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STIMULATION FACTORS FOR CHOLESTEROL SIDE-CHAIN CLEAVAGE IN THE CORPUS LUTEUM AND ADRENAL GLAND

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SUMMARY

A soluble buffer extract of an acetone powder of adrenal mitochondria loses enzymatic activity when heated at 100°C for 2 min but contains a protein factor which can stimulate the unheated active enzyme by 4- to 10-fold. This heat stable adrenal factor has been fractionated by chromatography on Sephadex G 100 and DEAE cellulose. The protein is assayed by a microfluorescence procedure, and activity is measured by its ability to stimulate the enzyme. With fractionation the activity of the factor becomes less stable and declines rapidly after 24 h

The cytosol from corpora lutea contains activity for stimulating the cholesterol side-chain cleavage enzyme. The cell sap is centrifuged at 48,000 *g* treated with 20% $(NH_4)_2SO_4$, and is extracted with 1-butanol. After chromatography on Sephadex G 100 and DEAE cellulose, a protein fraction is obtained which binds cholesterol and stimulates the enzyme. Both factors, purified from adrenal mitochondria and corpus luteum cytosol, contain a high ratio of non-heme iron protein. The relationship of these factors to cholesterol transport protein, lipoprotein, SCP and to adrenodoxin is under investigation.

INTRODUCTION

The cholesterol side-chain cleavage enzyme present in steroid hormone producing tissues has been purified to 3 components, flavoprotein, (FP), non-heme iron protein (NHIP) and cytochrome P 450 [1] a system which bears a close similarity to 11β-hydroxylase. As the rate limiting step in steroidogenesis, and therefore a likely site for control, hormone stimulation at this step has been postulated. One possibility for regulation is to increase the availability of cholesterol, a water insoluble substrate, to the catalytic site of the cleavage enzyme [2] in the rat adrenal. ACTH has been found to increase the amount of free cholesterol in mitochondria [3]; in corpus luteum, LH has been found to increase a stable intermediate in mitochondria which is thought to be "active" cholesterol [4]. A rapidly turning over protein in the adrenal cytoplasma has been proposed as a mediator for ACTH action as an agent for cholesterol transport [2, 4].

A sterol carrier protein (SCP) has been reported for the stimulation of liver cholesterol biosynthesis in the laboratory of Scallen[5] and Dempsey[6]. It is present in the cytosol and in microsomal membranes [5, 6]. Dempsey proposed that the supernatant SCP could be involved in steroidogenesis, [7] and later in collaborative study we reported the finding of a similar SCP fraction in adrenal [8] tissue.

A supernatant factor which activates steroid hydroxylations in steroid hormone producing tissues has been observed previously. Peron[9] reported that an adrenal supernatant stimulated corticosteroid production with mitochondria. Nakamura *et al.*[10] purified a supernatant protein from liver which stimulated 11β-hydroxylase in the adrenal. Yago *et al.*[11] reported that acetone extracted supernatant from corpus luteum enhanced cholesterol side-chain cleavage of mitochondrial acetone powder suspensions. In this laboratory we have reported a crude heat stable protein obtained from the extract of adrenal mitochondrial acetone powder which stimulates the enzyme contained in the acetone powder[12]. This fraction binds cholesterol but not other hydroxylated steroids. It was shown to be devoid of the flavoprotein as indicated by absence of TPNH diaphorase activity. The absence of cytochrome P_{450} was confirmed by difference spectrophotometry. The non-heme iron content of

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enzyme was found to be decreased by 75% after 90 s of heating at 100°C. Results of further studies are now described for this adrenal factor after partial purification by Sephadex and DEAE chromatography. In addition, the isolation and partial purification of a stimulatory fraction for cholesterol side-chain cleavage by the soluble cell sap from the bovine corpus luteum is described. A comparison has been made of these stimulating factors from adrenal and corpus luteum with the known 3 proteins of the cholesterol side-chain cleavage system.

METHODS

1. *Preparation of bovine corpus luteum mitochondrial enzyme*

Bovine corpora lutea were collected fresh from a nearby slaughter house, or were purchased from Pel Frez, shipped on ice. Fractionation of corpora lutea into mitochondria and its subsequent acetone powder preparation followed a similar procedure described for adrenals except that an initial 15% homogenate was substituted for a 50% homogenate [12]. Cholesterol side-chain cleavage enzyme in the acetone powder was extracted with a glass homogenizer into buffer solution of 0·02 M potassium phosphate, pH 7·4, 0·001 M magnesium sulfate, and remained in the supernatant after 48,000*g* centrifugation for 30 min. Enzyme activity was destroyed after 90 s heating in a boiling water bath, and after 48,000*g* centrifugation for 30 min the heated enzyme supernatant was tested for stimulatory activity.

