Intracellular Localization and Properties of 3β-Hydroxysteroid Dehydrogenase/Isomerase in the Adrenal Cortex*

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The intracellular locations and properties of the 3β-hydroxysteroid dehydrogenase/isomerase were studied in subcellular fractions isolated from homogenates of rat adrenal and calf adrenal cortex. The goal was to determine whether the enzyme has a dual localization in the microsomes and mitochondria in the adrenal cortex. Mitochondrial fractions isolated from these homogenates were found to contain 26% of the total 3β-hydroxysteroid dehydrogenase/isomerase activity of the homogenate. Sucrose density gradient centrifugation demonstrated a co-migration of 3β-hydroxysteroid dehydrogenase/isomerase activity with an adrenal mitochondrial marker enzyme. No artificial attachment or transfer of 3β-hydroxysteroid dehydrogenase/isomerase activity to liver mitochondria could be demonstrated when liver and adrenal tissues were homogenized. A portion of the 3β-hydroxysteroid dehydrogenase/isomerase activity associated with the mitochondrial fraction was active with matrix space NAD⁺. The microsomal enzyme was inactive in the absence of exogenous NAD⁺. When levels of intramitochondrial NAD⁺ were decreased either through reduction to NADH or by elution from calcium-swollen (10 mm CaCl₂) mitochondria, mitochondrial 3β-hydroxysteroid dehydrogenase/isomerase activity was inhibited. 3β-Hydroxysteroid dehydrogenase/isomerase activity in adrenal cortex microsomes was abolished by mersalyl (40 μM). The mitochondrial enzyme was unaffected by mersalyl except when the mitochondria were in a calcium-swollen condition. It is concluded that 3β-hydroxysteroid dehydrogenase/isomerase has a dual intracellular location in the adrenal cortex.

3β-Hydroxysteroid-dehydrogenase/isomerase* (HSD) is a membrane-bound enzyme found in adrenal cortex (1), ovary (2), testes (3), and placenta (4). The enzyme is involved in steroidogenesis and catalyzes the oxidative conversion of steroids with the Δ5,3β-hydroxy structure to the Δ4,3-keto configuration; e.g. pregnenolone + NAD⁺ → progesterone + NADH + H⁺. The intracellular location of HSD in bovine adrenal cortex tissue was examined by Beyer and Samuels in 1956 (1). Their initial enzyme distribution study revealed HSD activity in the microsomes (39%) as well as the nuclear (26%) and mitochondrial (14%) fractions. The HSD activity found in the nuclear fraction could be reduced to zero by repeated washing of the pellet through resuspension and recentrifugation. Applying the same technique to the microsomes and mitochondria was without effect and in these organelles the enzyme was removable only through detergent solubilization. On the basis of phase-contrast microscopic examination of the mitochondria Beyer and Samuels (1) concluded that HSD activity in this fraction could be accounted for by microsomal contamination and that in intact adrenal cortex tissue the HSD was a constituent only of the endoplasmic reticulum.

Despite the general acceptance of the conclusion of Beyer and Samuels (1), other investigators have reported results which suggest that mitochondrial fractions from all steroid hormone-producing tissues do contain HSD activity. Thus, HSD has been demonstrated in the mitochondrial fractions isolated from ovary (2, 5, 6), testes (3, 7), human term placenta (4, 8–10), and rat adrenal cortex (11–14). These reports included both enzyme distribution studies and the indirect evidence that mitochondria from steroid-producing tissues incubated with cholesterol produce progesterone as well as pregnenolone. Additionally, when rat adrenal cortex tissue slices were stained for HSD activity, the product of the reaction was found in the mitochondria as well as the smooth endoplasmic reticulum (15). Nevertheless, other investigators disputed the presence of HSD in the mitochondria either as microsomal contamination (16), or as an enzyme redistribution artifact that occurred during homogenization (17). Consequently, the exact intracellular localization of HSD has remained unsettled.

