ADRENAL CHOLESTEROL-BINDING PROTEIN: PROPERTIES AND PARTIAL PURIFICATION

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1. Introduction

The rate-limiting step of adrenal and gonadal steroidogenesis is the conversion of cholesterol to pregnenolone [1–3]. There is good evidence that most of the steroidogenic compounds activate the steroid biosynthetic pathway at a site prior the formation of pregnenolone [4–6]. This effect of the steroidogenic compounds in both adrenals and gonads requires protein synthesis, since cycloheximide inhibits the transformation of cholesterol to pregnenolone [1,7] and increases the accumulation of unconjugated cholesterol in the cytosol [8]. It has been postulated [1] that the steroidogenic compounds stimulate steroidogenesis by regulating a protein which translocates cholesterol from cytosol to mitochondria, but such a protein has not been identified.

We report here the presence of a heat stable protein in the cytosol of adrenal, testicle and ovary, which specifically binds cholesterol and some of its derivatives, but which does not recognize the compounds without the side chain of cholesterol.

Abbreviations: CBP, cholesterol-binding protein; cholesterol, 5-cholesten-3βol; (20 S) 20-hydroxycholesterol, (20 S) 5 cholesten-3β, 20-diol; (20 R, 22 R) 20,22-dihydroxycholesterol, (22 R) 5 cholesten-3β20α22-triol; (20 R, 22 S) 20,22-dihydroxycholesterol; (22 S) 5 cholesten-3β20α22-triol; pregnenolone, 5 pregnen-3βol-20-one; progesterone, 4-pregnene-3,20 dione; dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; testosterone, 17β hydroxyandrost-4-ene-3-one; cortisol, 4-pregnen-11β, 17α, 21 triol-3,20-dione; 17-oestradiol, 1,3,5(10) oestratriene-3,17β-diol; 25-hydroxycholesterol, 5-cholesten-3β, 25-diol

2. Materials and methods

2.1. *Partial purification of adrenal cholesterol-binding protein*

All tissues were homogenized in 50 mM Tris–HCl buffer, pH 7.4 and centrifuged to 105 000 × *g* for 90 min. The supernatant was slowly added to 10 vol. cold acetate (–60°C) and centrifuged to 10 000 × *g* for 10 min. The pellet was washed once with cold acetone and once with cold ethyl ether (–60°C), and finally dried under vacuum. The acetone powder was resuspended in Tris–HCl buffer heated at 100°C for 5 min and centrifuged. The supernatant was saved (heat-stable cytosol). The proteins of this fraction were precipitated by addition of ammonium sulfate to 30% saturation. After centrifugation, more ammonium sulfate was added to reach 60% saturation and centrifuged. The pellet of each centrifugation was redissolved in 50 mM Tris–HCl buffer, pH 7.4 and dialyzed against the same buffer. The fraction precipitated by 60% ammonium sulfate was chromatographed on a DEAE–Sephadex A 50 column (20 × 1.5 cm) pre-equilibrated in 50 mM Tris–HCl buffer. After washing the column was eluted by a continuous gradient of NaCl (0–0.5 M). In some cases, the fraction precipited by 60% ammonium sulfate was preincubated with [^{3}H]cholesterol (spec. act. 43 Ci/mmol; Amersham, England) before chromatography on DEAE–Sephadex.

2.2. *Binding assays*

Celite 454 was extensively washed with 6 N HCl, distilled water, and distilled methanol. After activa-

tion at 600°C for 12 h, 50 mg aliquots were distributed in small tubes. Labeled and unlabeled sterols dissolved in benzene were layered on celite and then the solvent was dried under vacuum. The protein fraction in 50 mM Tris–HCl buffer was added to the tubes containing the celite and sterols and shaken for the times and at the temperatures indicated in the fig.1–6 and tables 1,2. At the end of the incubation the tube was centrifuged at 10 000 × *g* for 10 min. An aliquot of the supernatant was used for determination of the radioactivity, another was layered over a sucrose gradient (5–20%) prepared in Tris–HCl buffer containing 10% glycerol (v/v) and centrifuged for 30–40 h at 250 000 × *g*.

All binding determinations using the celite system were performed in triplicate. Three additional samples were also measured in the presence of a 100-fold excess of unlabeled cholesterol.

