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PARTIAL PURIFICATION OF STEROID–RECEPTOR COMPLEXES BY DNA-CELLULOSE CHROMATOGRAPHY AND ISOELECTRIC FOCUSING

R. IRVING and W. I. P. MAINWARING

Androgen Physiology Department, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, England.

SUMMARY

1. The propensity of steroid–receptor complexes for native DNA immobilized on cellulose, together with their relatively acidic isoelectric point, may be exploited as means of partial purification.
2. A critical appraisal of these procedures is presented and potential future improvements are discussed in detail.
3. Overall, the facility with which DNA-cellulose chromatography and isoelectric focusing may be conducted suggests that they provide a suitable means for the preliminary fractionation of proteins from steroid-responsive cells prior to final purification of the steroid–receptor complexes by more discriminating analytical procedures, notably affinity chromatography.

INTRODUCTION

An almost invariant feature of steroid-responsive cells is the presence of proteins or "receptors" that avidly bind steroid hormones in a highly tissue- and steroid-specific manner[1]. With a few minor exceptions the receptors demonstrate a pronounced binding affinity only for the steroids that regulate the growth and function of a particular steroid-responsive tissue[1]. Widespread interest in the means by which steroid–receptor complexes regulate important metabolic processes, notably genetic transcription, has prompted the need for the purification of receptor complexes. This is a daunting task since the receptor complexes are exceedingly labile and present in only minute quantity; consequently, ideal methods of purification should be rapid and highly specific. Sequential chromatography on DNA-cellulose and isoelectric focusing was proposed for the partial purification of steroid–receptor complexes[2, 3] and the principal objective of the present paper is to appraise critically these and other currently available methods for receptor purification.

EXPERIMENTAL

For the main part, the present investigation was conducted using the procedures described in detail elsewhere[2]. Additionally, covalent attachment of calf thymus DNA to either Sepharose 4B[4] or Sephadex G-200[5] was performed as recommended by the innovators of the procedures. Denaturation of DNA prior to coupling to Sepharose 4B was accomplished by heating at 100°C for 10 min and then rapid cooling to 0°C.

RESULTS AND DISCUSSION

(a) *Purification of cytoplasmic receptor–steroid complexes*

The cytoplasmic androgen receptor has been selected to serve as a model for our preparative procedures; reference to other steroid–receptor complexes will be made in the text, where appropriate. A representative purification of cytoplasmic 8S androgen receptor is presented in Table 1. A substantial increase in the specific radioactivity of the final product was accomplished when purification was conducted in the presence of 1 nM [^{3}H]-5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one). Using this more protracted scheme, bound [^{3}H]-5α-dihydrotesterone in fractions from analytical procedures was assessed by gel exclusion chromatography on Sephadex G-25. The excess free [^{3}H] ligand presumably counteracts the dissociation of receptor–bound steroid during analysis particularly during desalting (stage IV; Table 1). Each stage of the purification will be critically evaluated. This isolation was conducted in 50 mM Tris–HCl buffer, containing 0·5 mM dithiothreitol, 0·25 mM EDTA and 10% (v/v) glycerol[2], except for isoelectric focusing in sucrose gradients (stage V; Table 1).

Table 1. Partial purification of 8S cytoplasmic 5α-dihydrotestosterone–receptor complex of rat prostate gland

		(a) Absence of excess [^{3}H]-5α-dihydrotestosterone throughout			(b) Presence of excess [^{3}H]-5α-dihydrotestosterone throughout		
	Stage of purification	Protein (mg)	^{3}H bound to 8S receptor (d.p.m.)	Specific radioactivity (d.p.m./mg of protein)	Protein (mg)	^{3}H bound to 8S receptor (d.p.m.)	Specific radioactivity (d.p.m./mg of protein)
I	Initial cytoplasmic extract	364	5.5×10^7	1.52×10^5	341	5.4×10^7	1.61×10^5
II	Precipitation in $(NH_4)_2SO_4$ (33% saturation)	30.4	4.9×10^7	1.62×10^6	32.2	5.4×10^7	1.68×10^6
III	DNA-cellulose chromatography	3.0	2.4×10^7	8.02×10^6	3.1	4.2×10^7	1.35×10^7
IV	Desalting; Sephadex G-25	2.9	7.4×10^6	2.55×10^6	3.0	4.0×10^7	1.29×10^7
V	Isoelectric focusing (pI 5.8)	0.10	5.8×10^6	5.28×10^7	0.09	3.4×10^7	3.69×10^8
	Degree of purification						
(a)	based on recovery of protein		3466			3706	
(b)	based on specific radioactivities		347			2291	