In this report we show that adrenal cortex mitochondria contain a substantial and consistent amount of the total homogenate HSD activity, and that this activity cannot be removed from the mitochondria either by repeated washings, or by sucrose density gradient centrifugation. Moreover, we show that the HSD activity associated with the mitochondria has characteristics which clearly differentiate it from the microsomal enzyme.

EXPERIMENTAL PROCEDURES

Materials

White male Sprague-Dawley descent rats (250 to 300 g) were obtained from Blue Spruce Farms, Altamont, N.Y. Calf adrenal glands were generously supplied by Parnett Packing Co., Bloomville, NY.
Adrenal Cortex 3β-Hydroxysteroid Dehydrogenase/Isomerase

Spectral grade heptane was purchased from Eastman Kodak. NAD⁺, NADase (Neospora crassa), digitonin, cytochrome c (horse heart), and glucose-6-PO₄ dehydrogenase (Leuconostoc mesenteroides) were purchased from Sigma Chemical Co. Glucose-6-PO₄, bovine serum albumin (Fraction V), progesterone, and [7-³H]pregnenolone (117 Ci/mmol) were purchased, respectively, from Calbiochem, Miles Laboratories, Steraloids, Inc., and New England Nuclear. All other chemicals were of reagent grade.

Methods

Preparation of Subcellular Fractions from Rat Adrenals—Six to ten male Sprague-Dawley rats weighing from 250 to 300 g were used in each experiment. The animals were killed by decapitation and the adrenals were removed and homogenized as described previously (18). The homogenate medium contained 0.25 mM succrose, 1 mM EDTA, 30 mM Tris/Cl, pH 7.4, and 0.1% w/v bovine serum albumin (6 mg/ml). The unbroken cell nuclear pellet was obtained by a 10 min at 700 x g centrifugation and was washed once in Medium A. The mitochondrial pellet was obtained by a 20 min at 5200 x g centrifugation of the postnuclear supernatant fluid and was washed four times by resuspension and recentrifugation (10 min at 5200 x g) in Medium A. The microsomal (pellet) and cytosol (supernatant fluid) fractions were obtained by centrifugation of the postmitochondrial supernatant fluid for 1 hr at 165,000 x g.

Preparation of Calf Adrenal Cortex Mitochondria—Adrenals, removed from slaughtered calves, were chilled in ice-cold, buffered salt solution (30 mM Tris/HCl, 6 mM KCl, 0.15 M NaCl, pH 7.4). The adherent fat was removed and the adrenals were packed on ice and transported to the laboratory. The time between death of the animal and arrival in the laboratory was about 75 min and the glands were processed immediately upon arrival at the laboratory. Preparation of the adrenal cortex tissue and homogenate, as well as isolation of the mitochondrial fraction, have all been described previously (19). For all experiments the crude mitochondrial pellet was resuspended in Medium A and washed four times.

Measurement of HSD Activity by Progesterone Formation—The rate of progesterone formation catalyzed by HSD was measured by a modification of the method described by Koritz (20). Aliquots of mitochondria, or other cellular fractions suspended in Medium A, were added to duplicate 15-ml incubation tubes containing 1 ml of a medium composed of 50 mM succrose, 20 mM KCl, 1 mM EDTA, 30 mM Tris/HCl, pH 7.4, and 0.1% w/v bovine serum albumin (Medium B). NAD⁺ when present, was 0.5 mM and other additions were as indicated in the tables and figures. The assay mixture was allowed to equilibrate at 37°C for 10 min and the reaction was started by the addition of 100 nmoles of progesterone in 10 μl of ethanol. After 15 min incubation the reaction was stopped by the addition of 2 ml of heptane followed by a 10 s blending on a Vortex mixer. The aqueous and organic phases were separated by centrifugation. The absorbance of the aqueous phase due to the Δ4,3-keto configuration of progesterone was measured at 233 nm. The product of the incubation extracted into the organic solvent was identified as progesterone by thin layer chromatography. The extraction efficiency of progesterone standards from Medium B was 89% ± 2% (± S.E.), n = 26. Progesterone concentration was estimated using a molar absorptivity of 17,000 at 240 nm with the extinction coefficient of progesterone is shifted to 233 with no change in the extinction coefficient. The amount of progesterone was linearly related to the enzyme concentration.

