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STUDIES OF CHOLESTEROL BINDING IN THE SOLUBLE FRACTION OF THE ADRENAL CORTEX OF THE GUINEA PIG

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SUMMARY

A cholesterol-binding component has been studied in the supernatant of guinea pig adrenocortical cytosol after heating for two minutes in a boiling water bath. Enzymatic degradation revealed that the binding component was a protein. On sucrose density gradient analysis the cholesterol-binding protein had an S value of approximately 2, and a similar binding component was not present in unheated cytosol. Cholesterol binding to heated adrenal cytosol was unaffected by sulfhydryl reactants. The equilibrium association constant at 0°C was approximately $10^4\ M^{-1}$ and the concentration of binding sites was 300×10^{-9} mol per mg protein. Heated adrenal cytosol was found to bind not only cholesterol but also cholesterol sulfate and 20α-hydroxycholesterol when examined by sucrose density gradient analysis. When examined by polyacrylamide gel electrophoresis, it appeared that cholesterol and cholesterol sulfate were bound to the same protein band. On the other hand binding of 20α-hydroxycholesterol was not detected with the multiphasic buffer system that was used. Heated adrenal cytosol did not bind cholesterol oleate, pregnenolone, and pregnenolone sulfate; nor did it bind cyclic AMP, phosphatidyl choline, NADP, acetyl coenzyme A, or prostaglandin E_2. Cholesterol binding in heated cytosol was not confined to the adrenal; similar binding was found in liver, kidney, lung, testis, muscle, gut, and brain.

INTRODUCTION

It is generally accepted that the regulation of adrenal steroidogenesis occurs between cholesterol and pregnenolone[1–3]. It is also generally accepted that this regulatory process involves the *de novo* synthesis of protein[3]. A regulatory protein with a rapid turnover time has been proposed[3, 4]. This latter consideration does not, of course, rule out an important role for more stable proteins. Until recently no specific protein had been identified or characterized. We have, therefore, attempted to examine specific protein–steroid interactions that may be involved in the regulation of steroid synthesis. This report deals with studies based on cholesterol binding in the soluble fraction of the guinea pig adrenal cortex. Since the regulation of steroid synthesis is believed to involve the reaction cholesterol → pregnenolone, it seemed reasonable to examine the interaction of protein and substrate for the reaction (cholesterol) as well as the product of the reaction (pregnenolone). A previous report from this laboratory has described certain properties of a specific pregnenolone-binding protein in the soluble fraction of the guinea pig adrenal cortex[5]. The studies reported here were also prompted by previous reports that the rat and cow adrenal cortex contained a similarly derived, heat-stable, cholesterol-binding protein which was capable of stimulating the conversion of cholesterol to pregnenolone in a cell-free system[6, 7].

EXPERIMENTAL

Radiolabeled compounds. [1,2-^{3}H]-cholesterol (60 Ci/mmol), [1,2-^{3}H]-cholesterol sulfate[1], ammonium salt (48.3 Ci/mmol), [4-^{14}C]-cholesterol oleate (56.1 mCi/mmol), [7-^{3}H]-20α-hydroxycholesterol (25 Ci/mmol), [7-^{3}H]-pregnenolone (15.8 Ci/mmol) and [7-^{3}H]-pregnenolone sulfate, ammoium salt (25 Ci/mmol) were purchased from New England Nuclear and their purity as judged by thin-layer chromatography was greater than 97%. Other radiolabeled compounds used were [^{3}H]-acetyl Coenzyme A (0.74 Ci/mmol), [5, 6, 8, 9, 11, 12, 14, 15 - ^{3}H]-arachidonic acid (72 Ci/mmol), [^{3}H]-adenosine-3′,5′-monophosphate ammonium salt (cAMP) (37.3 Ci/mmol), [1-^{14}C]-oleic acid (55.5 mCi/mmol), [choline-methyl-^{14}C]-phosphatidyl choline dipalmitoyl lecithin (57 mCi/mmol), [5, 6, 8, 11, 12, 14, 15-^{3}H]-prostaglandin E_2 (210 Ci/mmol), and [carbonyl-^{14}C]-nicotinamide adenine dinucleotide phosphate (NADP) (41 mCi/mmol). The latter compounds were used as obtained from New England Nuclear and their purity was not further evaluated. Ovalbumin (Worthington, 2× crystallized and lyophilized) and bovine γ-globulin (Nutritional Bio-

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1. Trivial steroid names used are: pregnenolone sulfate, 20-oxo-5-pregnen-3β-yl sulfate; cholesterol sulfate, 5-cholesten-3β-yl sulfate.

chemicals, fraction II, crystallized and lyophilized) were labeled with [^{14}C]-methyl groups attached to amino groups by reductive alkylation[8]. Labeled substrate used in this reaction was [^{14}C]-formaldehyde (10 mCi/mmol) purchased from New England Nuclear. Specific activities of $1–2 \times 10^6$ c.p.m./mg protein were obtained.

Crystalline steroids. Steroids were purchased from Research Plus Steroid Laboratories, Steraloids, and Schwarz-Mann. Their purity as evaluated by mass spectroscopy was determined to be greater than 98%.

Enzymes and chemicals. Phospholipase-A, trypsin, trypsin inhibitor, pronase, RNase, and DNase were purchased from Sigma. All chemicals were reagent grade.

Solutions. The following buffer systems were used: 0.25 M sucrose, 50 mM phosphate, 3 mM $MgCl_2$, pH 7.4 (buffer A); 10 mM Tris, 1.5 mM Na_2EDTA, pH 7.4 (buffer B); 0.1 M citric acid and 0.2 M dibasic sodium phosphate were mixed in appropriate proportions to produce solutions with a pH of 4.0, 5.0, 6.0, and 7.0 (buffer C); 0.2 M Tris and 0.2 M HCl were mixed in appropriate proportions to produce solutions with a pH of 8.0 and 9.0 (buffer D). All labeled and nonlabeled steroid stock solutions were made up in either absolute methanol or ethanol and stored at −15°C.

Glassware. All glassware used in steps following the preparation of cytosol was acid-washed, rinsed exhaustively with water, and siliconized with Dri-film SC-87 (Pierce Chemical Co.).