At 24 h after castration, a cytoplasmic extract (105,000 *g* supernatant) was prepared from glands pooled from 18 animals and labelled with 5 nM [^{3}H]-5α-dihydrotestosterone (S.A. 44 Ci/m mol) for 2 h at 0°C. Samples were analyzed either by Sephadex G-200 chromatography[6] or sucrose gradients[6] to assess the initial labelling of 8S (Stoke's radius 96 A) receptor complex. Purification was continued in (a) the absence or (b) the presence of 1 nM [^{3}H]-5α-dihydrotestosterone. In (b) individual fractions were analyzed in columns of Sephadex G-25 to measure bound radioactivity. Receptor complex was monitored by scintillation spectrometry; protein was determined only in peak fractions pooled at each stage. The data are taken from Mainwaring and Irving[2].

The initial extract (stage I) should be prepared at 0°C without excessive shearing during homogenization[6], otherwise the labile receptor is destroyed. Polytron or Ultraturrax homogenizers should be used at low speed and preferably avoided. [^{3}H]-5α-Dihydrotestosterone is added to a concentration of 5 nM in a min. vol. (5 μl per ml of extract) of ethanol: 1,2-propanediol 1:2 (v/v). There is little advantage in adding the [^{3}H]-5α-dihydrotestosterone to whole prostate homogenates, despite its stabilization of the receptor (Table 1), as it is rapidly metabolized by prostate microsomes to various 5α-androstane diols. Other receptor complexes, say rat uterine oestrogen receptor, seem more stable at this stage.

Stage II of the purification is accomplished by the classical procedure of $(NH_4)_2SO_4$ fraction[7]; alternatives, including protamine precipitation[6], were far from satisfactory[2]. However, the possibility of finding a suitable means of releasing receptor–complexes from protamine-containing precipitates should not be discounted. $(NH_4)_2SO_4$ fractionation at 30–33% saturation removes the bulk of non-specific (4S) androgen-binding proteins and provides a reasonable enrichment of specific (8S) receptor complex[2, 7, 8]. Similar procedures have been adopted in the preliminary purification of oestrogen receptor complexes[9, 10, 11] but all investigators have encountered problems of receptor aggregation. Better yields of receptor complexes are achieved when extracts are treated by the dropwise addition of a saturated solution of enzyme grade $(NH_4)_2SO_4$ (low in heavy metals; pH adjusted to 7.4 with 1 M-NH_4OH) with gentle stirring at 0°C rather than by addition of solid salt. Speed of manipulation, low temperature and the presence of EDTA appear mandatory for the preservation of 8S receptor complexes during $(NH_4)_2SO_4$ fractionation; without these provisions, receptor complexes can be transformed to a complex of sedimentation coefficient 4.65[12].

DNA has been implicated in the retention of steroid–receptor complexes by chromatin in cell-free systems [13, 14] and by nuclei[15]. While controversy exists concerning the involvement of non-histone nuclear-associated proteins in the nuclear retention of steroid–receptor complexes (see review in reference 1), it was proposed[16] that selective binding of steroid–receptor complexes to immobilized DNA may provide a profitable means of purification (stage IV; Table 1). This concept has been widely validated[2, 17–19] and the essential feature of DNA-cellulose chromatography is that steroid-protein complexes other than those containing receptor proteins are not retained. This is illustrated by the studies presented in Table 2 on the mouse kidney androgen receptor that preferentially binds testosterone rather than 5α-dihydrotestosterone [19]. This specificity[3, 19] is evident even with complexes containing testosterone bound with high affinity to the sex steroid-binding β-globulin[20, 21] of human serum.

