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Steroidogenesis in the Zona Glomerulosa of the Adrenal Cortex I. Isolation and Some Properties of Mitochondria from the Zona Glomerulosa of the Bovine Adrenal Cortex

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Abstract

Isolation procedures for mitochondria from the zona glomerulosa of the bovine adrenal cortex are described and the properties of the mitochondria thus prepared are compared with those isolated from the zona fasciculoreticularis. The cristal membranes of mitochondria in the zona glomerulosa in situ are tubular or tubulovesicular, whereas those of mitochondria in the zona fasciculoreticularis in situ are vesicular. When mitochondria are isolated from the former zone, they invariably showed the condensed configuration regardless of isolation media, whereas those isolated from the latter zone in an ST medium showed the orthodox configuration. When Ca^{2+} was added to mitochondria isolated either from the zona glomerulosa or the zona fasciculoreticularis in an STE medium in the condensed configuration, a transition from the condensed to the orthodox configuration took place; the cristal membranes of mitochondria from the zona glomerulosa became tubular or tubulovesicular and those of mitochondria from the zona fasciculoreticularis became vesicular. Contaminations of mitrochondria of the zona glomerulosa with other cellular organelles were examined using various marker enzymes. There was no difference in cytochrome content between mitochondria of the two zones specified above. The coupling efficiency of mitochondria of the zona glomerulosa was found to be remarkably effected by temperature during the isolation procedures.

Effects of various substrates, isolation media, and bovine serum albumin on the coupling efficiency of mitochondria of the zona glomerulosa are also described.

Introduction

Steroidogenesis in the adrenal cortical gland has been studied extensively by many workers using mitochondria or microsomes isolated from the bovine [1–19], the rat[20–28] or the pig[15, 22, 29–32] adrenal gland. For example, the enzyme system which catalyzes the 11β-hydroxylation of deoxycorticosterone is associated with the mitochondrial fraction of the adrenal cortex. Harding et al. [21] showed that cytochrome P-450 occurs in adrenocortical mitochondria. All these studies have been restricted to the zona fasciculata (including a portion of the zona reticularis) or to the whole cortex.

On the other hand, steroidogenesis in the zona glomerulosa has remained unsolved, since isolation procedures for subcellular fractions such as mitochondria or microsomes have not been established. Thus, the metabolic pathway of aldosterone in the zona glomerulosa of the adrenal cortex remains unsolved.

Previously we have reported the isolation procedures and the identification of mitochondria from the zona glomerulosa of the bovine adrenal cortex as a preliminary note[33].

Allmann et al. [34–36] have shown that mitochondria isolated from the zona fasciculata (plus a minor portion of the zona reticularis) are unique in that they can be isolated either in condensed (aggregated) or in the orthodox (vesicular) configuration depending on isolation media. Mitochondria isolated from the zona glomerulosa, on the other hand, are invariably isolated in the condensed (aggregated) configuration regardless of isolation media[33].

The present study was undertaken to learn more about the isolation procedures and coupling efficiency of mitochondria from the zona glomerulosa of bovine adrenal cortex in comparison with those from the zona fasciculoreticularis. In the present communication, which is the second of this series, the distribution of cytochrome P-450 in the zona glomerulosa together with some biochemical and ultrastructural characteristics of the microsomal fraction obtained from the zone will be described.

Experimental Procedures

Isolation of Mitochondria from the Zona Glomerulosa of Bovine Adrenal Cortex

The excised bovine adrenals of 13–15 animals which had been slaughtered were collected, placed in ice, and brought to the laboratory

within 1.0–1.5 hr of the death of the animal. The glands were trimmed to remove fat and cut in half longitudinally. The central medulla and a minor portion of the adjacent zona reticularis were scraped away and discarded. The rest of the cortex (mainly the zona fasciculata, a minor portion of the zona reticularis, and a minor portion of the zona glomerulosa) was scraped away and collected.

Finally the zona glomerulosa adhering to the capsule was obtained by scraping. In the course of the study it turned out that the temperatures at which the procedures described above were carried out (from the bisecting of the adrenals until scraping and collecting of the zona glomerulosa and the zona fasciculata) play a key role in determining the coupling efficiency of isolated adrenal cortex mitochondria. The following alternative methods were then employed: (1) The adrenals were bisected from the animal and kept at room temperature until the zona glomerulosa and the zona fasciculoreticularis were scraped and collected. (2) Adrenals were trimmed to remove fat immediately after they were bisected from the animal, placed in ice, and brought to the laboratory. They were kept in ice for additional 3–4 hr.