Preparation of the cytoplasmic supernatant fraction. Male guinea pigs (NIH strain) weighing between 600 and 800 g were used within 24 h after being received in the laboratory. The animals were killed by decapitation. The adrenal glands were immediately removed and placed in buffer A on ice. The extraneous fat and fibrous material were removed and the tissue weighed. The tissue was minced with a razor blade and placed in buffer B on ice (1 g/5 ml) and homogenized with a polytron Pt-10 homogenizer in a 4°C cold room. The homogenization was performed and the 235,000 g cytoplasmic supernatant fraction (cytosol) prepared as previously described[5]. Since the adrenal gland of a mature guinea pig (mean weight 700 g) has a cortex to medulla ratio of approximately 91:1[9], the contribution of medullary cytosol to the total adrenal cytosol preparation was ignored. When tissue other than the adrenals was used, it was processed in a similar fashion.

Formation of the cholesterol–macromolecular complex. Tubes containing freshly prepared cytosol were placed in a boiling water bath and left for two min following the first appearance of a coagulum. Following this the preparation was centrifuged for 30 min at 235,000 g and the clear supernatant (referred to hereafter as the heated cytosol) decanted for use in the cholesterol-binding studies, usually without further dilution. Protein concentrations in unheated cytosol and in the supernatant of cytosol after heat treatment ranged from 8–12 mg/ml and 0.8–1.2 mg/ml respectively. [^{3}H]-cholesterol was added to an aliquot of the heated cytosol (final methanol content was less than 1% v/v). Samples were vortexed gently and incubated at 0°C for 60 min. A time study demonstrated that equilibrium was achieved by 10 min and that binding remained stable for at least 16 h. When binding of other compounds was examined, samples were processed in a similar fashion. For steroid competition studies a thousand-fold excess of the nonlabeled steroid was added with labeled cholesterol.

Binding assay. Separation of bound from free cholesterol was achieved by gel filtration on sephadex G-25 columns at 4°C. Columns were prepared by packing 5 ml siliconized glass pipettes to the 1 ml mark with gel swollen in buffer B and degassed (column bed dimension = 14 × 0.6 cm). A 0.1 ml sample volume was applied and the columns were eluted with buffer B. Six drop fractions were collected directly into counting vials with the bound cholesterol emerging in the void volume.

Sucrose density gradient analysis. Samples (200 μl) were layered on 4.8 ml linear gradients of 5–20% sucrose. The sucrose was dissolved in buffer B for routine studies and in either buffer C or D for pH studies. [^{14}C]-labeled ovalbumin and bovine γ-globulin were used as markers. The gradients were centrifuged for 16 h at 40,000 rev/min in a Beckman SW 50.1 rotor at 2°C. Following centrifugation, the polyallomer tubes were placed in a piercing unit and fractions collected directly into counting vials. Ten ml of scintillation solution containing 10% Biosolve (Beckman) were added and the radioactivity determined.

Polyacrylamide gel electrophoresis. The conditions for polyacrylamide gel electrophoresis have been previously described[10]. Photopolymerization and electrophoresis were performed at 0°C. The multiphasic buffer system used consisted of bistris (bis-[2-hydroxyethyl] iminotris [hydroxymethyl] methane), acetate, chloride, and TES (*N*-Tris [hydroxymethyl] methyl-2-aminoethanesulfonate) operative at pH 7.38 (0°C). Stacking gels were made 3.125% total gel concentration (%T) with 20% crosslinking with methylene-bisacrylamide (%C). The separation gels were 16% T with 2% C. Bromphenol blue was used as the tracking dye. Following electrophoresis gels were either sectioned transversely for the determination of radioactivity or stained with Coomassie brilliant blue. For the determination of radioactivity, gels were sliced into 1 ± 0.1 mm sections which were placed into 10 ml of scintillation fluid (LSC-complete, Yorktown Research Co.). For staining, gels were prefixed in 12.5% trichloroacetic acid (40 ml/gel) for 30 min. One ml of a 0.25% aqueous solution of Coomassie brilliant

blue was added and the gels left overnight. Stained gels were stored in 5% acetic acid in the dark.

Protein determination. Trichloroacetic acid-precipitable material was assayed according to the method of Lowry *et al.*[11] using bovine serum albumin (BSA) as the standard.

Enzymatic release studies. Aliquots of heated cytosol (1 ml) were equilibrated with [^{3}H]-cholesterol and incubated for 60 min at 0°C with: (a) no addition, (b) 1 mg pronase or trypsin, (c) 1 mg of trypsin and trypsin inhibitor, (d) 1 mg RNase, and (e) 1 mg DNase plus 5 μl of 1 M $MgSO_4$. Studies with phospholipase A were performed according to a modification of the method of Fleischer and Fleischer[12]. Tris buffer (pH 7.4) was used in place of glycylglycine. Aliquots of heated cytosol (1 ml) were equilibrated with [^{3}H]-cholesterol after the addition of 0.4 M $CaCl_2$, 10 mg/ml BSA and finally 100 μl of the enzyme and incubations carried out at 37°C for 30 min.

RESULTS

Cholesterol-binding components in different tissues

Heated and unheated cytosol preparations from the adrenal, brain, kidney, liver, muscle, and gut were equilibrated with [^{3}H]-cholesterol and analyzed by sucrose density gradient centrifugation. As shown in Fig. 1, the binding patterns in unheated cytosol from the various tissues were similar, i.e., there was a major peak of radioactivity near the bottom of the gradient and a minor peak migrating in the region of the γ-globulin marker or between the two marker proteins (fractions 15–21) with little or no binding activity in the region from the ovalbumin marker to the top of the gradient. The *S* value of the minor peak was approximately 7–8[13]. The binding patterns in the heated cytosol from the various tissues were substantially different. For the heated cytosol of adrenal, kidney, and liver there was a major peak of radioactivity near the top of the gradient (fractions 22–27) corresponding to an *S* value of approximately 2. A minor peak again occurred in the region of the γ-globulin marker (fractions 15–21) which was of approximately the same location and magnitude as the minor peak seen in the unheated cytosols. For the heated cytosol of brain, muscle, and gut, the major radioactive peak migrated essentially between the two marker proteins (fractions 15–21). For brain and muscle there was no binding in the region from the ovalbumin marker to the top of the gradient (fractions 22–27) as occurred with adrenal, liver the kidney. For the gut there was a minor shoulder of radioactivity in