Measurement of HSD Activity by Pregnenolone Disappearance—The reaction mixture was prepared and incubated to measure the rate of pregnenolone disappearance catalyzed by HSD. Mitochondria were preincubated for 10 min at 37°C in 0.3 ml of Medium B. The reaction was started by the addition of 12.5 nmoles of [7-³H]pregnenolone (4 mCi/mmol) in 0.2 ml of Medium B and terminated by adding 0.050 ml of a 5% v/v HgCl₂ solution. Mercuric chloride added prior to pregnenolone established a “zero” time point. Aliquots (0.1 ml) were removed and pregnenolone was measured in a Beckman model LS-100C liquid scintillation counting. Counting efficiency was determined by the external standards ratio.

Other Enzyme Assays—The following marker enzyme assays were used in the characterization of cell fractions obtained by differential centrifugation: HSD and the steroid 21-hydroxylase as markers for microsomes (23); cytochrome c oxidase as a marker for mitochondria (24); and glucose-6-PO₄ dehydrogenase as a marker for cytosol (25). Succinate-cytochrome c reductase activity was measured by the method of Sottocasa et al. (26). Neutralized perchloric acid extracts of mitochondria were assayed for NAD⁺ and NADH by the method of Estabrook et al. (27). Mitochondrial NADH and NADPH were measured as described by Klingenberg (28). Protein was determined by the method of Lowry et al. (29), or by a biuret method (30).

Ca²⁺-Loading and Sucrose Density Gradient Centrifugation of Adrenal Cortex Mitochondria—Massive Ca²⁺-loading was performed as described by Greenberg et al. (31). The medium consisted of 10 mM succinate, 10 mM MgCl₂, 4 mM KHPO₄, 0.15 mM sucrose, 3 mM ATP, 25 mM Tris/Cl, pH 7.4, and 0.1% w/v bovine serum albumin. Two milliliters of this solution were added to a 25-ml flask and brought to 37°C in a shaking water bath. Five milligrams of mitochondrial protein were added and allowed to equilibrate for 2 min before the reaction was initiated by the addition of 2 mM CaCl₂. After various times, Ca²⁺ uptake was stopped by the addition of 20 mM EGTA (32), followed by chilling in ice. The complete reaction mixture, usually about 2.5 ml, was then added to the top of a 15 to 55% w/w continuous sucrose gradient and centrifuged for 3 h at 25,000 rpm in a SW 25.1 swinging bucket rotor in a Spinco model L centrifuge. Fractions were collected from the bottom of the tube and were either frozen or assayed immediately. Sucrose concentrations were determined using a Goldberg T/°C refractometer.

Ca²⁺ uptake associated with O₂ consumption was measured using a Clark oxygen electrode. Calf adrenal cortex mitochondria (4 mg of protein) were added to an oxygen-saturated medium contained in a 1-ml oxygen electrode chamber. The incubation medium used was that developed by Carafoli and Lehninger (33) and contained 225 mM mannitol, 55 mM sucrose, 5 mM succinate, 5 mM KHPO₄, 0.1% w/v bovine serum albumin, and 10 mM Tris/Cl, pH 7.4. Oxygen consumption was recorded and the effect of Ca²⁺ (700 μM) on mitochondrial respiration was observed. After incubation at 30°C the complete mixture was layered atop a 15 to 55% w/w continuous sucrose density gradient. Centrifugation, fraction collection, and assay were as described for the massive Ca²⁺-loading experiment.