2. *Preparation of an activating factor from cell sap of bovine corpus luteum*

A 15% homogenate of tissue was prepared in a medium containing 0·25 M sucrose, 0·001 M magnesium sulfate, 0·02 M potassium phosphate buffer, pH 7·4. Cell debris was removed at 900*g*. Mitochondria were obtained after centrifugation 2 times at 10,000*g* for 30 min each, and the microsomes were collected after centrifugation at 48,000*g* for 60 min. Thirty grams of ammonium sulfate were added to 200 ml of the supernatant with stirring. After the precipitation was complete, the supernatant was centrifuged at 48,000*g* for 30 min. From the original wet weight of 35 g of corpora lutea, 320 ml of the supernatant was obtained after dialysis against 1 mM phosphate buffer, pH 7·4. Butanol extraction was performed following the procedure of Morton[13]. 1-Butanol was added to the dialyzate slowly with stirring to a final butanol concentration of 25% (v/v). Magnetic stirring was continued for 30 min at 4°C. Centrifugation at 6000*g* for 10 min separated the emulsified mixture into 3 distinct layers, aqueous in the bottom, organic at the top, with a solid yellow layer in between. After aspiration of the butanol phase with a disposable pipet, the solid layer was removed carefully with a spatulla. The aqueous phase was decanted, dialyzed and tested for both enzyme activity and stimulatory activity. It was lyophilyzed and put on chromatographic columns for further purification.

3. *Assay of cholesterol side-chain cleavage*

Substrate and enzyme were incubated at 37°C in air for 15 min in 3·0 ml of a phosphate buffer 0·02 M, pH 7·4, containing 1 mM magnesium sulfate. TPNH was 0·1 mM, [1,2-^{3}H]-cholesterol [50 ng/0·5 μCi] was added in 50λ propylene glycol : methanol (1 : 4 v/v). The sequence of additions was buffer, enzyme, activating factor, TPNH and cholesterol. Incubations were terminated by addition to the flasks placed on ice of 50 μg of cold cholesterol and pregnenolone in 50λ methanol. Enzyme activity was followed by quantitation of pregnenolone formed, using thin layer chromatography and liquid scintillation counting as described in a previous report [12].

4. *Fractionation of enzyme and activating factor on gel filtration and cellulose ion exchanger*

Sephadex G 100 column (2·2 × 35 cm) was previously equilibrated with 0·02 M phosphate and 1 mM magnesium sulfate, pH 7·4. Protein added in 3·0 ml was eluted with the same buffer at a flow rate of 20 ml h. Protein profile was determined by absorption at 280 nm, or with a fluorimetric assay described in 6 below. The eluate from the Sephadex column which contained stimulating activity was rechromatographed on a DEAE cellulose [14] column (1 × 9 cm) equilibrated with 0·02 M phosphate pH 7·4, containing 1 mM magnesium sulfate and 0·1 mM calcium chloride. Protein fractions were eluted stepwise with 20 ml of the same buffer of 0·02 M phosphate pH 7·4, 1 mM magnesium sulfate, 0·1 mM calcium chloride, and with additions of 0·15 M, 0·5 M, and 1·0 M sodium chloride. Sodium chloride which interferes with enzyme activity at high ionic strength was removed by dialysis against 0·02 M potassium phosphate and 0·001 M magnesium. The protein eluates were tested for stimulatory of activity under standard conditions described above.

5. *Assay for non-heme iron*

Non-heme iron was determined as the ferrous *o*-phenanthroline complex by the procedure of Massey [15]. A sample of the protein was deproteinized with 5% TCA and then centrifuged to spin down the precipitate. A 3·0 ml aliquot of the supernatant was

added to a mixture containing 0·36 ml of water, 0·35 ml of a 0·1% solution of *o*-phenanthroline, 0·15 ml of a freshly prepared 0·06 N solution of ascorbic acid, and 0·14 ml of a saturated solution of ammonium acetate. Absorption was measured at 510 nm against an appropriate blank.

6. *Protein assay*

Fluram, (Roche Diagnostics, New Jersey) was used for microanalysis of protein, following the procedure recommended by the manufacturer. The protein sample in a volume of 100λ was adjusted to 1·5 ml with 0·2 M borate buffer pH 9·2, and 0·5 ml Fluram (25 mg in 100 ml acetone) was added with immediate and rapid mixing. Fluorescence was read ten minutes later at an excitation wavelength at 390 nm, and emission wavelength at 480 nm.

7. *NADPH diaphorase assay*

The NADPH diaphorase activity was assayed according to the procedure of Omura[16].