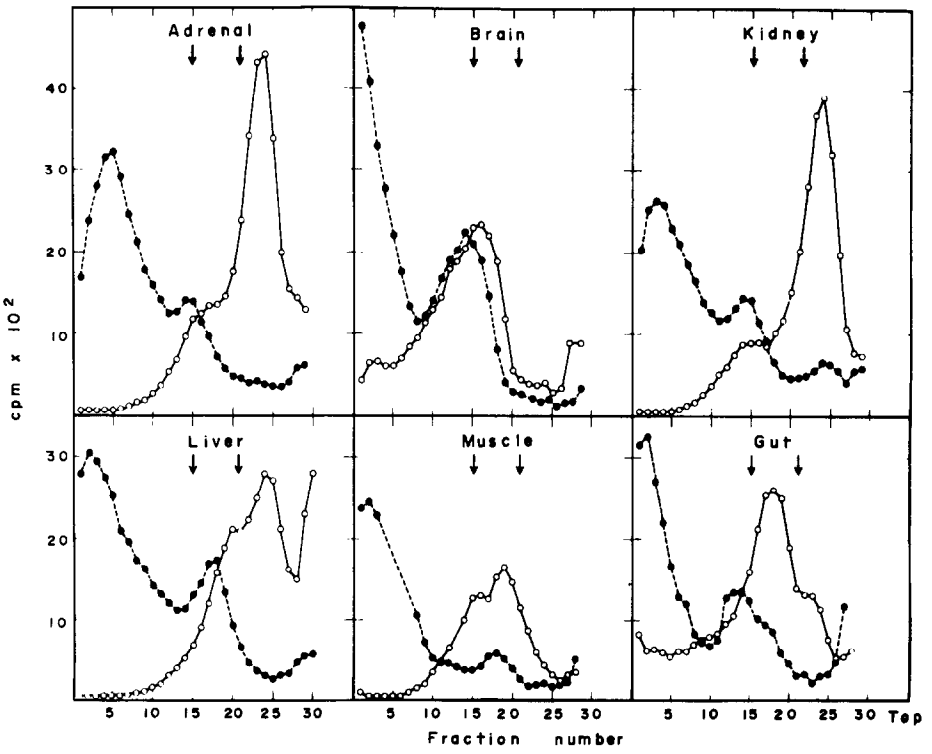

Fig. 1. Sucrose density gradient patterns of heated (○—○) and unheated (●----●) cytosol incubated for 60 min at 0°C with 3×10^{-9} M [^{3}H]-cholesterol. Samples [200 μl] were layered on 5–20% sucrose gradients (4.8 ml) and centrifuged at 149,000 *g* for 15–16 h in a SW 50.1 rotor at 2°C. Cytosols were prepared from guinea pig adrenal, brain, kidney, liver, muscle, and gut. The arrows indicate the peak positions for the [^{14}C]-ovalbumin and γ-globulin marker proteins. The tops of the gradients are to the right.

the region from the ovalbumin marker to the top of the gradient (fractions 22–27). The heated cytosols, from all tissues, in contrast to the unheated cytosols, did not demonstrate binding in the region towards the bottom of the gradient. When serum was heat-treated in a similar fashion and equilibrated with [^{3}H]-cholesterol a single radioactive peak was demonstrated near the top of the gradient (fractions 22–27) (data not shown). The serum peak was, on the average, 1/10 the magnitude of the adrenal peak.

Effect of an SH-reacting reagent on the heated adrenal cytosol cholesterol-binding component

The addition of *p*-hydroxymercuribenzoate to heated adrenal cytosol did not alter [^{3}H]-cholesterol binding (data not shown).

Enzymatic digestion of the cholesterol-binding component in heated cytosol

Treatment of heated adrenal cytosol with pronase or trypsin prior to equilibration with [^{3}H]-cholesterol destroyed binding activity while binding was preserved when trypsin inhibitor was added along with the trypsin (data not shown). RNase and DNase had no effect on the adrenal cholesterol-binding component. The results of the studies with phospholipase A were somewhat difficult to interpret but it was finally concluded that the cholesterol-binding activity was not destroyed by treatment with this enzyme. As controls for the phospholipase studies, the effect of either 0.4 M $CaCl_2$ or BSA on cholesterol-binding was examined. Neither 0.4 M $CaCl_2$ nor 0.4 M $MgCl_2$ had an appreciable effect on cholesterol binding (data not shown). Although BSA appeared to bind cholesterol, when BSA and heated cytosol were mixed, essentially all the cholesterol was bound to the heated cytosol with very little albumin binding (data not shown). Initially when the effect of phospholipase A on cholesterol binding was examined it appeared that the cholesterol binding activity was destroyed by the enzyme treatment. However, when these studies were repeated and the method modified so that after incubation with the enzyme, samples were reheated in a boiling water bath for 2 min and then dialyzed for 3–4 h at 4°C, cholesterol binding remained intact (data not shown).

Effect of phospholipid and cholesterol on [^{3}H]-cholesterol binding in heated adrenal cytosol

The effect of the phospholipids dimyristoyl lecithin, disteroyl lecithin, and dipalmitoyl lecithin (46 μM) in the presence and absence of cholesterol (3 μM) on [^{3}H]-cholesterol (3 nM) binding to heated adrenal cytosol was examined. The phospholipids were microdispersed in Tris-EDTA buffer by ultrasonic irradiation[12]. Ten μl of the microdispersed phospholipid solution were added to one ml of heated cytosol and nitrogen gas bubbled through the sample. The tubes were capped, incubated at 37°C for 60 min, and then put on ice. [^{3}H]-cholesterol and, in some experiments, nonlabeled cholesterol were added to the phospholipid, heated cytosol samples and incubated at 0°C for 60 min. Cholesterol binding was analyzed by sucrose density gradient centrifugation (Fig. 2). The lecithins alone caused a variable degree of aggregation which was markedly enhanced in the presence of nonlabeled cholesterol. The aggregates tended to migrate between fractions 10–20 with an associated decrease in binding activity in the region of fractions 22–27. At no time did we see any binding activity towards the bottom of the gradients as was seen when unheated cytosol was used (Fig. 1).