Many procedures have been advocated for the immobilization of DNA in a form suitable for column chromatography. In the procedures of Alberts[22] and

Table 2. The specific binding of mouse kidney testosterone–receptor complex to DNA cellulose

Source of protein	Steroid	Binding (c.p.m./0·25 g column) (a) Mouse kidney DNA	(b) Calf thymus DNA
Mouse kidney	^{3}H-Testosterone	329	303
Mouse kidney control	^{3}H-Testosterone	80	99
Mouse kidney	^{3}H-Testosterone and 200 nM testosterone	44	58
Mouse spleen	^{2}H-Testosterone	40	55
Rat prostate	^{3}H-5α–Dihydrotestosterone	529	550
Human plasma	^{3}H-Testosterone	41	34
None (medium A alone)	^{3}H-Testosterone	40	54

Samples of various tissues or plasma (0·7 ml, 7–10 mg of protein) were labelled with 2 nM ^{3}H-steroids and applied to columns containing 0·25 g of DNA-cellulose (70 μg of purified DNA from calf thymus or mouse kidney). All preparations and equilibration of the columns were conducted in medium A (50 nM Tris–HCl buffer, pH 7·4 containing 0·25 mM EDTA, 0·5 mM dithiothreitol and 10% v/v glycerol). After washing with 10 ml of medium A, columns were eluted with medium A containing 0·5 M KCl. Input of ^{3}H throughout was 5000 c.pm. Mouse kidney control contained only 0·25 g of cellulose. Data are from reference [19].

Litmann[23] the precise nature of the linkage of DNA is unknown but possibly involves hydrogen bonds; in other cases[4, 5] covalent linkage is indubitably involved. DNA-cellulose prepared either with[23] or without[22] an ultraviolet irradiation step has the advantage of ease of preparation but in our hands, the binding of cytoplasmic oestrogen–receptor complexes is not as extensive as found with androgen receptors; this is also the experience of other investigators[17, 24]. The Litmann[23] procedure promotes the immobilization of DNA to approximately 1·5 mg/g wet weight of cellulose as against only the 0·5/g of cellulose achieved by the Alberts procedure[22]. Covalent attachment of DNA is clearly an advantage since the columns may theoretically be used indefinitely and such matrices will clearly be predominant in future work. However, in this laboratory at least, it has not proved possible to prepare covalently linked DNA to Sephadex G-200 [5] in other than small quantities, with a maximum of 50 mg wet wt. of Sephadex per synthesis. The technical difficulties encountered during the large scale synthesis of this DNA-containing matrix regrettably remain unsolved. The covalent linkage of denatured DNA to CNBr-activated Sepharose 4B[4] must be conducted with care. An excess of CNBr during the activation of Sepharose 4B, prior to the covalent coupling of the DNA, can lead to irreversible dissociation (or denaturation) of steroid–receptor complexes. Nevertheless, this matrix is perhaps the most attractive of presently available means of immobilizing DNA and will probably be the method of choice in future work. This is indicated by its striking efficiency in purifying other proteins with an elective affinity for DNA, such as DNA polymerase[4], as compared to DNA-cellulose[22, 23]. The use of denatured (single stranded) DNA does not impose a limitation to the DNA-Sepharose 4B technique since steroid–receptor complexes bind as satisfactorily[16] or perhaps even better[25] to denatured DNA than to native (helical) DNA. The introduction of single stranded DNA, generating a form suitable for coupling to CNBr-activated Sepharose 4B, is probably best achieved by digestion with exonucleases[4], especially the enzyme from λ phage[26]. We have used thermally denatured DNA for coupling to Sepharose 4B but only a fifth of the amount of DNA was covalently bound as compared to exonuclease-digested DNA[4]. We have not explored the potential of DNA-acrylamide columns [27] and these may also be of value for the isolation of steroid–receptor complexes.