RESULTS

1. *Cholesterol side-chain cleavage activity in the corpus luteum*

An acetone powder preparation of mitochondria was obtained from the bovine corpus luteum similar to that reported for the bovine adrenal [12]. The enzyme activity curve (Fig. 1a) for the acetone powder extract was identical to that observed for the adrenal. From 1 mg of acetone powder, an extract converted 5–20% of the 50 ng cholesterol substrate to pregnenolone in 15 min. The heated enzyme extract (activator fraction) was considerably less active than the corresponding adrenal fraction, a stimulation of 2–3-fold was usually observed in the former as compared to a 5–10-fold stimulation of the latter. Heat treatment in the presence of 1–5 mM mercaptoethanol, 1 mM dithiothreitol, and 10–20% ammonium sulfate or 10% glycerol failed to maintain a higher stimulatory activity in the heated corpus luteum fraction.

The cell sap of fresh corpus luteum was removed after centrifugation at 48,000*g* and after treatment by addition of ammonium sulfate to 20% saturation. The addition of this supernatant to enzyme was inhibitory, but following extraction with 1-butanol (25% v/v) which removed lipid and dissociated lipoprotein material, the aqueous extract was capable of stimulating enzyme activity (Fig. 1b). The butanol extracted supernatant by itself had no enzyme activity (Table 1).

The heated extract of corpus luteum (Fig. 2) acetone powder stimulated corpus luteum enzyme as did the heated extract of adrenal acetone powder (adrenal activator). As reported previously [12] higher concentrations of adrenal activator (above 8 mg) become inhibitory.

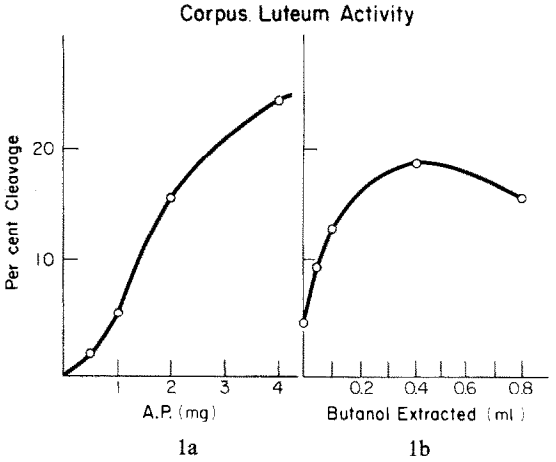

Fig. 1a. (Left) Corpus luteum enzyme concentration curve. Buffer extract of corpus luteum mitochondrial acetone powder was used as source of enzyme. Enzyme was assayed in a mixture containing 0·02 M phosphate buffer, pH.7·4, NADPH 0·1 mM, magnesium sulfate 0·001 M, [1,2H^3]-cholesterol 50 ng 0·5 μCi added in 50λ propylene glycol–methanol (1:4), and the amount of enzyme as indicated in the Fig. Incubation was in air at 37°C for 15 min. Values in graph were obtained from duplicate experiments done on different days.

Fig. 1b. (Right) Effect of corpus luteum supernatant on enzyme activity.

The butanol extracted fraction prepared as described in Methods was the source of supernatant. Enzyme was assayed under standard conditions with varying supernatant concentrations. Buffer extract of 0·3 mg bovine adrenal mitochondrial acetone powder was used as enzyme. Curve was plotted through the average of duplicate experimental values obtained on different days (1 ml of superantant had 1·1 mg BSA equivalent of protein).

Table 1. Corpus luteum cytosol fraction stimulation of adrenal cholesterol side-chain cleavage enzyme*

Additions to adrenal enzyme	Cholesterol conversion (per cent)
1. 0·3 mg enzyme –	3·6
2. 0·3 mg enzyme + 0·3 ml crude cytosol	0·7
3. 0·3 mg enzyme + 0·3 ml extracted cytosol	12·5
4. 0·4 ml extracted cytosol alone	0·05

* Cytosol was obtained by centrifugation of corpus luteum homogenate at 48,000*g*. The supernatant was heated with 20% $(NH_4)_2SO_4$, dialyzed, and extracted by 1-butanol (25% v/v). Protein content of extracted cytosol was 1·10 mg/ml (Fluram procedure). Adrenal enzyme obtained from 0·3 mg Acetone powder ≈0·03 mg protein.

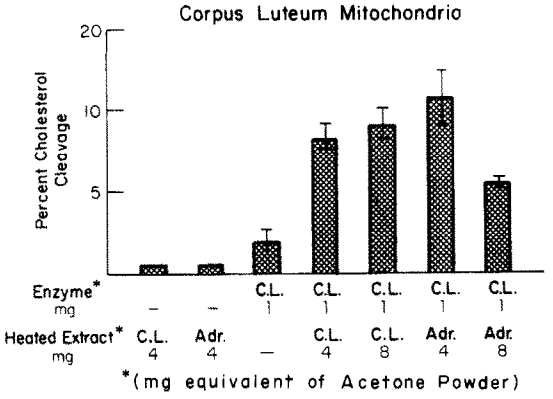

Fig. 2. Stimulation of enzyme with heat-treated enzyme from adrenal and corpus luteum.