The effect of nonlabeled cholesterol (3 μM) alone on [^{3}H]-cholesterol (3 nM) binding is shown in Fig. 3. Again, as shown in Fig. 1, [^{3}H]-cholesterol in the absence of nonlabeled cholesterol was bound in a single major peak between fractions 22–27 with a slight shoulder of radioactivity in the region of fractions 15–21. In the presence of 3 μM

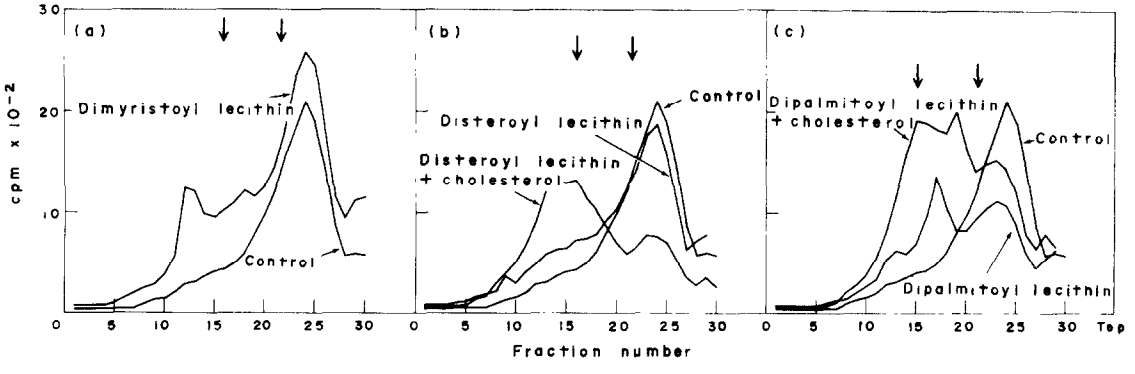

Fig. 2. Sucrose density gradient patterns of heated adrenal cytosol incubated for 60 min at 0°C with 3×10^{-9} M [^{3}H]-cholesterol in the presence of absence of 46 μM dimyristoyl lecithin (Panel A), 46 μM disteroyl lecithin with and without 3 μM cholesterol (Panel B), 46 μM dipalmitoyl lecithin with and without 3 μM cholesterol (Panel C). Samples (200 μl) were layered on 5–20% sucrose gradients (4.8 ml) and centrifuged at 149,000 *g* for 15–16 h in a SW 50.1 rotor at 2°C. The arrows indicate the peak positions for the [^{14}C]-ovalbumin and γ-globulin marker proteins.

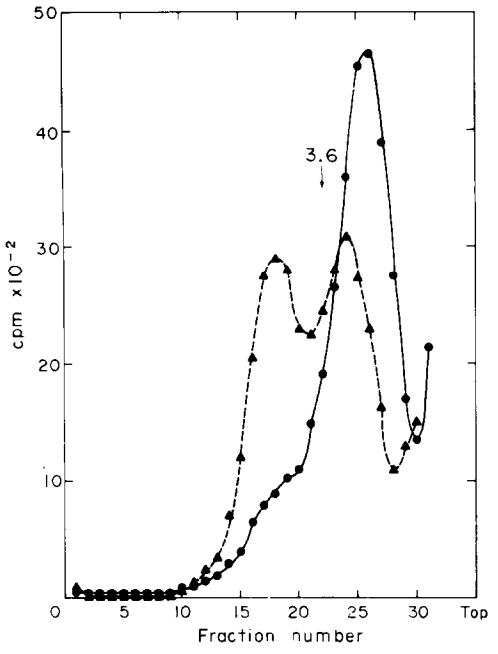

Fig. 3. Sucrose density gradient patterns of heated adrenal cytosol incubated with 3×10^{-9} M [^{3}H]-cholesterol for 60 min at 0°C in the presence (▲---▲) or absence (●—●) of 3×10^{-6} M cholesterol. Samples (200 μl) were layered on 5–20% sucrose gradients (4.8 ml) and centrifuged at 149,000 *g* for 15–16 h in a SW 50.1 rotor at 2°C. The peak position of the [^{14}C]-ovalbumin marker protein is indicated by the arrow (3.6*S*).

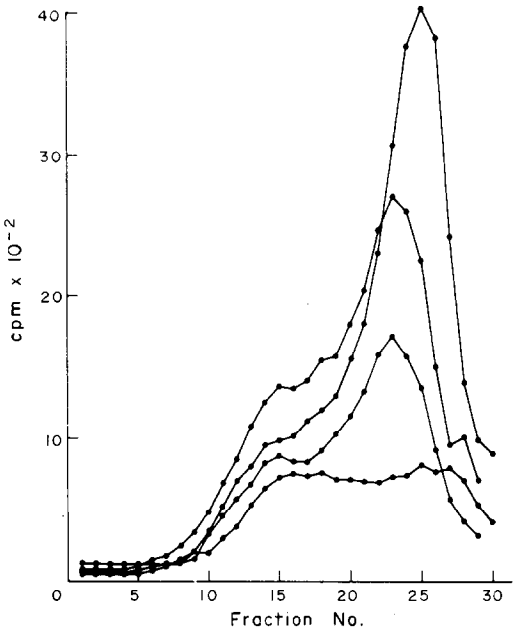

Fig. 4. Sucrose density gradient patterns of heated adrenal cytosol incubated with 3×10^{-9} M [^{3}H]-cholesterol for 60 min at 0°C. The protein concentrations were 1.0, 0.5, 0.25, and 0.125 mg/ml. Samples (200 μl) were layered on 5–20% sucrose gradients (4.8 ml) and centrifuged at 149,000 *g* for 15–16 h in a SW 50.1 rotor at 2°C.

nonlabeled cholesterol a second major peak of radioactivity appeared in the region between fractions 15–21, the region associated with the shoulder of radioactivity which occurred in the absence of added cholesterol. The appearance of this second peak was associated with a decrease in binding in the region of the gradient between fractions 22–27.