3. Results

Cholesterol content of sheep adrenal cytosol is very high (4–8 μg/mg protein, 5 expt). Therefore, to study the binding of [^{3}H]cholesterol to adrenal cytosol, it was crucial to extract endogenous cholesterol without protein degradation. After acetone precipitation, more than 99% cytosol cholesterol was recovered in the pool of organic solvents and cholesterol was undetectable in the acetone powder. Heating at 100°C for 4 min precipitated 90–95% resuspended acetone powder proteins. The recovery of the cholesterol-binding protein was almost complete as indicated by the measurement of its binding activity (table 1).

3.1. *Binding of [^{3}H]cholesterol to heat stable adrenal cytosol proteins*

The classical methods used to separate unbound from bound steroids, such as dextran-coated charcoal, ammonium sulfate precipitation, or equilibrium dialysis do not give reproductible results when used to separate bound from unbound cholesterol. Nor did gel filtration on Sephadex G-75 or G-100 columns (1×10 cm) separate bound from unbound cholesterol. When heat-stable cytosol was incubated at 4°C or 37°C with [^{3}H]cholesterol (10^{-9} M) in the absence or presence of unlabeled cholesterol (10^{-7} M), then chromatographed on Sephadex at 4°C most of the radioactivity came out in the void volume. Moreover, similar results were obtained when 50 mM Tris-buffer alone, rather than the heat-stable cytosol was used.

Table 1
Specific binding of [^{3}H]cholesterol by several preparations of sheep adrenal cytosol

	fmol/0.1 mg protein
Acetone powder	20 ± 10
Heat-stable cytosol acetone powder	1080 ± 204
Ammonium sulfate precipitated (0–30% satn)	220 ± 40
Ammonium sulfate precipitated (30–60% satn)	2580 ± 400
DEAE–Sephadex	4240 ± 500

Tris–HCl buffer, 1 ml 50 mM, containing 0.5 mg protein of the indicated cytosol preparation, was incubated with 50 mg celite containing 10 pmol [^{3}H]cholesterol, without or with 1 nmol unlabeled cholesterol at 4°C for 16 h and centrifuged at 10 000 × *g* for 10 min. An aliquot was taken for protein determination, another was layered over a sucrose gradient (5–20%) and centrifuged at 250 000 × *g* for 36 h. The specific binding represents the integration of radioactivity under the 3 S peak. The results are the mean ± SD of 3 different determinations

When the celite system was used [^{3}H]cholesterol bound to heat-stable cytosol was displaced by unlabeled cholesterol (fig.1). Sucrose density gradient ultracentrifugation of the soluble complex formed during incubation of the heat stable cytosol with celite containing [^{3}H]cholesterol reveals that most of the radioactivity is bound to a macromolecule in the region of 3 S (fig.2). The specificity of the binding was inferred by the fact that a 100-fold excess of unlabeled cholesterol completely inhibits the radioactive peak in the 3 S region (fig.2).

Preincubation of heat stable cytosol with trypsin or pronase at 37°C for 30 min completely abolished the radioactive peak (fig.2). This result demonstrates that the macromolecule which binds [^{3}H]cholesterol is a protein.

It should be emphasized that the combined celite–sucrose gradient assay is more specific than celite alone, since a 100-fold excess of unlabeled cholesterol completely inhibits the binding in the former system but not in the latter (compare the displacement produced by 10^{-7} M in fig.1 with fig.2).

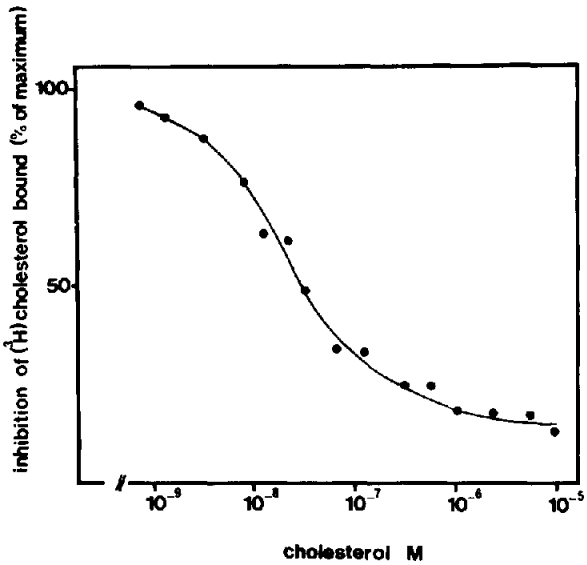

Fig.1. Displacement of [^{3}H]cholesterol bound to heat-stable sheep adrenal cytosol by increasing concentrations of unlabeled cholesterol. Heat stable adrenal cytosol, 1 ml (0.5 mg protein) was incubated at 4°C for 16 h with 50 mg celite containing [^{3}H]cholesterol and the indicated concentrations of cholesterol. At the end of the incubation the samples were centrifuged and 2 aliquots of each of triplicate incubations were taken for determination the radioactivity.