Incubations contained extract of 1·0 mg corpus luteum mitochondrial acetone powder as enzyme in a medium of 0·02 M phosphate buffer, pH 7·4, magnesium sulfate 0·001 M, NADPH 0·1 mM and 50 ng/0·5 μCi of [1,2H^3]-cholesterol. Heated enzyme fraction was added in amounts as indicated. Enzymic reaction was carried out for 15 min at 37°C in air.

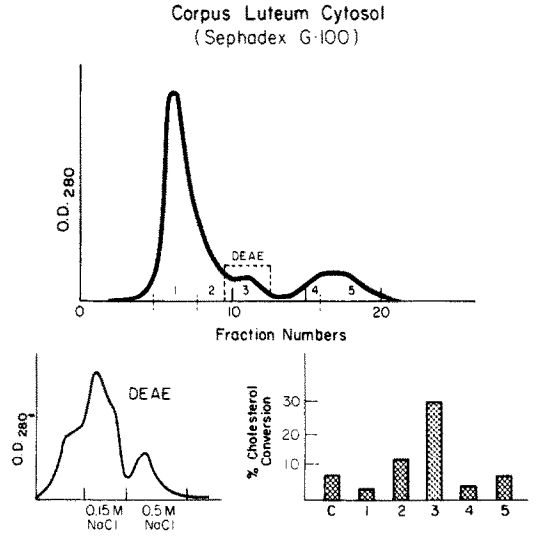

Fig. 3. Fractionation of corpus luteum cell sap.

Butanol extracted corpus luteum cell sap was lyophilized to give 200 mg powder from 110 ml. Protein content was 0·6 mg BSA protein/1 mg powder. 150 mg powder, dissolved in 3·0 ml 0·002 M phosphate buffer pH 7·4, 0·001 M magnesium sulfate, was added to a Sephadex G 100 column (2·2 × 35 cm) which was previously equilibrated with 0·02 M phosphate buffer pH 7·4, 0·001 M magnesium, and was eluted with the same buffer. Protein profile was determined with absorbance at 280 nm. Fractions were pooled and aliquots tested for stimulatory activity with 0·4 mg adrenal mitochondrial acetone powder extract as enzyme.

Pooled fraction 3 from Sephadex column was rechromatographed on DEAE column (1 × 9 cm) previously equilibrated with 0·02 M phosphate pH 7·4, 0·001 M magnesium sulfate, 0·1 mM calcium chloride, and was eluted with sodium chloride 0·15 M, 0·5 M, 1·0 M in the same buffer solution.

Table 2. Activity of corpus luteum cytosol fraction after chromatography on Sephadex G 100 and DEAE

Additions to adrenal enzyme (0·3 mg A.P.)	Cholesterol conversion (per cent)	NHI Content (nms)
1. Enzyme Control	2·7	—
2. Fraction B, 0·3 ml (30 μg)	14·4	14·0
3. Fraction B, 0·6 ml (60 μg)	16·2	30·0
4. Fraction A, 0·3 ml (78 μg)	5·5	1·5
5. Fraction A, 0·6 ml (152 μg)	7·1	2·3

Extracted Corpus luteum cytosol was fractionated first on Sephadex G 100. Elution from DEAE cellulose column with 0·15 M NaCl gave protein fraction *A*, elution with 0·50 M NaCl gave protein fraction *B*. Adrenal enzyme obtained from 0·3 mg acetone powder contained 0·03 mg of protein (Fluram procedure).

NHI = Non-heme iron protein assay by the procedure of Massey as the ferrous O-phenanthroline complex.

2. *Column chromatography*

Purification of corpus luteum supernatant fraction and adrenal mitochondrial acetone powder was achieved by chromatography in columns containing Sephadex G 100 and DEAE cellulose.

The corpus luteum cell sap after butanol extraction and dialysis was lyophilized and fractionated on a column (2·2 × 35 cm) of Sephadex G 100. The eluate corresponding to fractions 9–12 contained the major stimulatory activity. These eluates were pooled and chromatographed on 1 × 9 cm DEAE cellulose columns. The fractions eluted with 0·5 M NaCl contained the enzyme stimulatory activity (Fig. 3).