Adequate controls of the type presented in Table 2 should always be included in studies with immobilized DNA to ensure the specificity of the process. It is also advisable to check independently the binding efficiency of every new batch of DNA-containing adsorbent before studies on steroid–receptor complexes are attempted; both DNA polymerase[2, 4, 22, 23] and RNA polymerase[22, 28] have been advocated for this purpose.

One contentious aspect of using DNA-containing adsorbent is that the molecular interaction between the DNA and the steroid–receptor complex, or indeed any protein with an affinity for DNA, remains ill-defined. Little if any specificity has been attributed to the source of DNA[2, 17, 18, 25] in the retention of cytoplasmic receptor complexes and hence commercially available calf thymus DNA is widely used. D-2-deoxyribose has not a critical involvement since polyribonucleotides[29] and even ribonucleoprotein particles[30] are known to bind receptor complexes.

Guanine residues are seemingly involved for binding of receptor complexes occurs to poly (G) but not poly (I); furthermore, hydroxymethylation of 6-amino groups abolishes binding[29]. However, the activity of the guanosine residues is clearly influenced by adjacent nucleotide groups for actinomycin D, which binds to guanosine, does not impair the binding of any type of receptor complex to either chromatin or DNA[1]. With native DNA, it is not known whether the receptor complexes preferentially occupy the wide or narrow grooves within the helical structure. Studies with N,N,N-trimethyl-N′,N′-dimethyl-N′-(β-2,4-dinitroanilinoethyl)-1,3-diammoniumpropanedibromide may be relevant in this context since this molecule specifically occupies the minor (narrow) groove of DNA[31].

For the final step in the purification scheme (stage V: Table 1), isoelectric focusing was used[2] since cytoplasmic receptor complexes have a relatively acidic isoelectric point (pI 5·8). The satisfactory performance of this procedure requires the maintenance of temperatures as close to 0°C as possible and this was most readily accomplished in columns made to the design of Osterman[32]. Rigorous checks on the validity and reproducibility of this technique were reported at length in our original study[2] using several proteins of known pI. The satisfactory performance of isoelectric focusing may be followed visually by the addition of coloured marker proteins to the samples under analysis; we recommend the use of horse spleen ferritin (pI 5·0), bovine heart catalase (pI 6·0) and bovine haemoglobin (two bands; pI 7·2 and 7·6). It cannot be over emphasized that isoelectric focusing is conducted in the absence of any visible precipitation of protein. When this phenomenon occurs, extreme variations are observed in the pI of the steroid–receptor complexes. Presumably the receptor complex becomes entrapped within the precipitate which, on slowly settling under gravity, results in the aberrant recovery of receptor complex in the lower region of the apparatus.

Our proposed scheme (Table 2) has proven applicability for the partial purification of androgen receptors, oestrogen receptors and progesterone receptors[2]. However, low recoveries of oestrogen–receptor complexes were found, due to losses by aggregation at stage II and a low binding to DNA-cellulose at stage III. The physicochemical properties of these receptors were remarkably similar[2] and these findings are harmonious with the reports by other investigators who purified steroid–receptor complexes by somewhat different means[10, 11]. Constancy in the form (or configuration) of the receptor complexes during their partial purification was evident from the analysis of material recovered at each stage by polyacrylamide electrophoresis[2]. In addition, the material recovered at stage V (Table 1) can still fulfil one fundamental function of cytoplasmic androgen receptor protein[2], namely the transfer of 5α-dihydrotestosterone into chromatin in a reconstituted cell-free system[7]. Other studies[8, 33] have indicated that cytoplasmic androgen–receptor complexes at stage II purity can stimulate RNA synthesis on prostate chromatin, *in vitro*; it is imperative that such investigations be repeated with the highly purified material recovered at stage V. This is currently in progress.

(b) *Purification of nuclear steroid–receptor complexes*

In the main, the purification scheme represented in Table 1 is also applicable to nuclear receptor complexes. Three major points of difference should be stressed. First, nuclear receptor complexes can only be extensively labelled with [^{3}H]-steroids in whole tissue *in vitro* or *in vivo*[1]. Secondly, there is certain evidence that nuclear–receptor complexes demonstrate tissue specificity in their retention by immobilized DNA[17], the tissue of origin of the receptor being the most suitable source of DNA. We have not rigorously attempted to confirm this interesting finding, but it should be borne in mind in future investigations. Thirdly, an additional step involving the use of Dextran sulphate to remove contaminant basic proteins may be required before isoelectric focusing of nuclear–steroid receptor complexes[2].

