surprising that the aldosterone-induced changes in whole bladder phospholipid fatty acid composition are small. If the observed changes were to occur specifically in the serosal plasma membrane of the epithelial cells, they could easily account for the observed effects of aldosterone on toad bladder function.

Acknowledgments

The authors thank Mrs. Heidi Renger and Mrs. Boo Shik Yoon for their expert technical assistance. Dr. Peter Braun provided much helpful advice during the course of the studies

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Studies on the Soluble 17β-Hydroxysteroid Dehydrogenase from Human Placenta. Evidence for a Subunit Structure*

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ABSTRACT: The molecular weight of the 17β-hydroxysteroid dehydrogenase, estimated from polyacrylamide electrophoresis in 0.1% sodium dodecyl sulfate, is 35,000. This is approximately one-half the value obtained in an earlier study of the active enzyme and suggests that the enzyme is composed of subunits. Cross-linking with dimethyl suberimidate followed by electrophoresis in sodium dodecyl sulfate gives, in addition to a band of molecular weight 37,000, a second protein band with a molecular weight of approximately 73,000. The molecular weight calculated using the Stokes radius and the sedimentation coefficient of the active enzyme is 72,000. The elution volume of the 17β-hydroxysteroid dehydrogenase on Sephadex G-200 filtration is markedly affected by the conditions of filtration: acidic pH or low temperature (4°) produces aggregation of the enzyme while sodium dodecyl sulfate leads to dissociation.

Early attempts to purify the 17β-hydroxysteroid dehydrogenase from human placenta were hindered by its instability (Langer and Engel, 1958; Talalay *et al.*, 1958), but addition of glycerol to solutions of the enzyme protected it from inactivation and permitted an extensive purification (Jarabak *et al.*, 1962). Further studies revealed that the instability of the enzyme was due to a partially reversible cold inactivation, during which the enzyme underwent aggregation (Jarabak *et al.*, 1966). Taking precautions to protect the enzyme from cold inactivation, three groups of investigators (Descomps *et al.*, 1968; Jarabak, 1969; Karavolas *et al.*, 1970)

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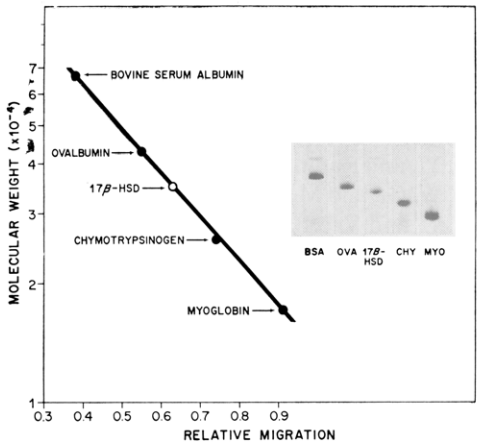

FIGURE 1: Molecular weight estimation from electrophoretic mobility in the presence of sodium dodecyl sulfate. The migration is relative to that of bromophenol blue. The insert shows the polyacrylamide gels used for this determination. Abbreviations used are: BSA, bovine serum albumin; OVA, ovalbumin; 17β-HSD, 17β-hydroxysteroid dehydrogenase; CHY, chymotrypsinogen; MYO, myoglobin. Direction of migration is from top to bottom.

have isolated the 17β-hydroxysteroid dehydrogenase in an apparently homogeneous state, but their estimates of its molecular weight have ranged from 48,000 (Karavolas *et al.*, 1970) to 92,000 (Descomps *et al.*, 1968). The present study was undertaken to examine these discrepancies and to investigate the possibility that the enzyme is composed of subunits.