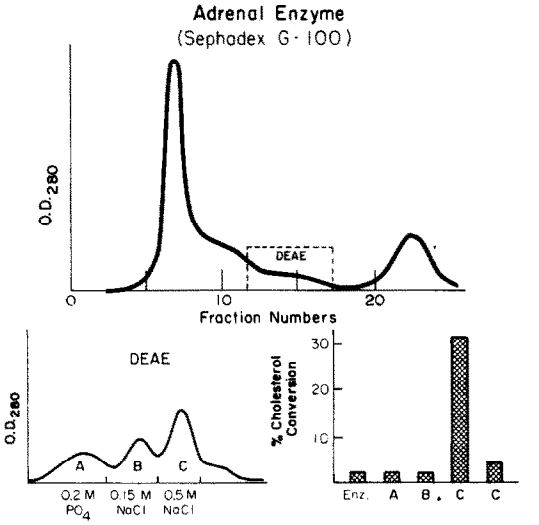

Fig. 4. Fractionation of adrenal enzyme on Sephadex G 100 and DEAE column. Buffer extract of 500 mg bovine adrenal mitochondrial acetone powder was fractionated on Sephadex and DEAE chromatographic column as described in legend of Fig. 3.

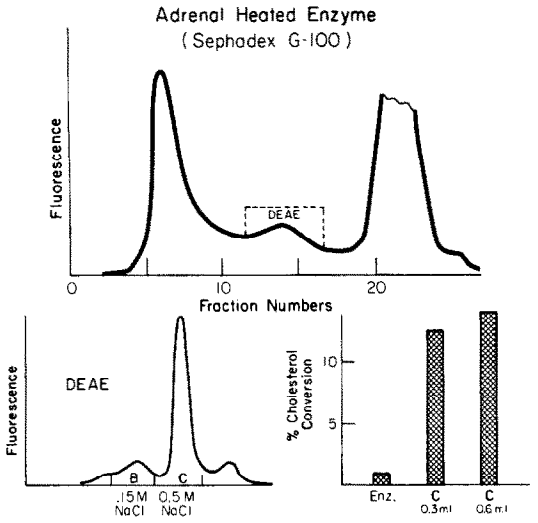

Fig. 5. Fractionation of adrenal activator (heated extract) on Sephadex G 100 and DEAE column.

Heated buffer extract from 920 mg of bovine adrenal mitochondrial acetone powder was fractionated on Sephadex and DEAE chromatographic column as described in legend of Fig. 3.

The enzyme activity, in terms of cholesterol conversion to pregnenolone and the non-heme iron protein content (NHI) of the DEAE purified fractions are shown in Table 2.

Similar purification of the adrenal activator fraction is shown for the mitochondrial adrenal acetone powder extract (Fig. 4) and for the heated extract of the acetone powder (Fig. 5).

A Sephadex G 100 column standardized for proteins of known molecular weight was used to estimate the size of the purified adrenal fractions. A molecular weight of 14,000 daltons was estimated (Fig. 6) for the activator obtained from both heated and unheated pools of adrenal acetone powders. The estimates were consistent with the estimates based on mobility of the purified activator samples previously observed with SDS gel electrophoresis [12].

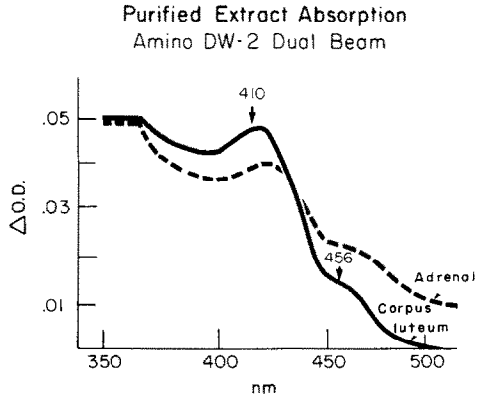

Fig. 6. Estimation of molecular weight of stimulatory protein by Sephadex G 100 chromatography. Standard proteins were separately chromatographed on the same Sephadex G 100 column as the column purified adrenal activator. Protein concentrations were 10 mg per sample.

Table 3. Non-heme iron content of bovine adrenal and corpus luteum

Adrenal	NHI (nMols/mgP)	Corpus Luteum	NHI (nMols/mgP)
105,000g S_N	0·9	48,000g S_N	1·8
Microsomes	1·3	—	
Mitochondria	3·6	Mitochondria	3·5
* Purified fraction	52·0	* Purified fraction	48·0

Non-heme iron protein was determined by the procedure of Massey as the ferrous O-phenanthroline complex.

Protein was determined by the Fluram fluorimetric procedure.

* Purified fraction—Active stimulator of cholesterol side-chain cleavage after chromatography on Sephadex G 100 and DEAE cellulose.