Sucrose Density Gradient Centrifugation for the Separation of Liver and Adrenal Mitochondria—Liver and adrenals were removed from rats killed by decapitation and placed in ice-cold 0.25 M sucrose. The adrenal glands were trimmed of adherent fat, blotted on tissue paper, and weighed. The liver was rinsed of blood prior to blotting and weighing. The cleaned adrenals (150 mg) were combined with an equal amount of liver tissue, minced together with scissors, and homogenized in Medium A. The homogenization procedure and isolation of the mitochondria were as described for the adrenal alone. The resulting mitochondrial pellet, which contained both liver and adrenal mitochondria, was washed twice by resuspension and recentrifugation (10 min at 5200 x g) in Medium A and was suspended in 30 mM Tris/HCl, pH 7.4, and 0.1% w/v bovine serum albumin. Mitochondrial NADH and NADPH were assayed for NAD⁺ and NADH by the method of Estabrook et al. (27). Mitochondrial NADH and NADPH were measured as described by Klingenberg (28). Protein was determined by the method of Lowry et al. (29) or by a biuret method (30). Ca²⁺-Loading and Sucrose Density Gradient Centrifugation of Adrenal Cortex Mitochondria—Massive Ca²⁺-loading was performed as described by Greenberg et al. (31). The medium consisted of 10 mM succinate, 10 mM MgCl₂, 4 mM KHPO₄, 0.15 mM sucrose, 3 mM ATP, 25 mM Tris/Cl, pH 7.4, and 0.1% w/v bovine serum albumin. Two milliliters of this solution were added to a 25-ml flask and brought to 37°C in a shaking water bath. Five milligrams of mitochondrial protein were added and allowed to equilibrate for 2 min before the reaction was initiated by the addition of 2 mM CaCl₂. After various times, Ca²⁺ uptake was stopped by the addition of 20 mM EGTA (32), followed by chilling in ice. The complete reaction mixture, usually about 2.5 ml, was then added to the top of a 15 to 55% w/w continuous sucrose gradient and centrifuged for 3 h at 25,000 rpm in a SW 25.1 swinging bucket rotor in a Spinco model L centrifuge. Fractions were collected from the bottom of the tube and were either frozen or assayed immediately. Sucrose concentrations were determined using a Goldberg T/°C refractometer.

RESULTS AND DISCUSSION

Enzyme Distribution—Table I lists the results of three experiments on the subcellular distribution of HSD in rat adrenal cortex tissue. As shown, adrenal cortex mitochondria washed four times contain 26% of the total homogenate HSD activity. Contribution to the mitochondrial HSD activity by microsomes was judged not to be a significant factor. When the mitochondria were assayed for microsomal contamination only 6% of the microsomal marker enzyme, 21-hydroxylase, was found. The ratio of HSD activity to 21-hydroxylase activity was calculated in both subcellular fractions. This is an important comparison, for if mitochondrial HSD activity resulted simply from microsomal contamination one should expect the ratio of HSD to 21-hydroxylase in the mitochondria to parallel its pronounced microsomal source closely. In the microsomes the ratio of the two enzymes is 2.8:1 whereas in the mitochondria it is 19.6:1. Moustafa and Koritz (16) suggested that this disparity is the result of microsomal heterogeneity, with microsomes richer in HSD activity selectively contaminating the mitochondrial fraction. To our knowledge differences in enzyme distributions have not been demon-
in adrenal cortex microsomes.

In a preliminary report Kream and Sauer (14) suggested that rat adrenal cortex mitochondria could utilize the NAD* in the matrix space as a cofactor for HSD activity. Using this information we reexamined the intracellular distribution of HSD, this time in the absence of added NAD* (Table II). HSD activity, active with endogenous NAD*, is clearly associated with the mitochondria. Even HSD activity found in the crude nuclear and microsomal fractions appears to be of mitochondrial origin. In the nuclear and microsomal fractions the percentage of mitochondrial contamination, as judged by cytochrome c oxidase activity, is almost identical with the percentage of HSD activity (with endogenous NAD*). Two separate controls were employed to ensure that HSD activity in the absence of added NAD* resulted solely from the utilization of matrix space NAD*. In one, glucose-6-PO4 and NAD*-linked glucose-6-PO4 dehydrogenase were added to reduce available NAD* to NADH. In the other experiment NADase (N. crassa) was added. Each one of these enzyme systems abolished or severely reduced HSD activity in microsomes supplemented with NAD* (not shown) and therefore should have inhibited HSD activity in the mitochondrial fraction if the activity were due to microsomes and exogenous NAD*. The results of these experiments are also shown in Table II. Neither system influenced the mitochondrial HSD distribution, suggesting that the endogenous NAD* used by the mitochondria remained enclosed within and protected by the inner membrane for the duration of the incubation and did not exit and become available to a microsomal contaminant.