Binding affinity of the cholesterol binding component in heated adrenal cytosol

Because 3 μM nonlabeled cholesterol caused little, if any, reduction in 3 nM [^{3}H]-cholesterol binding, it was apparent that the cholesterol-binding component had a large capacity for binding cholesterol and that the binding affinity would be quite low. We first attempted to dilute out the binding capacity and obtained the results shown in Fig. 4. As the total protein concentration was decreased from 1.0 mg/ml to 0.06 mg/ml, there was a progressive decrease in [^{3}H]-cholesterol binding as determined by sucrose density gradient analysis. Saturation studies were performed using undiluted and diluted heated adrenal cytosol. The results of two such studies are shown in Fig. 5. These studies were performed with undiluted heated adrenal cytosol [1.0 mg/ml] and [^{3}H]-cholesterol covering the concentration range of either 0.863–17.260 μM (Fig. 5A) or 8.63–172.60 μM (Fig. 5B). Over the lower of the two concentration ranges saturation did not occur (Fig. 5A$_1$) and the Scatchard analysis gave a flat curve (Fig. 5A$_2$). At the higher concentration range, however, saturation began to occur (Fig. 5B$_1$) and the Scatchard analysis gave a negative slope (Fig. 5B$_2$). The apparent equilibrium association constant [K_A] at 0°C was 1.75×10^4 M^{-1}. The concentration of cholesterol-binding sites as estimated from the Scatchard analysis was 300×10^{-9} mol per mg protein. In another experiment when the protein concentration was reduced to 0.2 mg/ml the Scatchard analysis gave an apparent K_A of 5×10^4 M^{-1} at 0°C and a cholesterol binding site concentration per mg protein which was essentially the same as obtained using undiluted heated adrenal cytosol. As a control for these binding studies buffer B only [no cytosol] was equilibrated with [^{3}H]-cholesterol covering the concentration range 8.62–172.60 μM at 0°C for 60 min and analyzed in the same fashion as reported here for the cytosol preparation. When this was done it was found that less than 0.1% of the applied cholesterol emerged in the void volume. We thus concluded that with this technique micelles were not being excluded from the columns. This indicated that the radioactivity emerging in the void volume when heated adrenal cytosol was used was probably protein bound and not bound by cholesterol micelles.

Effect of hydrogen ion concentration on [^{3}H]-cholesterol binding in heated adrenal cytosol

These studies were performed at six hydrogen ion concentrations giving the results shown in Fig.

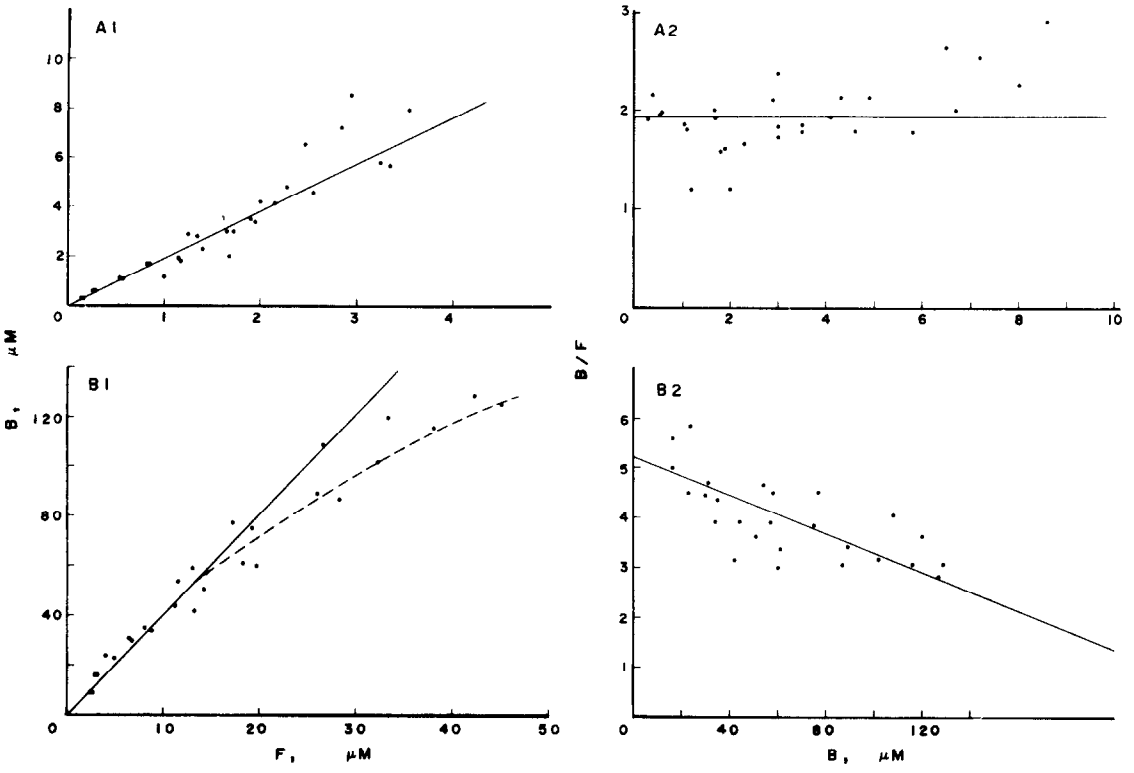

Fig. 5. Saturation studies of heated adrenal cytosol incubated with [^{3}H]-cholesterol for 60 min at 0°C. Samples (0.1 ml) were applied to G-25 sephadex columns (0.6 × 14 cm) and eluted with buffer B at 4°C. The protein-bound [^{3}H]-cholesterol appeared in the void volume. Fractions (0.2 ml) were collected directly into counting vials and the radioactivity determined. A = [^{3}H]-cholesterol (S.A. 52 mCi/mmol); B = [^{3}H]-cholesterol (S.A. 5.2 mCi/mmol). A_1 and B_1 relate the bound (B) and free (F) concentrations of [^{3}H]-cholesterol. A_2 and B_2 are Scatchard analyses.

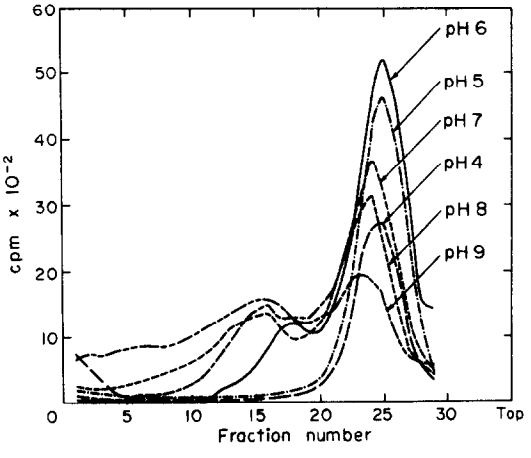

Fig. 6. Sucrose density gradient patterns of heated adrenal cytosol incubated with 3×10^{-9} M [^{3}H]-cholesterol at pH 4, 5, 6, 7, 8, and 9 for 60 min at 0°C. Adrenal glands were homogenized in either buffer C at pH 4, 5, 6, and 7 or buffer D at pH 8 and 9. The gradients were prepared with the same buffer systems at the appropriate pH. Samples (200 μl) were layered on 5–20% sucrose gradients (4.8 ml) and centrifuged at 149,000 g for 15–16 h in a SW 50.1 rotor at 2°C.