A comparison of the non-heme iron content (NHI) of the corpus luteum cell sap and adrenal mitochondrial acetone powder through the purification procedures indicates that the most purified fractions have a 50-fold concentration of NHI. (Table 3). The absorption spectra

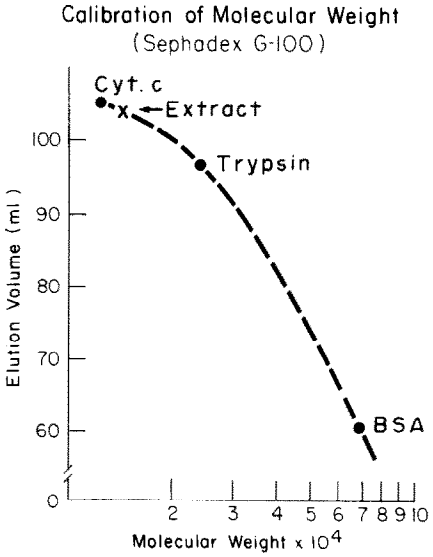

Fig. 7. Visible light spectrum of partially purified protein from bovine heated enzyme and corpus luteum cell sap.

Adrenal and corpus luteum protein with stimulating activity were prepared and purified on Sephadex G 100 and DEAE as described in the Methods section. Visible light absorption spectrum of adrenal protein (200 μg protein/ml, 52 nm NHI/mg protein) and corpus luteum protein (250 μg protein/ml, 48 nm NHI/mg protein) were scanned in Aminco dual wavelength beam DW-2 U.V./vis spectrophotometer.

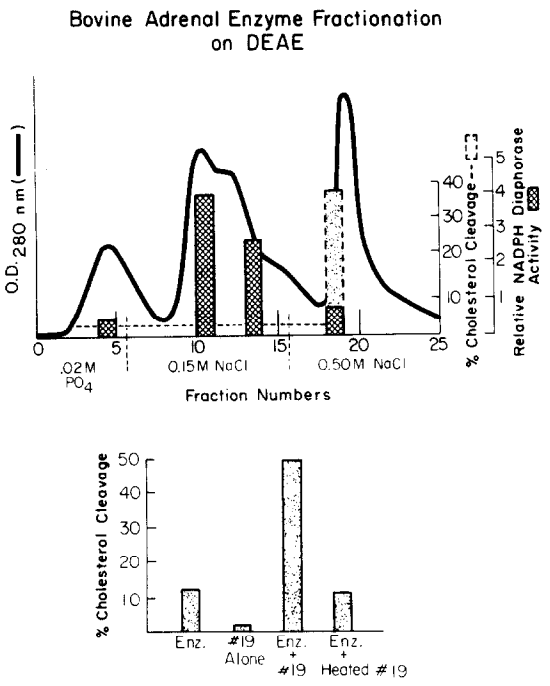

Fig. 8. Fractionation of Bovine Adrenal Enzyme. Buffer extract of 500 mg bovine adrenal mitochondrial extract (unheated) was fractionated on Sephadex G 100 and on DEAE as described in legends of Fig. 3 and 4. Aliquots of the DEAE eluates were analyzed for NADPH diaphorase activity and for cholesterol side-chain cleavage activity by procedures listed in Methods.

Stimulation of side-chain cleavage activity was not observed in eluates of 0·02 M PO_4 buffer alone or with 0·15 M NaCl. Stimulatory activity (49% conversion) was observed only in the first eluate with 0·5 M NaCl (Fraction #19). Fraction #19 did not itself contain enzyme activity. Unlike the heated buffer extract of acetone powder which will stimulate enzyme activity, the stimulatory activity of eluate #19 was lost after heating. Activator activity after Sephadex or DEAE chromatography loses its activity at 4°C after 24 h, NADPH diaphorase activity was observed only in the eluates eluted with 0·02 M PO_4 containing 0·15 M NaCl.

indicative of iron protein content of purified concentrates of corpus luteum cell sap and adrenal acetone powder are shown in Fig. 7.

The separation of NADPH diaphorase activity from cholesterol side chain cleavage stimulating activity in the DEAE purified adrenal enzyme fractions is demonstrated in Fig. 8. Stimulating activity (Tube #19) in fraction C (0·5 m NaCl) contains the highest concentration of NHI and also would be the fraction to contain adrenodoxin, if present [14]. The heat stability of the stimulating factor originally observed in the acetone powder extract, is no longer maintained after chromatography.

3. *Cholesterol binding*

The association of cholesterol with protein fractions on Sephadex and DEAE columns is depicted in Fig. 9. Adrenal protein (100 mg) was incubated with [12-^{3}H]-Cholesterol (1000 ng/10 μCi in 100λ methanol) at 4°C for 10 min prior to chromatography. Most of the radioactivity was eluted at the void volume and with the first protein peak. A small level of radioactivity persisted in the DEAE column which was eluted with the protein with 0·5 M NaCl. A corresponding cholesterol binding to a similar DEAE protein fraction was observed with the corpus luteum cell sap.