**Calcium-loading Studies**—Greenawalt et al. (31) have shown that intact mitochondria are able to accumulate large amounts of Ca2+ in an energy-dependent process. Mitochondria sequestering Ca2+ in this manner become more dense and can be clearly differentiated from control mitochondria when both are subjected to sucrose density gradient centrifugation. This aspect of mitochondrial function was chosen to further define the nature of the mitochondrial HSD. Sucrose density gradient centrifugation is a well recognized technique for increasing the homogeneity of subfractionated organelles. Therefore, combining this technique with the known mitochondrial capacity of Ca2+ uptake could further differentiate between mitochondrial HSD activity and HSD activity thought to originate through microsomal contamination. In these experiments calf adrenal cortex mitochondria were first incubated under conditions for massive Ca2+-loading (see "Methods") and then centrifuged to equilibrium in sucrose density gradients. Fig. 1a is a photograph of the tubes after removal from the centrifuge and demonstrates the fact that Ca2+ accumulation increases mitochondrial density. Fig. 1b

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### Table I

**The distribution of 3β-hydroxy steroid dehydrogenase/isomerase (HSD) and marker enzymes in subcellular fractions of rat adrenal**

|                | Homogenate | Nuclear | Mitochondria | Microsomes | Cytosol | Washes | Recovery % |
|----------------|------------|---------|--------------|------------|---------|--------|------------|
| HSD            | 210.5 ± 42.9 | 37.6 ± 11.7 | 54.9 ± 13.8  | 80.1 ± 15.8 | N.D.*   | 30.4 ± 7.8 | 95         |
| Steroid 21-hydroxylase | 44.1 ± 3.1 | 6.9 ± 1.0  | 2.8 ± 0.49   | 28.6 ± 4.16 | N.D.    | 6.29 ± 2.26 | 98         |
| Cytochrome c oxidase    | 187.4 ± 36.9 | 40.6 ± 10.1 | 123.6 ± 18.2 | 2.8 ± 0.75  | N.D.    | 4.9 ± 0.88  | 93         |
| Glucose-6-PO4 dehydrogenase | 502.3 | 40 (21) | 28 (66) | 212 (1) | 4353 (1) | 99 (3) | 94         |
| Protein                      | 43.5 ± 1 | 9.6 ± 1.3 | 7.1 ± 0.5   | 3.2 ± 0.5   | 12.3 ± 2.5 | 4.4 ± 0.8 | 91         |

* N.D., not detectable

### Table II

**The distribution of cytochrome c oxidase and HSD activity (without added NAD*) among subcellular fractions of rat adrenal**

HSD activity was measured as described under "Methods", under "Measurement of HSD Activity by Progesterone Formation" except that NAD* was not added. Enzyme activities are given as nanomoles of progesterone per min per total fraction for the HSD assay and first order rate constant per min per total fraction for cytochrome c oxidase. The values represent means ± S.E. for three experiments. The numbers in parentheses refer to per cent recovery as compared to the total homogenate.

|                | Homogenate | Nuclear | Mitochondria | Microsomes | Cytosol | Recovery % |
|----------------|------------|---------|--------------|------------|---------|------------|
| Cytochrome c oxidase | 112.7 ± 25.6 | 26.1 ± 5.0 | 71.6 ± 18.3  | 2.0 ± 0.5   | N.D.*   | 91         |
| HSD            | 43.2 ± 3.0  | 10.8 ± 0.13 | 20.2 ± 1.8   | 1.35       | N.D.    | 75         |
| HSD*           | 43.3 ± 5.0  | 11.6 ± 0.6 | 22.3 ± 3.4   | 1.45       | N.D.    | 79         |
| HSD*           | 45.1        | 12.8    | 21.5         | 1.0        |         | 78         |

* N.D., not detectable.