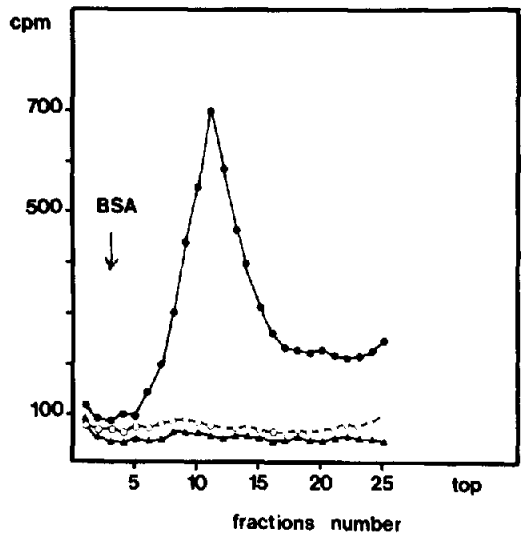

Fig.2. Sucrose density gradient ultracentrifugation profiles of adrenal heat-stable cytosol (0.4 mg protein/ml) (•, ▲), adrenal heat-stable cytosol treated with trypsin or pronase (○), preincubated with celite containing [^{3}H]cholesterol (1 pmol) alone (•, ○), or with 100-fold excess of unlabeled cholesterol (▲). At the end of incubation (14 h, 4°C) the samples were centrifuged and an aliquot of the supernatant was layered on 5–20% sucrose gradient and centrifuged at 250 000 × *g* for 38 h.

3.2. *Partial purification of adrenal cholesterol binding protein (CBP)*

Most of the cholesterol binding activity is recovered in the protein fraction precipitated between 30–60% saturation of ammonium sulfate (table 1).

The elution profile of the DEAE Sephadex A-50 column chromatography shows that the cholesterol-binding activity was associated with the fraction eluted with about 0.3 M NaCl (fig.3). About 200-fold purification was reached at this step (table 1).

3.3. *Specificity of binding*

Competition studies using the combined celite–sucrose gradient system were carried out in order to determine the degree of specificity of cholesterol binding to CBP.

The addition of a 100-fold excess of non-radioactive steroids without the side chain of cholesterol had no inhibitory effects (table 2). In addition, when CBP was incubated with [^{3}H]pregnenolone or [^{3}H]de-

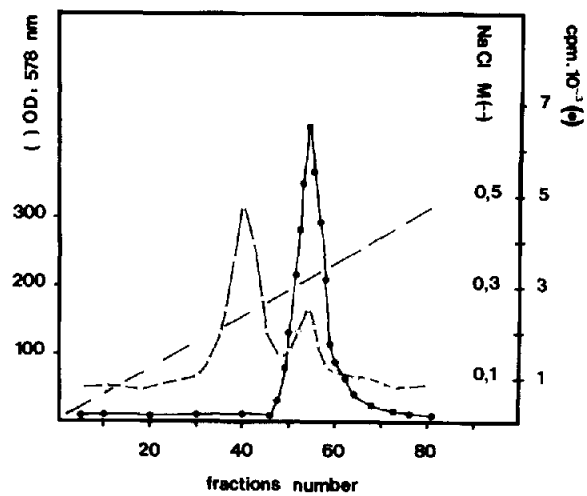

Fig.3. Elution profile of cholesterol-binding protein on DEAE Sephadex A-50 column. Proteins of adrenal heat stable cytosol precipitated by ammonium sulfate (30–60% saturation) were incubated with [^{3}H]cholesterol (10^{-9} M) at 4°C for 16 h and placed on 1.5 × 20 cm DEAE–Sephadex column. Elution was monitored at 280 nm (○) and aliquots from each fraction analyzed for radioactivity (•).

