a) trans-retinol acid (C), retinol (B), retinal (D); (b) RO 10-1670 (F), RO 13-7410 (E), cis-retinol acid (E); (c) 17-β-dihydrotestosterone (B), testosterone (F), trans-retinol acid (D). The binding of [%H]retinol acid was assessed by sucrose gradient centrifugation as described in "Materials and Methods." Each experiment was repeated 3 times.

Chart 7. Inactivation of [%H]retinol acid binding by heat and trypsin treatment: sucrose gradient analysis. Cytosol was treated with either 0.05% trypsin or briefly heated to 60°C. Untreated (A), heated (B), trypsin (B), and 25-fold excess unlabeled retinol acid (B)-treated cytosols were then incubated with 100 nM [%H]retinol acid and overlayed on sucrose gradients. Each experiment was performed in triplicate.

Our initial fear that this competition may arise from the metabolism of either retinol or retinoic acid was not confirmed, since metabolism of either ligand was not manifested under conditions identical to those used for the receptor assay. Therefore, we believe that the competition by the retinol is genuine and may reflect the particular characteristics of the retinoic acid binding protein in the human prostate gland.

It is clear from the results reported in this paper that the prostate probably possesses 2 specific cytosol-binding sites for the acid and alcohol analogues of vitamin A; preliminary studies indicate that the expression of these retinoid binding proteins is suppressed in cancer. Since these analogues are known to maintain cell differentiation in the prostate gland (12), it remains to be seen whether such effects could be induced by the presence of these receptors in the nucleus, a possibility which we are presently investigating.

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