**HSD activity was assayed as described above except that 2 mM glucose-6-PO4 and 1 unit of NAD* -linked glucose-6-PO4 dehydrogenase (L. mesenteroides) were added to each incubation.**

**The results of a single experiment in which all HSD activity was assayed as described above except that 0.025 unit of NADase (N. crassa) was added to each incubation.**
mitochondria was isolated from the combined tissue homogenate (not shown). b, the distribution of HSD and succinate-cytochrome c reductase activities versus fraction density following isopycnic sucrose density gradient centrifugation of the 0 (-----0), 45 (-----B), and 90 (-----C) s CaC2-loaded calf adrenal cortex mitochondria shown in a. HSD activity was measured as described under "Methods," under "Measurement of HSD Activity by Progesterone Formation" except that the incubation medium consisted of 50 mm Tris/HCl pH 7.4, 5 mm EGTA, 1 mm KCN, 0.012% sodium deoxycholate, 0.05 mM NAD+, 1 mM pyruvate, 1 unit of lactic dehydrogenase, and the incubation was for 30 min.

Figure 2. The distribution of HSD and succinate-cytochrome c reductase activities versus fraction density following isopycnic sucrose density gradient centrifugation of control (-----0) and CaC2-loaded (-----C) calf adrenal cortex mitochondria. Mitochondria were loaded with calcium by the procedure described under "Methods," under "CaC2 Loading and Sucrose Density Gradient Centrifugation of Adrenal Cortex Mitochondria." Calf adrenal cortex mitochondria (4 mg of protein) were incubated in a 1-m1 oxygen electrode chamber and the rate of mitochondrial respiration was stimulated by two additions of 350 nmol of CaC2. These CaC2-treated mitochondria and mitochondria from a control incubation which received an ADP addition (300 nmol, no CaC2) were centrifuged to equilibrium in a 15 to 55% w/w continuous sucrose density gradient for 3 h at 25,000 rpm in a SW 25.1 swinging bucket rotor. HSD activity was measured under the conditions described in the legend for Fig. 1b.
Effect of Reduction of Intramitochondrial NAD(P)+ on Mitochondrial HSD Activity—Mitochondrial HSD activity was found to be decreased by over 75% by the addition of citric acid cycle substrates (14) suggesting that reduction of the pyridine nucleotides in the matrix space inhibited mitochondrial HSD. To test this mechanism in more detail we measured the mitochondrial pyridine nucleotide content in rat adrenal cortex mitochondria in the presence of an uncoupler of oxidative phosphorylation ("1799"), and in the presence of pregnenolone as well as pregnenolone + succinate and α-ketoglutarate. The measured NADP+ + NADPH and NAD+ + NADH values agreed well with the values reported by Purvis et al. (34). Mitochondria (1.1 mg of protein/ml) incubated with 4 μM "1799" (pregnenolone was not added) contained 2.2, 1.1, 2.9, and 0 nmol/mg of protein of NADP+, NADPH, NAD+, and NADH, respectively. Mitochondria incubated with 0.1 mM pregnenolone contained 2.5, 1.1, 2.9, and 0 nmol/mg of protein of NADP+, NADPH, NAD+, and NADH, respectively. Mitochondria incubated with 0.1 mM pregnenolone plus 10 mM α-ketoglutarate and 10 mM succinate contained 0, 2.5, 0.4, and 2.7 nmol/mg of protein of NADP+, NADPH, NAD+, and NADH, respectively. Pregnenolone did not alter the oxidation-reduction state of the pyridine nucleotides probably because the rate of HSD activity (3.0 nmol of progesterone·min⁻¹·mg⁻¹) is only about one-seventeenth that of the rate of respiration linked to NADH oxidation measured under these conditions (18). Fig. 4 shows that α-ketoglutarate and succinate inhibited progesterone formation by over 80%. Under these conditions only about 0.4 nmol of NAD+/mg of protein remained available to the HSD.