Methods

Materials. Deionized glass-distilled water was used for all solutions. Reagents were obtained from the following sources: NAD from P-L Laboratories, 17β-estradiol from Steraloids, crystalline bovine serum albumin from Armour, crystallized ovalbumin from Sigma, α-chymotrypsin, three-times-crystallized ribonuclease, and five-times-crystallized chymotrypsinogen A from Worthington, two-times-crystallized myoglobin from Mann, Spectroscopic grade glycerol from Matheson, Coleman & Bell, coomassie brilliant blue from Colab, Bio-Gel P-100 from Bio-Rad, Sephadex G-200 (superfine) and Blue Dextran 2000 from Pharmacia, and sodium dodecyl sulfate from Eastman. The 17β-hydroxysteroid dehydrogenase was prepared by two methods (Jarabak, 1969; Karavolas *et al.*, 1970) and had specific activities between 1.5 and 2.0 units per mg of protein when assayed by the rate of NAD reduction (Jarabak, 1969). Unless specified the enzyme used was prepared by the method of Karavolas *et al.* (1970) and was stored at a protein concentration of 4 mg/ml in a buffer containing 50% glycerol, 5 mM potassium phosphate, and 1 mM EDTA at pH 7.0. The storage temperature was 4°.

Enzyme Assay. Enzyme activity was measured spectrophotometrically. The assay has been previously described (Jarabak, 1969). One unit of enzyme is defined as the amount which reduces 1 μmole of NAD/min under the stated conditions.

Protein Concentrations. The values given for protein concentrations were obtained by the method of Lowry *et al.* (1951).

Electrophoresis in Polyacrylamide Gels. Electrophoresis in polyacrylamide gels (5–10%) containing 0.1% sodium dodecyl sulfate was performed as described by Weber and Osborn (1969) or Marshall and Zamecnik (1969) except that samples were not dialyzed prior to electrophoresis. The samples contained 10–20 μg of each protein. The gels were fixed and stained with coomassie brilliant blue according to the procedure of Chrambach *et al.* (1967).

Amidination. The 17β-hydroxysteroid dehydrogenase was treated with dimethyl suberimidate according to the procedure of Davies and Stark (1970), and then electrophoresis was performed in polyacrylamide gels containing 0.1% sodium dodecyl sulfate, as described above.

Gel Filtration. One-hundred-microliter samples containing bovine serum albumin, ovalbumin, myoglobin, 17β-hydroxysteroid dehydrogenase (approximately 100 μg of each protein), and Blue Dextran 2000 were applied to a 9 × 200 mm column of Sephadex G-200 (superfine) which had been equilibrated with respect to buffer and temperature. Fractions (0.25–0.3 ml) were collected and samples were assayed for enzyme activity. Electrophoresis was performed in 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate, as described above.

The Stokes radius of the enzyme was determined by the method of Ackers and Steere (1967). For this purpose a 10 × 880 mm Bio-Gel P-100 column was equilibrated at 25° with buffer containing 0.2 M potassium phosphate and 1 mM EDTA (pH 7.0). Ribonuclease, α-chymotrypsin, ovalbumin, and bovine serum albumin were used as standard proteins while potassium chromate and Blue Dextran 2000 were used to determine the internal volume and void volume of the column, respectively.

Ultracentrifugation was performed in a Spinco Model E analytical ultracentrifuge equipped with a schlieren optical system. The enzyme (10 mg of protein/ml) was dialyzed for 16 hr at 4° against several changes of buffer containing 5 mM sodium phosphate, 100 mM sodium chloride, and 10^{-5} M NADP at pH 7.0. Under these conditions the enzyme was stable in the absence of glycerol. Centrifugation was done at 4° and 59,780 rpm; photographs were taken at 16-min intervals after the maximum velocity was obtained.

Amino Acid Analysis. Samples containing 144 μg of protein were dialyzed for 48 hr against several 1-l. changes of distilled water, transferred to hydrolysis tubes and evaporated to dryness in a stream of nitrogen. Constant-boiling HCl was added and the tubes were evacuated and sealed. The samples were hydrolyzed for 20, 48, and 72 hr at 110 ± 4°. They then were taken to dryness, 25 mμmoles of norleucine was added to each as a standard and the volume of each sample was brought to 1.2 ml with 0.01 N HCl. Samples of 0.5 ml were applied to each column of a Beckman 120C amino acid analyzer.