Table 2
Effects of unlabeled sterols and steroids on specific binding of [^{3}H]cholesterol to CBP prepared from sheep adrenal

Unlabeled compounds	Binding (% control)
None	100
Dehydroepiandrosterone	100
Progesterone	100
Pregnenolone	100
Testosterone.	100
Cortisol	100
17β-Oestradiol	100
25-Hydroxycholesterol	30
(20 S)20 hydroxycholesterol	21
(20 R, 22 S)20-22 dihydroxycholesterol	18
(20 R, 22 R)20-22 dihydroxycholesterol	9
7-Carboxy-methyl-oxime-cholesterol	0
4-Methyl-4-aza-5α-cholestane	0
Cholesterol sulfate	0
Cholesterol	0

CBP, 1 ml (0.7 mg protein) was incubated at 4°C for 16 h with 50 mg celite containing [^{3}H]cholesterol (1 pmol) alone, or with a 100-fold excess of unlabeled compounds. After centrifugation, aliquots of the supernatant were layered on a 5–20% sucrose gradient, and centrifuged at 250 000 × *g* for 38 h. The radioactivity in the 3 S region was integrated, and expressed as % control

hydroepiandrosterone no specific binding was observed in the 3 S region (data not shown).

On the other hand, cholesterol derivatives with modifications in the A and B rings such as 7-carboxy-methyloxime-cholesterol, 4 methyl-4aza-5α-cholestane and cholesterol sulfate have the same inhibitory potency as those observed with the cholesterol itself (table 2).

The inhibitory effect of cholesterol derivatives with hydroxylated groups on the side-chain is shown in table 2, fig.4 and fig.5. (20 R, 22 R)20-22 dihydroxy-

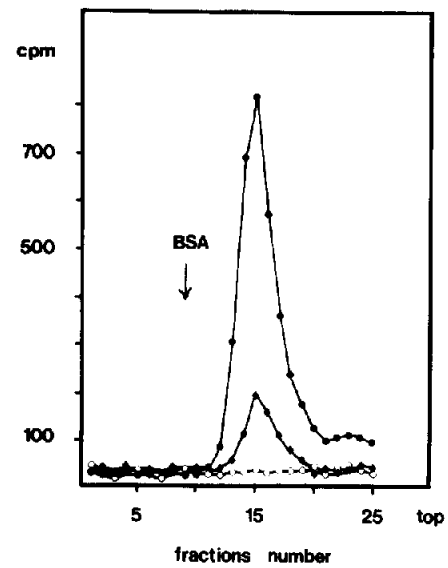

Fig.4. Inhibition of [^{3}H]cholesterol bound to CBP by several cholesterol derivatives. Partial purified CBP, 1 ml (0.58 mg protein) was incubated with 50 mg celite containing [^{3}H]-cholesterol (1 pmol) alone (•), or in the presence of 100 pmol (20 S)20-hydroxycholesterol (♦), and cholesterol (○). At the end of incubation (14 h, 4°C) the samples were centrifuged and an aliquot of the supernatant was layered on 5–20% sucrose gradient and centrifuged at 260 000 × *g* for 38 h.

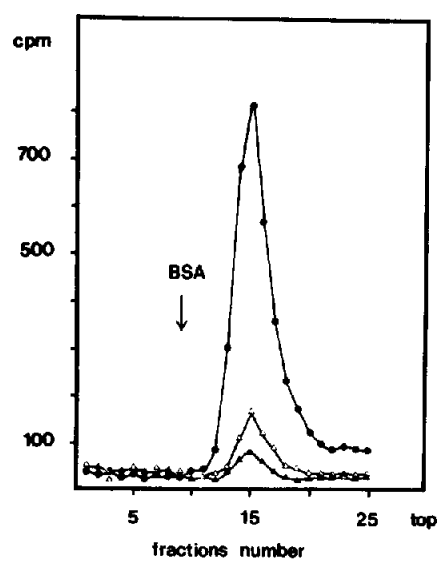

Fig.5. Inhibition of [^{3}H]cholesterol bound to CBP by several cholesterol derivatives. Partial purified CBP, 1 ml (0.58 mg protein) was incubated with 50 mg celite containing [^{3}H]cholesterol (1 pmol), alone (•), or in the presence of 100 pmol (20 R, 22 S)20,22-dihydroxycholesterol (△), and (20 R, 22 R)20,22-dihydroxycholesterol (▲). At the end of incubation (14 h, 4°C) the samples were centrifuged and an aliquot of the supernatant was layered on 5–20% sucrose gradient and centrifuged at 260 000 × *g* for 38 h.

cholesterol is a better competitor than its stereoisomer (20 R, 22 S)20-22 dihydroxycholesterol. The inhibitory effect of 25 hydroxycholesterol and (20 S) 20-hydroxy-cholesterol is lower than that of cholesterol hydroxylated in C_{20} and C_{22}, which is in turn lower that of cholesterol itself.