Fig. 4 also shows that malonate, a competitive inhibitor of succinic dehydrogenase, reverses the inhibition of mitochondrial HSD activity due to succinate. This result is further evidence for the mitochondrial location of the HSD activity. These results suggest that the rate of mitochondrial HSD activity is dependent on the oxidation-reduction state of intramitochondrial NAD+, the dynamics of which are regulated through substrate selection and availability as well as energy requirements of the cell.

Moustafa and Koritz (16) examined the intracellular distribution of HSD in the rat adrenal and reported that mitochondria contain 13% of the total homogenate HSD activity, a value somewhat smaller than our result (Table I). The assay procedure used by Moustafa and Koritz (16) employed an NAD+ regenerating system consisting of lactic dehydrogenase and pyruvate. These agents removed the inhibitor NADH and sustained Vmax, HSD activity in microsomes (20). However, pyruvate is capable of reducing intramitochondrial NAD+ which in turn could inhibit mitochondrial HSD activity. As Fig. 4 shows, the addition of pyruvate to a preparation of intact adrenal cortex mitochondria did inhibit mitochondrial HSD activity by about 50%. Thus, reduction of intramitochondrial NAD+ following addition of pyruvate may explain...
the lower percentage of HSD activity found in the mitochondrial fraction by these investigators.

Rat adrenal cortex mitochondria can form 11β-hydroxyprogesterone from progesterone in the presence of citric acid cycle substrates (35). This steroid, which is more polar and therefore is not extracted by heptane as well as is progesterone, could lead to errors in measurement of HSD activity based on extractable progesterone. Therefore, we also assessed the effect of citric acid cycle substrates on mitochondrial HSD activity as measured by the rate of pregnenolone disappearance (22). No differences were noted in the results obtained by either assay method (not shown). Citric acid cycle substrates inhibited mitochondrial HSD activity measured by pregnenolone disappearance as well as by pregnenolone formation.

Effect of Mitochondrial Swelling on HSD Activity—Calcium is known to promote swelling of the mitochondrial inner membrane (36). Through this action otherwise impenetrable metabolites have free access to, and exit from, the mitochondrial matrix area. For example, adrenal cortex mitochondria in the presence of 10 mM CaCl₂ can utilize exogenous NADPH to support high rates of steroid hydroxylation (37). Pyridine nucleotides do not normally penetrate intact mitochondria. As Fig. 5 shows, rat adrenal cortex mitochondria swollen by 10 mM CaCl₂ lose most of their HSD activity (active with endogenous NAD⁺). We interpret the loss in activity to be the result of exit and subsequent dilution of intramitochondrial NAD⁺, a conclusion reinforced by the observation that HSD activity can be restored to the swollen mitochondria by the addition of exogenous NAD⁺. When NAD⁺ was added to "intact" adrenal cortex mitochondria HSD activity was enhanced 2- to 3-fold. The reason for this increase in HSD activity is not completely understood. Certainly, a part of the HSD activity (23% of the increment as calculated from Tables I and II) dependent on added NAD⁺ is due to microsomal contamination. Microsomes plus a contribution from damaged mitochondria could be the reason for this added HSD activity. Further research on mitochondrial HSD activity in the presence of added NAD⁺ will be presented in a subsequent paper.

Mersalyl is an organic mercurial that has been used as a tool to demonstrate the presence and location of mitochondrial inner membrane carrier systems (38). Because of its charge and size mersalyl does not rapidly penetrate an intact mitochondrial inner membrane. Therefore, loss in enzymatic or transport activity through the use of mersalyl suggests that the active site is on the outside of the inner membrane.

Mersalyl at a concentration of 40 μM destroys all HSD activity in rat adrenal cortex microsomes (Fig. 6A). Results were similar in the presence or absence of Ca²⁺. However, when mersalyl was added to adrenal cortex mitochondria only HSD activity dependent on exogenous NAD⁺ (0.5 mM) was affected, and not the HSD active with intramitochondrial NAD⁺ (Fig. 6B). Support for this conclusion is obtained by comparing mitochondrial HSD activity (3.2 nmol of pregnenolone formed/min/mg) in the absence of both exogenous NAD⁺ and mersalyl (top arrow, Fig. 6B) to mitochondrial HSD activity (3.8 nmol of pregnenolone formed/min/mg) at 40 μM mersalyl plus 0.5 mM NAD⁺ (Fig. 6B). In separate experiments, mersalyl at concentrations as high as 50 μM had no effect on mitochondrial HSD activity dependent on matrix area NAD⁺ (data not shown).