DISCUSSION

While the cholesterol side-chain cleavage enzyme in adrenal tissue has been extensively studied and its enzyme components purified, much less is known of the similar enzyme system in corpus luteum. Hall and Koritz[17] used an acetone powder of corpus luteum mitochondria to study cholesterol side-chain cleavage. They found that the 100,000g supernatant of the acetone powder had little enzyme activity. Our preparation from different batches of corpus luteum had good activity in the 48,000g buffer extract. The enzyme activity was comparable to that of bovine adrenal mitochondria prepared in the same way. The corpus luteum enzyme concentration curve using an acetone powder extract and exogenous cholesterol (Fig. 1) corresponded to that previously observed with adrenal enzyme [18, 12]. The sigmoidal nature of the enzyme curve was considered to be a property of an enzyme with multiple components [19]. The similarity of corpus luteum and adrenal mitochondrial enzyme was further demonstrated by the fact that a heat stable fraction could be obtained from extracts of acetone powders of both tissues (Fig. 2), and also by the stimulation of corpus luteum enzyme with heated extracts of adrenal. However, we could not consistently prepare a good stimulatory fraction from heated corpus luteum enzyme, either due to presence of inhibitors (lipids) in the extract or other factors causing the activity to be heat labile. Accordingly, attempts were made to isolate the stimulatory activity in the supernatant fraction, based on the observation that an SCP-like protein for stimulation of cholesterol biosynthesis is present in liver supernatant [5, 6], and that cholesterol transport from lipid droplet to mitochondria may be rate limiting for steroidogenesis in adrenal [2] and corpus luteum [4] tissues. The unextracted cell sap was inhibitory, apparently due to dilution from tissue sources of the tritiated cholesterol. After butanol extraction, the same preparation of cell sap was stimulatory although it had no enzyme activity by itself. A similar observation was made by Yago *et al.*[11] in a study of the intracellular

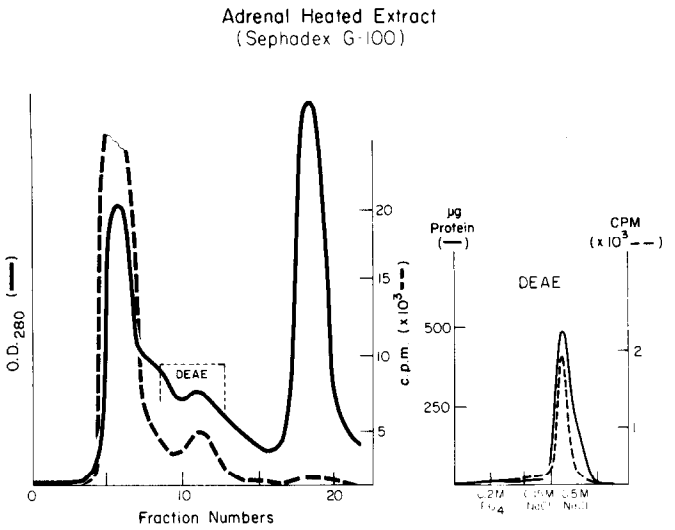

Fig. 9. Binding of [1,2-H^3]-cholesterol with adrenal heated enzyme fraction. Heated enzyme extract from 1·0 g of adrenal mitochondrial acetone powder was preincubated with 1000 ng (3·9 × 10^6) tritiated cholesterol at 4°C for ten min in a vol. of 2·6 ml. Two and a half ml of protein was fractionated on Sephadex G 100 equilibrated with 0·02 M phosphate buffer, pH 7·4. Fractions active in stimulating enzyme were pooled and rechromatographed on DEAE (1 × 9 cm) equilibrated with 0·02 M phosphate buffer, pH 7·4, and eluted with increasing salt fractions. Protein was determined with fluorimetric method, and radioactivity in 1·0 ml aqueous aliquot was counted in Bray's solution.