Results

Electrophoresis in Polyacrylamide Gels. When electrophoresis of the 17β-hydroxysteroid dehydrogenase was performed in polyacrylamide gels containing 0.1% sodium dodecyl sulfate, a single protein band was obtained. A plot of migration distance *vs.* molecular weight, using proteins of known molecular weight as standards, gives a molecular weight of 35,000 for the enzyme (Figure 1). Similar results were obtained from gels prepared from varying concentrations of acrylamide using either the method of Weber and Osborn (1969) or that of Marshall and Zamecnik (1969). Enzyme prepared by a procedure which included a heating step (Jarabak, 1969) and that prepared without the use of this step (Karavolas *et al.*, 1970) gave identical results in these electrophoresis experiments.

Amidination. Davies and Stark (1970) have demonstrated

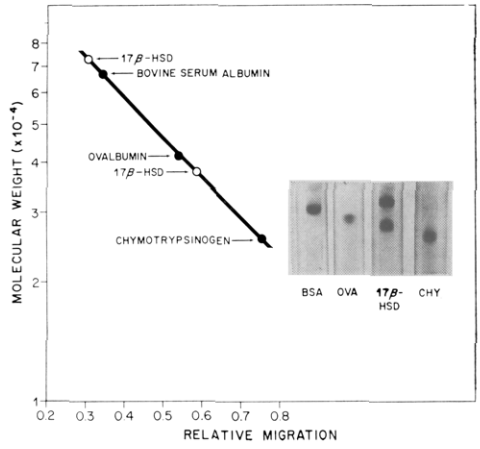

FIGURE 2: Estimation of the molecular weight of the amidinated 17β-hydroxysteroid dehydrogenase by electrophoretic mobility in the presence of sodium dodecyl sulfate. The 17β-hydroxysteroid dehydrogenase and each standard protein were treated as follows: 20 μg of the protein in 20 μl of buffer containing 50% glycerol and 5 mM potassium phosphate at pH 7 was incubated for 2 hr at 25° with 25 μl of 0.2 M triethanolamine at pH 8.6 containing 75 μg of dimethyl suberimidate. Then 0.1 ml of 0.2% sodium dodecyl sulfate was added and the mixture was allowed to stand at 25° for 1 hr more before electrophoresis was performed. The insert shows the polyacrylamide gels obtained in this experiment.

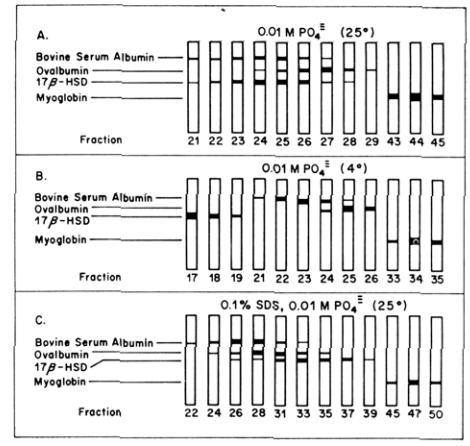

FIGURE 3: Polyacrylamide electrophoresis in 0.1% sodium dodecyl sulfate of fractions from Sephadex G-200 gel filtration experiments. (A) Results obtained when the protein mixture was applied to a 9 × 200 mm Sephadex G-200 column which had been equilibrated at 25° with 0.01 M sodium phosphate (pH 7.0). Elution was with the same buffer. Blue Dextran appeared in fractions 15 and 16. (B) Results of a similar filtration experiment performed at 4°. Blue Dextran appeared in fractions 17 and 18. (C) Results of a similar filtration experiment performed at 25° with the equilibrating and eluting buffer containing 0.1% sodium dodecyl sulfate and 0.01 M sodium phosphate (pH 7.0). Blue Dextran appeared in fractions 16–18. The fractions (0.25–0.3 ml) were collected by a drop-actuated fraction collector. Since the conditions of the experiment affected the size of the drops, fraction numbers corresponding to the void and elution volumes vary from column to column.

that dimethyl suberimidate may be used to cross-link oligomeric proteins. Polyacrylamide electrophoresis of these cross-linked proteins in sodium dodecyl sulfate gives a set of bands having molecular weights equal to integral multiples of the protomer molecular weight. After reaction of the 17β-hydroxysteroid dehydrogenase with dimethyl suberimidate, two protein bands were observed on polyacrylamide gels (Figure 2). The more rapidly moving band had a molecular weight of 37,000 in this experiment, while the more slowly moving one had a molecular weight of 73,000. Varying the amount of protein, dimethyl suberimidate, or prolonging the incubation with the reagent did not cause formation of more protein bands in samples containing the enzyme.