6. At all pH values there was a major peak of radioactivity migrating in the sucrose density gradient between fractions 22–27. Binding progressively increased from pH 4 to 6, then decreased from pH 6 to 9. Another feature of these studies was that from pH 6–9 in addition to the major peak of radioactivity between fractions 22–27, a shoulder of radioactivity occurred between fractions 15–21. Whereas, at pH 4 and 5, there was but a single peak of radioactivity between fractions 22–27 with no demonstrable binding below fraction 21.

Binding specificity for heated adrenal cytosol

We examined a variety of compounds, including steroids, fatty acids, cholesterol esters, phospholipid, and adenine nucleotides, for their ability to bind to heated adrenal cytosol using sucrose density gradient analysis. When [^{3}H]-cholesterol sulfate was tested a peak of radioactivity was found with an S value of approximately 2, essentially the same as for cholesterol (see below). When [^{3}H]-pregnenolone sulfate and [^{3}H]-pregnenolone were tested, no radioactive peaks were observed (data not shown). Although there was binding of oleic acid to heated adrenal cytosol, cholesterol oleate did not bind (data not shown). Like oleic acid, arachidonic acid also bound to heated adrenal cytosol, whereas prostaglandin E_2 did not bind (data not shown). The fatty acid radioactive peaks migrated more rapidly ($S = 4$) than the cholesterol peak ($S = 2$). In addition,

when [^{3}H]-acetyl coenzyme A, [^{3}H]-cyclic AMP, [^{14}C]-phosphatidyl choline, and [^{14}C]-NADP were tested, no demonstrable binding was found (data not shown). The cholesterol metabolite, 20α-hydroxycholesterol, was tested and was found to bind to heated adrenal cytosol (see below).

Studies comparing binding of cholesterol, cholesterol-sulfate, and 20α-hydroxycholesterol to heated adrenal cytosol using sucrose density gradient analysis and polyacrylamide gel electrophoresis

On sucrose density gradient analysis, the major radioactive peak for all three compounds had an *S* value of approximately 2 (Fig. 7). When a 1000-fold molar excess of the respective nonlabeled steroid was equilibrated with trace, only a slight decrease in the major binding peak (fractions 22–27) occurred (Fig. 7). As shown in Fig. 3, the excess nonlabeled cholesterol caused the appearance of a second major peak between fractions 15–21 (Fig. 7A); this latter phenomenon was much less with cholesterol sulfate (Fig. 7B) and did not occur with 20α-hydroxycholesterol (Fig. 7C).

Binding activity of these three steroids was next examined using polyacrylamide gel electrophoresis. In these studies, parallel gels were analyzed for steroid binding activity and protein banding. [^{3}H]-cholesterol bound to a protein with an R_F of 0.42 (Fig. 8) and [^{3}H]-cholesterol sulfate bound to a protein with an R_F of 0.42 (Fig. 9). Though the radioactive peaks for cholesterol and cholesterol sulfate migrated with the same protein band, cholesterol sulfate was always bound to a greater extent than cholesterol in these studies (40% vs 4% respectively).

Notwithstanding the fact that [^{3}H]-20α-hydroxycholesterol was bound to heated adrenal cytosol when examined by sucrose density gradient centrifugation (Fig. 7C), there was no binding of this steroid when polyacrylamide gel electrophoresis was used to analyze binding.

DISCUSSION

The data presented in this report indicate that the soluble fraction of the guinea pig adrenal contains a heat-stable protein factor which is capable of binding cholesterol. Cholesterol-binding activity, however, was not confined to the adrenal. All tissues tested, *viz.*, adrenal, kidney, liver, gut, muscle, brain, lung, and testis demonstrated cholesterol binding by a heat stable factor. On the basis of sucrose density gradient analysis, the cholesterol-binding activities of unheated as well as heated cytosols from the guinea pig adrenal, kidney, and liver were remarkably similar (Fig. 1). A similarly derived, heat-stable protein capable of binding cholesterol has been previously reported for the rat liver [19] and cow adrenal cortex [7]. In addition, cholesterol-binding studies using unheated cytosol from rat liver and brain have also been reported[15]. The distinction between cholesterol-binding in unheated vs heated cytosol is unclear. The cholesterol binding affinity for rat liver and brain unheated cytosol has been reported to be approximately $10^6\,M^{-1}$ at 37°C with the number of binding sites being $6–10 \times 19^{-9}$ mol/mg protein[15]. We found for guinea pig adrenal heated cytosol a binding affinity of approximately $10^4\,M^{-1}$ at 0°C with the concentration of binding sites being 300×10^{-9} mol/mg protein. In our experience, cholesterol-saturation studies using heated adrenal cytosol were difficult to perform by both gel exclusion and adsorption techniques; the difficulty was related, in part, to the limited solubility of cholesterol in aqueous solution (4,7 μM)[16], to the apparent high capacity for cholesterol binding, and to cholesterol interaction with various matrices. The binding affinity repor-