The inhibition of mitochondrial HSD in the presence of Ca²⁺ plus mersalyl further indicates that the NAD⁺ active site for mitochondrial HSD is located on the matrix side of the inner membrane. In the absence of calcium, mersalyl affects only the exogenous NAD⁺-dependent mitochondrial HSD activity suggesting that this HSD activity is present in microsomes or damaged mitochondria and is available to react with mersalyl. When calcium is present the mitochondria swell (36) and all mitochondrial HSD activity is abolished by mersalyl (see HSD activity at 40 μM mersalyl + 10 mM CaCl₂, Fig. 6B).

On the basis of these studies we conclude that adrenal cortex mitochondria contain an HSD that is distinct from that found in the endoplasmic reticulum. This conclusion is reinforced by the following observations. Mitochondria contain a substantial percentage of the total homogenate HSD activity; the mitochondrial HSD activity is not removed either by repeated washing or by sucrose density gradient centrifugation; the active site of the mitochondrial HSD faces the matrix space and utilizes matrix space NAD⁺; mersalyl inhibits microsomal HSD activity but not mitochondrial HSD activity dependent on matrix NAD⁺.

The role of mitochondrial HSD in vivo and its relationship to microsomal HSD are unknown. The data we have presented here suggest that mitochondrial HSD is regulated by the oxidation-reduction state of the matrix space NAD⁺. Hochberg et al. (13) reported that the addition of succinate influenced the products of cholesterol side chain cleavage catalyzed by rat adrenal mitochondria. The addition of succinate changed the ratio of pregnenolone to progesterone formed from 0.71 to 5.1. The total amount of C₂₀ steroids produced was unaltered. In view of our results, these data suggest that mitochondrial HSD is regulated by the ratio of matrix space NAD⁺ to NADH.

Fig. 6. A, effect of mersalyl on rat adrenal microsomal HSD activity in the presence or absence of Ca²⁺. Rat adrenal microsomes (43 μg of protein) were incubated for 15 min in Medium B containing 0.5 mM NAD⁺ and 0.1 mM pregnenolone. Mersalyl was present at the concentration indicated on the abscissa. The points connected by the dashed line represent incubations that also contained 10 mM CaCl₂. B, effect of mersalyl on rat adrenal mitochondrial HSD activity in the presence or absence of Ca²⁺. Rat adrenal mitochondria (119 μg of protein) were incubated for 15 min in Medium B containing 0.5 mM NAD⁺ and 0.1 mM pregnenolone. Mersalyl was present at the concentrations shown on the abscissa. CaCl₂ (10 mM) was present in the incubations connected by the dashed line. The two points on the ordinate shown by the arrows indicate the HSD activity of the mitochondria in the absence of added NAD⁺ (□), and in the presence of 10 mM CaCl₂ and the absence of NAD⁺ (△).
may be interpreted to mean that succinate, by reducing intra-mitochondrial NAD⁺, inhibited the mitochondrial HSD and decreased conversion of pregnenolone to progesterone. The importance of mitochondrial HSD in controlling the product of cholesterol side chain cleavage which leaves the mitochondrion cannot be determined at this time. However, HSD is located at a branch point in steroidogenesis through which steroid precursors for glucocorticoid and mineralocorticoid synthesis must pass. Therefore, a mitochondrial location for HSD in close proximity to the initial rate limiting, cholesterol side chain cleavage step, could be a deciding factor in determining the ultimate steroid products of the adrenal cortex.

Finally, because of their different intracellular locations and properties, mitochondrial inner membrane HSD and microsomal HSD may be considered as isoenzymes. The proteins may be coded for by different genes and be inserted into their respective membrane sites by different mechanisms. Consequently, it will be of great interest to determine whether the two HSD apoenzymes are identical or different.

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