Gel Filtration. Figure 3 illustrates the profound effect that filtration conditions have on the elution volume of the 17β-hydroxysteroid dehydrogenase on a Sephadex G-200 column. When filtration was done at 25° and the buffer contained 10 mM sodium phosphate at pH 7.0 (Figure 3A), most of the enzyme activity was found in fractions 24 and 25. Polyacrylamide electrophoresis in sodium dodecyl sulfate revealed that the major portions of both the 17β-hydroxysteroid dehydrogenase and of bovine serum albumin were in these fractions. When the filtration was done in the same buffer but at 4° (Figure 3B), cold inactivation occurred. No enzyme activity was detected and the inactive enzyme was eluted (at the void volume of the column) before bovine serum albumin. Similar results were obtained if the filtration was performed at 25° with the phosphate buffer at pH 4.7. When the filtration was done at 25° and the elution buffer contained 0.1% sodium dodecyl sulfate in 10 mM sodium phosphate (pH 7.0) (Figure 3C), no enzyme activity was detectable and the major portion of the inactive enzyme was eluted after the major portion of ovalbumin.

Molecular Weight Calculation. The 17β-hydroxysteroid dehydrogenase sedimented as a single symmetrical peak on ultracentrifugation with a sedimentation coefficient ($s_{20,w}$) of 5.24 S. The Stokes radius of the enzyme, obtained from gel filtration on Bio-Gel P-100, was 32.6 Å; the apparent partial specific volume, calculated from the amino acid composition (Cohn and Edsall, 1943), was 0.738 mg/ml. From these parameters, the molecular weight of the protein was calculated (Ackers and Steere, 1967; Notides and Williams-Ashman, 1967) to be 72,000.

Amino Acid Analysis. The amino acid analysis of the enzyme is presented in Table I. The amount of each amino acid obtained from 60 μg of protein was divided by the quantity of isoleucine[1] to give molar ratios. These were multiplied by seven and the products adjusted to give the nearest integral values. The molecular weight, calculated from the number of residues of each amino acid, is 68,370. Although 60 μg of protein (by Lowry determination) was used for each analysis, the sum of the weights of the constituent amino acids (after correction for water of hydrolysis) is only 32 μg. This suggests that determining the protein concentration by the method of Warburg and Christian (1941) may give a more accurate value than the method of Lowry *et al.* (1951), since the enzyme protein measured by the former method is 0.48 times that determined by the latter (Jarabak and Sack, 1969).

Discussion

The molecular weight of the 17β-hydroxysteroid dehydrogenase estimated from polyacrylamide gel electrophoresis in sodium dodecyl sulfate is 35,000. Since this value is approximately half that obtained from previous gel filtration and titration studies, it suggests that the enzyme is composed of two subunits. This is supported by the observation that after

[1] Isoleucine was chosen rather than methionine or tryptophan because of its stability on hydrolysis.