(c) *Future applications of the purification scheme*

It is evident that, at best, only a partial purification of receptor complexes is achieved by a scheme utilizing DNA-cellulose chromatography and isoelectric focusing[2, 3]. Final purification may possibly be achieved by repeated cycles of polyacrylamide electrophoresis or by affinity chromatography. The enormous potential of the latter technique is illustrated by the particularly elegant study conducted by Sica and his collaborators[34] on oestrogen–receptor complexes. Suitable matrices for the affinity chromatography of glucocorticoid[35] and androgen[36] receptor complexes are possibly available even now and their potential should be investigated as a matter of priority.

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REFERENCES

1. King R. J. B. and Mainwaring W. I. P.: *Steroid Cell Interactions*. Butterworths, London (1974).
2. Mainwaring W. I. P. and Irving R.: *Biochem. J.* **134** (1973) 113–127.
3. Mainwaring W. I. P. and Irving R.: *Methods in Enzymology* (Edited by B. W. O'Malley and D. G. Hardman): in press.
4. Poonian M. R., Schlabach A. J. and Weissbach A.: *Biochemistry* **10** (1971) 424–427.

5. Rickwood D.: *Biochim. biophys. Acta* **269** (1972) 47–50.
6. Mainwaring W. I. P.: *J. Endocr.* **45** (1969) 531–541.
7. Mainwaring W. I. P. and Peterken B. M.: *Biochem. J.* **125** (1971) 285–295.
8. Davies P. and Griffiths K.: *Biochem. J.* **136** (1973) 611–622.
9. Erdos T.: *Biochem. biophys. Res. Commun.* **32** (1968) 338–343.
10. de Sombre E. R., Puca G. A. and Jensen E. V.: *Proc. natn. Acad. Sci. U.S.A.* **64** (1969) 148–154.
11. Puca G. A., Nola N., Sica V. and Bresciani F.: *Biochemistry* **10** (1971) 3769–3779.
12. de Sombre E. R., Mohla S. and Jensen E. V.: *Biochem. biophys. Res. Commun.* **48** (1972) 1601–1608.
13. Musliner T. A. and Chader G. J.: *Biochem. biophys. Res. Commun.* **45** (1971) 998–1101.
14. Marver D., Goodman D. and Edelman I. S.: *Kidney Int.* **1** (1972) 210–230.
15. Harris G. S.: *Nature, New Biol.* **231** (1971) 246–248.
16. Mainwaring W. I. P. and Mangan F. R.: *Advanc. Biosci.* **7** (1971) 165–172.
17. Clemens L. E. and Kleinsmith L. J.: *Nature, New Biol.* **237** (1972) 204–206.
18. Yamamoto K. R. and Alberts B. M.: *Proc. natn. Acad. Sci. U.S.A.* **69** (1972) 2105–2109.
19. Bullock L. P., Mainwaring W. I. P. and Bardin C. W.: *Endocr. Res. Commun.* (1974): in press.
20. Daughaday W. H.: *J. clin. Invest.* **37** (1958) 511–519.
21. Rosner W. and Deakins S. M.: *J. clin. Invest.* **47** (1968) 2109–2116.
22. Alberts B. M., Amodio F. J., Jenkins M., Gutman E. D. and Ferris F. L.: *Cold Spring Harb. Symp. quant. Biol.* **33** (1968) 289–305.
23. Litmann R.: *J. biol. Chem.* **243** (1968) 6222–6233.
24. Puca G. A.: personal communication to the authors.
25. King R. J. B. and Gordon J.: *Nature, New Biol.* **240** (1972) 185–187.
26. Korn D. and Weissbach A.: *J. biol. Chem.* **238** (1963) 3390–3394
27. Cavalieri L. F. and Carroll E.: *Proc. natn. Acad. Sci. U.S.A.* **67** (1971) 807–812.
28. Humphries P., McConnell D. J. and Gordon R. L.: *Biochem. J.* **133** (1973) 201–203.
29. King R. J. B.: In *Effects of Drugs on Cellular Control Mechanisms* (Edited by B. R. Rabin and R. B. Freedman) 1973. Macmillan, London pp. 11–20.
30. Liao S., Liang T. and Tymoczko J. L.: *Nature, New* Biol. **241** (1973) 211–213.
31. Parker J., Baserga R. and Gabbay E. J.: *Biochem. biophys. Res. Commun.* **43** (1971) 675–681.
32. Osterman L.: *Sci. Tools* **17** (1970 31–33.
33. Davies P. and Griffiths K. *Biochem. biophys. Res. Commun.* **53** (1973) 373–382.
34. Sica V., Parikh I., Nola E., Puca G. A. and Cuatracasas P.: *J. biol. Chem.* **248** (1973) 6543–6558.
35. Trapp G. A., Seal U. S. and Doe R. P.: *Steroids* **18** (1971) 421–432.
36. Burstein S. H.: *Steroids* **14** (1969) 263–268.