3.4. *CBP tissue distribution*

Heat-stable cytosol was prepared from several tissues and tested for their ability to bind cholesterol. Using the combined celite–sucrose density gradient system, cholesterol-binding activity was found in adrenals (human, rat, sheep), testis and ovaries (rats, sheep) (fig.6). On the other hand, no binding activity was found in the heat-stable cytosol prepared from liver, kidney, heart, brain and plasma of rats or sheep (fig.6).

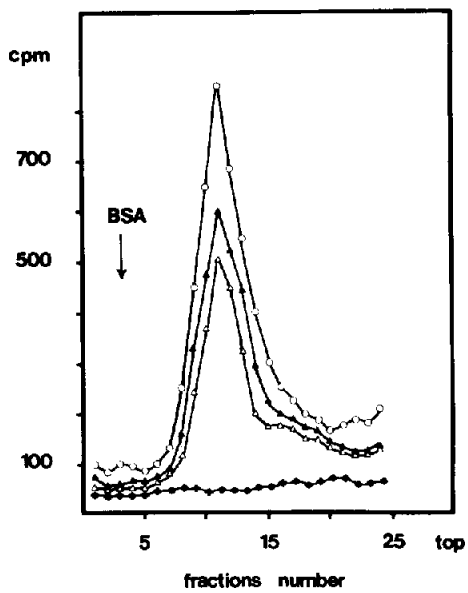

Fig.6. Sucrose density gradient ultracentrifugation profiles of heat stable cytosol (0.4 mg protein/ml) from rat adrenals (○), testis (▲), ovaries (△), and liver, heart, kidney, brain and plasma (♦), preincubated with celite containing [^{3}H]cholesterol (1 pmol). At the end of incubation (14 h, 4°C), the samples were centrifuged and an aliquot of the supernatant was layered on 5–20% sucrose gradient and centrifuged at 250 000 × *g* for 38 h.

4. Discussion

In this report, we have shown the presence in the cytosol of adrenal, testis, and ovaries of a heat-stable protein that specifically binds tritiated cholesterol. As determined by sucrose density gradient ultracentrifugation, the sedimentation coefficient of the complex formed is about 3 S. This cholesterol-binding protein (CBP) has been partially purified, about 200-fold, from the acetone powder by heat treatment, ammonium sulfate precipitation, and DEAE Sephadex A-50 chromatography.

Quantitation of binding affinity was rendered difficult by the affinity of cholesterol for glass and plastic and its tendency to self-association with a critical micelle concentration of about 35 nM at 25°C [9]. For these reasons, and because in the celite system there are at least two binding molecules (celite, CBP), competing for the same ligand, mathematical analyses of competitive displacement curves (fig.1) or saturation curves (data not shown) was not possible. It must be noted that similar difficulties have been found for other sterols [10].

None of the steroids without side-chain that were tested were able to displace bound radioactive cholesterol to CBP. On the other hand, sterols with modification in the rings A or B inhibited the binding of tritiated cholesterol as well as unlabeled cholesterol itself, suggesting that the side-chain is essential for binding. In this respect it is interesting to note that (20 R, 22 R)20,22 dihydroxycholesterol, which is the natural intermediate in the enzymatic conversion of cholesterol to pregnenolone [11], is a better competitor than its stereoisomer, (20 R, 22 S)-20,22 dihydroxycholesterol, which has no metabolic role.

A heat-stable sterol carrier protein which binds cholesterol has been isolated from the soluble fraction of liver homogenates [12–14]. This heat-stable factor stimulates the synthesis of cholesterol by liver microsomal enzymes [12,14] and the cholesterol side-chain cleavage of an acetone powder of adrenal mitochondria [15]. A similar heat stable sterol carrier protein in adrenal mitochondria is reported [16,17]. As indicated, using a different method we were able to demonstrate a cholesterol-binding activity in the heat-stable cytosol prepared from adrenal, ovary and testis, but not from liver, kidney, brain and heart. Therefore, despite the fact that some of the properties

of liver and adrenal heat-stable factors [12,14,16] are close to those of CBP, some are different, i.e., CBP has mol. wt ~ 70 000 (in preparation) while that of liver sterol-carrier protein is ~ 16 000 [10]. The specificity of CBP binding is localized mainly to the side chain of cholesterol, while that of liver sterol carrier protein seems to be less specific [10].

The role of CBP on hormone induced steroidogenesis of both adrenals and gonads is unknown. However, the binding stereospecificity and the tissue distribution suggest that CBP might play an important role. However purification to homogeneity of CBP in view to set up a specific and sensitive method of measurement is the next necessary step for studying its precise role in vivo.

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