TABLE I: Amino Acid Analysis of the 17β-Hydroxysteroid Dehydrogenase.[a]

Amino Acid	Most Probable Value (mμmoles)	Molar Ratio	Molar Ratio × 7	Nearest Integral No. of Residues
Asp	17.3	5.78	40.4	40
Thr	12.3	4.11	28.8	29
Ser	14.3	4.79	33.5	34
Glu	21.8	7.30	51.1	51
Pro	13.2	4.41	30.9	31
Gly	21.4	7.16	50.1	50
Ala	23.3	7.78	54.4	54
Val	20.1	6.72	47.0	47
Cys	4.45	1.49	10.4	10
Met	2.06	0.67	4.8	5
Ile	2.99	1.00	7.0	7
Leu	28.3	9.46	66.2	66
Tyr	3.80	1.27	8.9	9
Phe	8.60	2.87	20.2	20
Lys	8.47	2.83	19.8	20
His	4.88	1.63	11.4	11
Arg	15.6	5.22	36.5	37
Trp				2

[a] The amount of each amino acid represents the maximum yield of the amino acid or the value corrected for hydrolytic loss by extrapolation to zero-time hydrolysis (threonine, serine, and tyrosine). Tryptophan, which is destroyed by prolonged acid hydrolysis, was determined by the method of Goodwin and Morton (1946) while cystine was determined as cysteic acid by the method of Moore (1963). The enzyme used for this determination was prepared by the method of Jarabak (1969); 60 μg was used in each analysis. The results represent a single set of determinations.

cross-linking of the enzyme with dimethyl suberimidate a new, more slowly migrating protein band (mol wt 73,000) is seen in addition to the rapidly migrating band. Although these results would suggest that the subunits are identical, further studies will be necessary to confirm this.

When gel filtration experiments are performed under a variety of conditions, using standard proteins as internal markers, it is apparent that the "molecular weight" of the enzyme may vary from values of less than 43,000 (ovalbumin) to greater than 100,000,[2] with the fully active enzyme having approximately the same "molecular weight" as serum albumin. Gel filtration methods provide a more accurate estimate of the Stokes radius of a molecule than of its molecular weight (Ackers and Steere, 1967) since estimates of the latter value are profoundly affected by the shape of the molecule. Using the Stokes radius and sedimentation coefficient of the active enzyme, the molecular weight was calculated to be 72,000. This value is in reasonably good agreement with that obtained by titration of the enzyme with NADPH. It seems likely, however, that the latter value represents the amount of protein combining with two molecules of NADPH (and 17β-estradiol) rather than one, as previously assumed. This change results from the fact that the amount of protein added in the titration experiments had been determined by the method of Lowry *et al.* (1951).

The observation by Descomps *et al.* (1968) that the molecular weight of the 17β-hydroxysteroid dehydrogenase is 92,000 is open to question since it was apparently based on gel filtration experiments with a Sephadex G-100 column that had been calibrated with only a single protein (another steroid dehydrogenase). Although various buffers, concentrations of acrylamide, as well as unheated (Karavolas *et al.*, 1970) and heat-treated (Jarabak, 1969) enzyme have been used, we have always found the 17β-hydroxysteroid dehydrogenase to migrate more rapidly than ovalbumin in polyacrylamide gels containing sodium dodecyl sulfate. Hence we are not able to confirm the observation by Karavolas *et al.* (1970) that the molecular weight of the enzyme was 48,000 under such electrophoretic conditions.

[2] It seems likely that the observation (Hagerman, 1969) that the 17β-hydroxysteroid dehydrogenase undergoes a change in molecular weight ("trimerization") on Sephadex columns equilibrated at 4° with buffer containing 17β-estradiol is a reflection of this phenomenon, since 17β-estradiol does not completely protect the enzyme from cold inactivation (Jarabak *et al.*, 1966).

Acknowledgments

The authors wish to express their appreciation to Dr. Henry Paulus for his suggestions and generous gift of dimethyl suberimidate and to acknowledge the valuable advice given by Dr. H. G. Williams-Ashman and Dr. E. Goldwasser. In addition we thank Dr. P. Talalay for his support in certain phases of this study, Dr. F. P. Zuspan, Dr. A. C. Barnes, and their staffs for assistance in the collection of placentas, Mr. C. Kung for his determination of the sedimentation coefficient, and Mrs. Beatrix Carpenter for the performance of amino acid analyses.