distribution of different enzymes in bovine corpus luteum. They found that while the supernatant had no enzyme activity of its own, an acetone powder extract of that supernatant could enhance the enzyme activity of a mitochondrial acetone powder suspension. When crude cell sap preincubated with tritiated cholesterol was fractionated on Sephadex G 100, (Fig. 4), the bulk of the radioactivity came out at the void volume with approximately 20% associated with the second and third peaks. The specific binding, expressed in CPM/mg protein, was increased in the protein at the void volume and the third protein shoulder peak. The binding of radioactivity with protein at the void volume presumably was non-specific, since cholesterol alone was not eluted from the Sephadex column, while addition of large molecules like albumin, or aggregates of small molecules like phospholipid led to cholesterol elution at void volume. Only the third protein shoulder peak has been found to stimulate the cleavage of cholesterol side-chain (Fig. 5). The void volume protein did not stimulate and usually was found to be inhibitory. The enzyme components in the corpus luteum have not been purified, but are assumed to consist of the 3 proteins of the cytochrome P_{450} system as in the adrenal. The shoulder peak did not contain flavoprotein diaphorase activity. The third protein shoulder peak had a mobility of molecular size of 14,000 daltons on a Sephadex column which was calibrated with pure standard proteins, Although the P_{450} protein with a M.W. of 800,000 was excluded as a possibility, adrenodoxin, reported to have a M.W. of 12,700, (14) was considered a good prospect. The shoulder peak protein was further fractionated on a DEAE column to yield a protein peak fraction with a high non-heme iron protein content (Table 4). Comparison of non-heme iron protein content per mg protein in Tables 4 and 5 indicates that the crude corpus luteum cell sap had a level (2·2 nmols) comparable to adrenal enzyme extract (3·6 nmols). The corpus luteum protein material binds cholesterol, stimulates side-chain cleavage, and has high non-heme iron content. Whether or not it is a component of the enzyme system which would correspond to adrenodoxin of the adrenal ("luteodoxin") remains to be proven. Also, it is not known whether this large quantity of non-heme iron protein originally was part of the membrane system which was released into the supernatant by the tissue fractionation procedure.

The high content of non-heme iron protein in the active peak eluted from the DEAE column does not provide sufficient information to establish the identity of the stimulatory factor for cholesterol side-chain cleavage. The factor appears to be a low molecular weight protein which is associated with or contains non-heme iron. Adrenodoxin may be present in the adrenal enzyme fractions but it is probable that some other factor with or without non-heme iron is the stimulatory factor in the heated adrenal extract and in the corpus luteum cell sap.

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DISCUSSION

Villee:

In these incubations where you use exogenous cholesterol, have you used a detergent in the medium?

Ungar:

No, we did not use a detergent. We, like everybody else working with cholesterol, had a problem getting the cholesterol into a soluble form. We used Tween, methanol, and propylene glycol as a base. The secret here, I think, is to keep the cholesterol in a soluble form before it has a chance to aggregate. The factor we're adding perhaps is a protein that allows the cholesterol to get to the P-450 system before it can aggregate and this would increase conversion.

Neher:

I didn't quite follow this purification procedure. Am I correct in assuming that the material was heated before purification?

Ungar:

Yes it was heated but we have looked at the unheated also. One of the curves you saw was for unheated material, the other was for the heated extract.

Neher:

If you heat, isn't adrenodoxin then destroyed?

Ungar:

Well, that's what we were counting on. It has been reported that adrenodoxin is destroyed with heat. We've lost 75% of our iron content when we heat.

Neher:

The effect you see may be due to something else. As you mentioned, with increasing purification you're losing this supporting activity. Could it be that you have a complex of some protein or lipo-protein with an unknown precursor and by purifying this you are losing your precursor, a non-cholesterol precursor.

Ungar:

We feel there is a complex between the cholesterol and the protein. This protein, SCP, for example, is known to bind NADPH and in the transport you have cholesterol with NADPH on this protein complex. That may be one way in which you get the NADPH in the preliminary cleavage step.

Neher:

Do you see any action of, for example, bovine serum albumin in your system?

Ungar:

Yes, albumin, rat serum and a number of other agents will inhibit in high enough concentrations. The albumin at the protein concentrations we're using won't stimulate. Rat serum will and other kinds of protein will interfere with the enzyme activity. This is a little different from intact mitochondria where it has been reported that albumin might, in fact, stimulate. In our system, protein and phospholipid will inhibit.

Neher:

But it depends very much on the "brand" of bovine serum albumin.

Schrader:

Have you ever tried to prepare this material from a tissue that is not involved in steroid biosynthesis?

Ungar:

As you saw, the liver SCP fraction can stimulate.

McKerns:

Several years ago we did have a reconstructed system from bovine adrenal consisting of purified mitochondria in-

cubated with cytosol preparation where our substrates were glucose-6-phosphate and NADP. The addition of ACTH was able to stimulate glucose-6-phosphate dehydrogenase, the production of NADPH and the generation of corticosteroids from added cholesterol. Later it turned out that this was fortuitous because there are a lot of things present in cytosol. One was the shuttle systems, such as the malate shuttle, for transfer of reducing equivalents to the mitochondria. Now it would seem that we also had carrier protein for the added cholesterol.

Ungar:

There have been, perhaps, 3 or 4 other laboratories that have reported some stimulation by using the supernatant in their adrenal or corpus luteum preparation. Farese, R. V. (*Biochem.* **6** (1967) 2052) had several papers on ACTH stimulation of intact cells and then observing an effect of the homogenate. These observations are consistent with the possibility of a factor that could be involved in the transport of cholesterol. We can't say that transport is involved at this time nor can we say that we have specific binding.