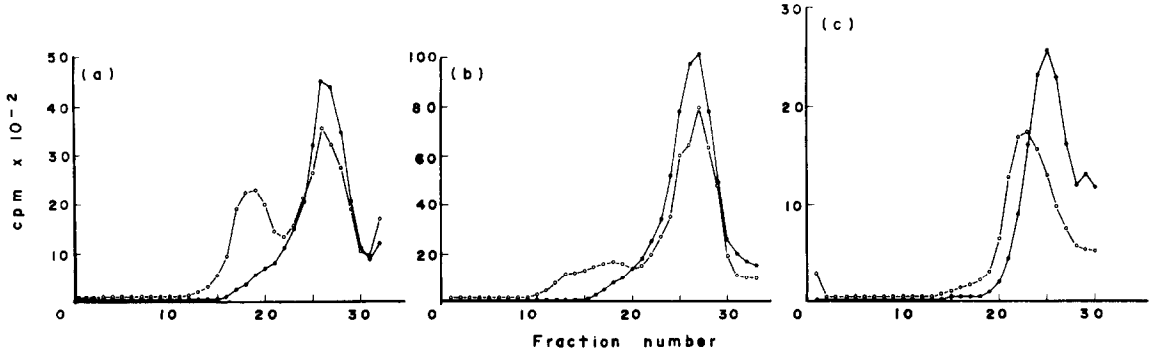

Fig. 7. Sucrose density gradient patterns of the same heated adrenal cytosol preparation incubated for 60 min at 0°C with [^{3}H]-cholesterol (panel a), [^{3}H]-cholesterol sulfate (panel b), and [^{3}H]-20α-hydroxycholesterol (panel c). The concentrations for each compound were: cholesterol, 2.5×10^{-9} M (●——●) and 2.5×10^{-6} M (○——○); cholesterol sulfate, 4×10^{-9} M (●——●) and 4×10^{-6} M (○——○); 20α-hydroxycholesterol, 5×10^{-9} M (●——●) and 5×10^{-6} M (○——○). Samples (200 μl) were layered on 5–20% sucrose gradients (4.8 ml) and centrifuged at 149,000 *g* for 15–16 h in a SW 50.1 rotor at 2°C.

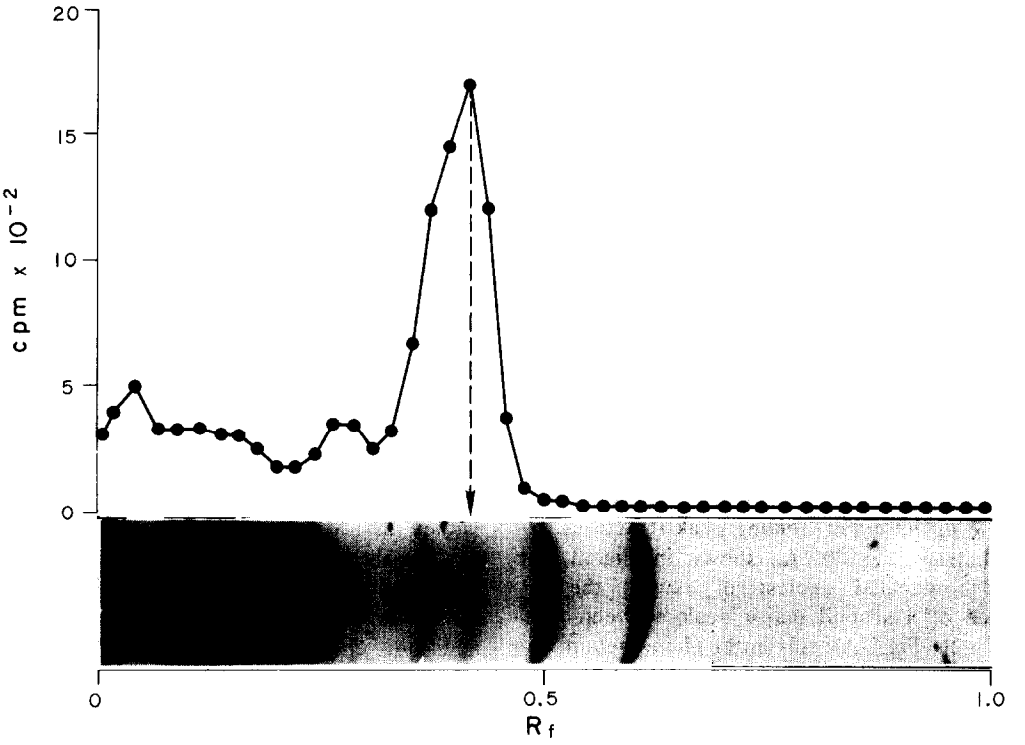

Fig. 8. Polyacrylamide gel electrophoresis of heated adrenal cytosol after incubation for 60 min at 0°C with and without 5×10^{-9} M [^{3}H]-cholesterol. Details of this procedure are described in the methods section. Parallel gels were layered with the same heated cytosol preparation: one set of gels was sliced and the radioactivity determined in each section; one set of gels was stained with Coomassie brilliant blue. Bromphenol blue was the tracking dye and determined the gel fronts. The pattern of radioactivity was aligned with the stained protein bands according to the dye fronts.

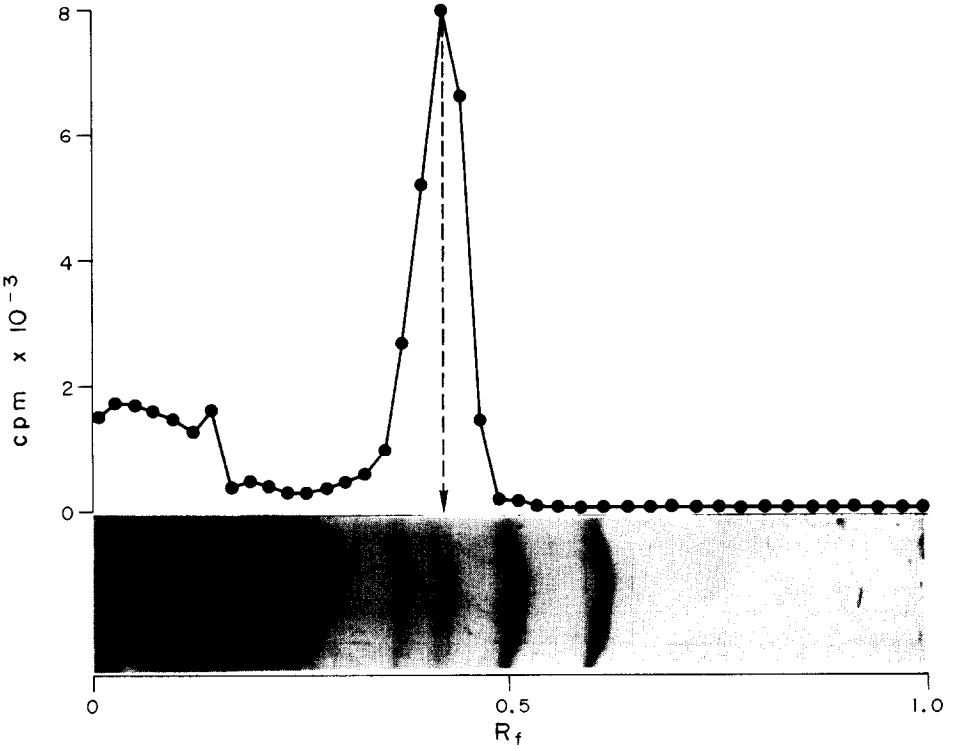

Fig. 9. Polyacrylamide gel electrophoresis of heated adrenal cytosol after incubation for 60 min at 0°C with and without 6×10^{-9} M [^{3}H]-cholesterol sulfate. Details of this procedure are described in the methods section. Parallel gels were layered with the same heated cytosol preparation: one set of gels was sliced and the radioactivity determined in each section; one set of gels was stained with Coomassie brilliant blue. Bromphenol blue was the tracking dye and determined the gel fronts. The pattern of radioactivity was aligned with the stained protein bands according to the dye fronts.

ted here of 10^4 at 0°C represents, at best, an approximation.