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Estradiol Binding of Exceptionally High Affinity by a Nonhistone Chromatin Protein Fraction*

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ABSTRACT: An estradiol binding system has been found in the nonhistone chromatin fraction of purified nuclei of calf endometrium which had not been exposed to estradiol. It exhibits hormonal and steric specificity since estradiol and diethylstilbestrol are bound to a more significant extent than 17α-estradiol, and neither testosterone nor progesterone compete for the estrogen binding sites. Studies with proteolytic enzymes and nucleases have shown that the binding component is at least in part a protein. Organ specificity is also probable as no specific binding of the hormone could be demonstrated in a similar preparation from calf liver nuclei. With the use of a charcoal adsorbent technique for removing unbound and bound with relatively low-affinity ligand, the binding parameters were studied with the computerized "proportion graph" method. The binding component was found to have an exceptionally high affinity for estradiol ($K \simeq 10^{14}$ M^{-1} at 4°), assuming that it follows the law of mass action, and that it is present only in a small number of sites (<10) per cell. The characteristics (affinity, cellular concentration, and molecular size) which differentiate it from the cytosol and the "neonuclear" estradiol receptors are discussed.

The presence of a receptor[1] for estradiol in the uterine cytosol of the castrated or immature animal is now well established (Talwar *et al.*, 1965; Toft and Gorski, 1966; Jensen *et al.*, 1967; Toft *et al.*, 1967; Baulieu *et al.*, 1967). In nuclei, there is an estradiol receptor, the formation of which appears to be dependent on the presence of both the cytosol receptor and the hormone (Brecher *et al.*, 1967; Jensen *et al.*, 1968; Shyamala and Gorski, 1968) and these dependencies justify its designation as the "neonuclear" receptor (Baulieu *et al.*, 1971). Both cytosol and neonuclear receptors can be detected in *in vitro* experiments using a radioactive estradiol concentration of $1 \times 10^{-9\pm1}$ M, and they have, at equilibrium, an apparent intrinsic association constant of the order of 10^{10} M^{-1} at 4° (Shyamala and Gorski, 1968; Puca and Bresciani, 1969; Truong and Baulieu, 1971). Calculations have shown that the number of binding sites per cell for these receptors is of the order of 10^3.

The possibility of a preexisting nuclear receptor, independent of the arrival of estradiol in the uterine cells and of the cytosol receptor, was considered, and a search was undertaken for its existence. Working with various nuclear extracts at the usual 1-nM level of the hormone, all attempts to find such a specific binding protein were negative. It was felt that this failure may have been due to the concentration of the hormone used in the experiments because if a preexisting receptor was present and had a fairly small number of sites per cell (*i.e.*, <100), the large amount of nonspecific binding found at relatively high levels of estradiol in all nuclear extracts would possibly mask this receptor. In addition, since isolation of the nuclei had to be performed in the absence of hormone and since such a receptor could be sensitive to proteolysis in the absence of its ligand (as is the case for other uterine receptors), its detection was likely to be more difficult after a rather long preparative procedure. Conversely, any exposure of the uterus or homogenate to estradiol in order to provide some degree of protection for the protein could provoke the formation of the neonuclear receptor, which in turn would conceal the presence of any preexisting receptor, especially if per cell, these are very few in numbers.

In order to minimize the effect of these interfering factors in the quest for a nuclear receptor in uteri not previously exposed to estradiol, the binding ability of various nuclear fractions was examined at very low concentrations of estradiol (the order of 1 pM). By so doing, it was possible to demonstrate an estradiol specific binding system in the chromatin nonhistone protein fraction of calf endometrium purified nuclei. The qualitative specificity was derived from studies with various hormones and from its absence in a similar preparation of calf liver nuceli. No such binding system was found in any other uterine nuclear fraction and in addition, there was some evidence that the molecular weight of this receptor was less than that of the cytosol and neonuclear receptors.

The binding parameters were the most striking characteristics of this system. They were determined by the use of a charcoal adsorbent technique and the computerized proportion graph method. The calculated number of estradiol binding sites per cell was found to be less than 10. Assuming that

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[1] An review of the terms cytosol, receptor, neonuclear, specific binding, etc., is found in Baulieu *et al.* (1971).