DISCUSSION

Jensen:

When you say you tested your purified steroid receptor complex by "transfer into chromatin", could you tell us just what you mean by this? How do you do these experiments?

Irving:

If we take chromatin and add our [^{3}H]-5α-dihydrotestosterone-receptor complex to the chromatin, we can show that there is a specific binding or association between them or a transfer of the steroid into the complex.

Jensen:

Actually what you're testing is whether it sticks to chromatin. But you would expect it to do so because you purified it by binding to DNA. Unless it becomes degraded, you might expect that anything that will bind to DNA would bind to chromatin. So this really is not a criterion that it is the original receptor in its physiologic form, only that it has not lost the property to bind to DNA by which you pulled it out of the mixture in the first place.

Irving:

Yes, but chromatin has a large amount of protein associated with it and we've shown that it is to *prostate* chromatin that it will bind or transfer the steroid specifically.

Jensen:

One other short question: when you first precipitate your material with ammonium sulfate, do you find there is an alteration of the receptor from its native form?

Irving:

No, there isn't. Our step one material seems to be exactly the same as the later steps.

Schrader:

I wonder what the problems are in removing the ampholytes that you use in isoelectric focusing from your receptor protein since they are highly charged.* I wonder if you have any problem getting them off and how you do it?

Irving:

The biggest problems we find are with the electrophoresis, in fact. There seems to be a band of ampholytes which binds the stain we use, Coomassie blue, and we have to use a large number of 5% TCA washes to remove this band, but we've shown that this band does disappear with washing. There appear to be no other problems.

Schrader:

Have you determined the *S* value for the purified protein after you have ascertained that all of the ampholytes have been removed from the protein? That is to say for example, by running sucrose density gradient centrifugation on material after electrophoresis.

Irving:

No, we haven't done this, but we have done it after the isoelectric focusing with ampholytes still associated with it and find that an *S* value of 8 is retained.

Villee:

Have you considered doing an experiment in which you take your purified prostatic 5α-dihydrotestosterone receptor complex and add it to prostatic nuclei to see whether this complex can enter the nucleus and work physiologically?

Irving:

No, we haven't tried this yet.

Vorob'ev:

Have you seen any difference in the binding of steroid receptor complexes to native and de-natured DNA?

Irving:

Yes, we have compared the binding to native and de-natured DNA and the values are very similar, perhaps the binding being marginally better with the native rather than the de-natured DNA.

Vorob'ev:

What is the difference between the binding of steroid-receptor complexes to chromatin from the same tissue (to prostatic chromatin) and to liver or kidney chromatin? Have you found any specificity in the binding to chromatin?

Irving:

Yes, there is some specificity in the binding to chromatin, for prostate chromatin.

Vorob'ev:

Have you tried to dissociate the chromatin complexes and to study the binding of hormone-receptor complexes to partial nucleoproteins?

Irving:

No.