The fact that the apparent binding affinities are different for unheated and heated cytosol is not surprising since the binding patterns on density gradient analysis are also quite different (Fig. 1). What happens as a result of the heat treatment to convert the unheated pattern (as shown in Fig. 1) to the pattern obtained after heat treatment is not known. We were not able to reverse the binding pattern starting with heated adrenal cytosol. The addition of phospholipid to the heated adrenal cytosol caused a shift of radioactive cholesterol into the denser region of the sucrose gradient. This effect was more pronounced when nonlabeled cholesterol was mixed with the phospholipid (Fig. 2), but the binding pattern seen with unheated cytosol did not occur. Whether the phospholipid and cholesterol caused the cholesterol-binding protein to aggregate or phospholipid-cholesterol micelles formed which incorporated some of the [^{3}H]-cholesterol was not determined for these studies. The fact that cholesterol micelles incorporating [^{3}H]-cholesterol will form and migrate into a sucrose density gradient as a well defined peak has been demonstrated in our laboratory and has been reported[16]. It has been reported that the cholesterol-binding protein protomer (MW = 16,000) in the heated cytosol from rat liver will aggregate to higher molecular weight species during binding to various lipids[14]. The fact that the heated adrenal cytosol did not bind radiolabeled phosphotidyl choline (see above) would indicate that phospholipid-cholesterol micelle formation rather than protein aggregation was occurring.

The heated adrenal cytosol was found to bind not only cholesterol but also cholesterol sulfate and 20α-hydroxycholesterol (Fig. 7). Binding studies with the latter two compounds have not been previously reported. Cholesterol oleate did not bind to heated adrenal cytosol, a finding which is consistent with a previous report for rat liver heated cytosol[14]. Cholesterol and cholesterol sulfate may be binding to the same protein as suggested by the electrophoretic and protein staining data in Figs. 8 and 9 while the binding of 20α-hydroxycholesterol appears to be to a different protein. The reason for the latter conclusion is the fact that while all three steroids demonstrated binding on sucrose gradient analysis only cholesterol and cholesterol sulfate-binding activity was demonstrated using polyacrylamide gel electrophoresis, and 20α-hydroxycholesterol binding was not demonstrable with the multiphasic buffer system that was used. From the standpoint of adrenal steroidogenesis, this is potentially important because cholesterol and cholesterol sulfate are both substrates for the cholesterol lyase system[17, 18] and 20α-hydroxycholesterol is considered a key intermediate in the lyase reaction[19]. It is the cholesterol lyase reaction which is considered rate-limiting in steroid synthesis[1, 2]. While cholesterol and cholesterol-sulfate were bound by heated adrenal cytosol, pregnenolone and pregnenolone sulfate (products of the cholesterol lyase reaction) were not apparently bound when examined by sucrose density gradient analysis. The lack of pregnenolone and pregnenolone sulfate binding to heated adrenal cytosol was also demonstrated using the more sensitive technique of gel filtration (data not shown). Pregnenolone has been shown to bind, however, to a heat-labile protein in the guinea pig adrenal cortex[5]. Pregnenolone sulfate has also been shown to bind to a heat-labile protein in the guinea pig adrenal cortex (unpublished data).

Several compounds were examined for their ability to bind to heated adrenal cytosol because of their potential importance in control and regulation of steroidogenesis. These compounds included cAMP, NADP, and prostaglandin E_2. cAMP is a potent stimulus of adrenal steroid synthesis[20] and has been proposed as a second messenger in ACTH regulated adrenal steroidogenesis[21]. It is known that cAMP binds to a heat sensitive soluble rat adrenocortical protein[22]. NADPH is a required cofactor for the mixed function oxidase systems involved in adrenal steroid synthesis including those systems involved in the cholesterol lyase reaction[23]. Prostaglandin E_2 has been reported to stimulate steroid synthesis in rat adrenal quarters[24]. Prostaglandin E_2 binding to rat and human adrenocortical membrane preparations has been reported[25]. None of these compounds, however, was found to bind to heated adrenal cytosol when examined by sucrose density gradient analysis.

The physiological importance of the heat-stable, cholesterol-binding protein in the guinea pig adrenal cortex is not known. Whether it plays any role in steroidogenesis must await further experimentation. The fact that the guinea pig adrenal heat-stable protein binds cholesterol and cholesterol sulfate (but not other cholesterol conjugates and not pregnenolone or pregnenolone sulfate), both of which are substrates for the cholesterol side-chain cleavage reaction (the rate-limiting step in steroidogenesis) cannot be overlooked. In addition, similarly derived, heat-stable adrenal cytosol preparations from the rat[6] and cow[7] have been shown to stimulate the cholesterol lyase reaction 4–10 fold. The known components of the cholesterol side-chain cleavage enzyme system, *viz.*, flavoprotein, non-heme iron protein, and cytochrome P_{450} have been shown not to be active in the cytosol of the cow adrenal cortex after boiling for two minutes[7]. If a cholesterol-binding protein in the adrenal cortex is found to play a role in steroid synthesis, the lack of tissue specificity will need to be clarified. It has been reported that a heat-stable cytosol preparation from the rat liver will also stimulate the rat adrenal mitochondrial cholesterol side-chain cleavage enzyme step[6]. A similarly prepared heat-stable cholesterol lyase

activator has been reported for the cow corpus luteum[26]. It must be pointed out that heat-stable cytosol preparations from the rat adrenal[6] and liver[27] have also been shown to augment specific enzymatic steps in cholesterol synthesis. In fact, it now appears that there is a family of proteins (both heat stable and heat sensitive) which function as indispensable cofactors in the conversion of squalene to cholesterol[28].

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