Mitochondria were always prepared both from the zona glomerulosa and the zona fasciculoreticularis at the same time in each experiment for cross comparison. In some experiments, however, mitochondria were prepared from the whole cortex (the zona glomerulosa + the zona fasciculoreticularis) without separating the above described two zones.

The yields of the zona glomerulosa and the zona fasciculoreticularis thus obtained were 1.05 ± 0.06 g and 5.21 ± 0.28 g, respectively (Table 1). The zona glomerulosa and the zona fasciculoreticularis were then suspended in the medium. The suspension was homogenized in a glass–teflon homogenizer. The isolation of mitochondria was accomplished in the following various media: (a) a solution which was 0.25 M sucrose and 10 mM Tris-Cl, pH 7.5 (ST); (b) a solution which was 0.25 M sucrose, which had been treated with cation-exchange resin (Amberlite MB-3), and 10 mM Tris-Cl, pH 7.5 [ST (Ca^{2+}-free)] [34]; (c) a solution

TABLE I. Yield of mitochondria isolated from the zona glomerulosa [a]

	Zona glomerulosa	Zona fasciculoreticularis
Scraped tissues/one gland (g)	1.05 ± 0.06	5.21 ± 0.28
R_2'''/one gland (mg)	12.84 ± 2.28	36.20 ± 13.10
R_2'''/scraped tissues (%)	1.02 ± 0.40[b]	0.69 ± 0.24
R_2'''/homogenates (%)	4.73 ± 1.58[b]	3.70 ± 1.02

[a] Data were obtained from 10 different experiments. Each experiment consisted of 15–20 adrenal glands. Values are expressed as mean ± SD.

[b] The difference was statistically significant in comparison to the zona fasciculoreticularis ($0.001 < P < 0.01$).

which was 0.25 M sucrose, 10 mM Tris-Cl, pH 7.5, and 0.1 mM NaK–EDTA (STE); and (d) a solution which was 0.21 M mannitol, 0.07 M sucrose, 5 mM Tris-Cl, pH 7.5, and 0.1 mM NaK–EDTA (MSTE). In some experiments, bovine serum albumin (BSA) (Fraction V, essentially fatty acid-free, Sigma Chemical Co.) was added to the various isolation media, specified above, at a concentration of 0.1%.

The homogenates obtained by the procedures described above were centrifuged for 10 min at 900 *g* in a Spinco No. 30 rotor, and the residue was discarded. The supernatant fluid was then centrifuged for 10 min at 9000 *g* in the same rotor. The fluffy layer was discarded, and the heavy mitochondrial pellet was washed two times at the same speed in the same rotor. The pellet thus obtained was finally suspended in the medium and centrifuged for 10 min at 9000 *g* in a Spinco No. 40 rotor. The fluffy layer was again discarded, and the residue was suspended with sufficient medium so that the final protein content was 30–50 mg/ml. The protein concentration was determined by the biuret method of Gornall et al. [37] in the presence of deoxycholate. Heavy beef heart mitochondria were prepared by Nagase digestion of beef heart muscle by the method of Hatefi et al. [38] as modified by Ozawa [39]. Mitochondria were also prepared as a control from mouse liver by the method of Hogeboom et al. [40].

Biochemical Assays and Probes

Concentration of reagents. The final concentrations of the reagents which were added are shown in paranthesis: rotenone (2 μg/mg of protein), ADP (500–1000 nmol/mg of protein), potassium phosphate, pH 7.4 (10 mM), mCl–CCP (1 μM), and 2,4-dinitrophenol (20–40 μM).

Measurement of Oxygen Uptake. The rate of consumption of oxygen was measured at 25° C with a Clark-type electrode (Beckman Co., Fullerton, California). Unless stated otherwise, the experiments were carried out in 5 ml of medium, 0.25 M sucrose (Ca^{2+}-free), 0.01 M Tris-Cl, pH 7.4, and 3 mM $MgCl_2$, and containing 5 mg of mitochondria. The oxidizable substrate in most experiments was succinate (5 mM, potassium salt). Other oxidizable substrates were L-glutamate, DL-β-hydroxybutylate, malate, and pyruvate. They were potassium salts of pH 7.4.

Assay of Oxidative Phosphorylation. Oxidative phosphorylation was measured by the conversion of inorganic phosphate to esterified phosphate concomitant with the oxidation of succinate at 25° C.

Cytochrome Content. Cytochrome content of the mitochondrial fraction was measured essentially according to the method of Blair et al. [42] using the Shimazu multipurpose recording spectrophotometer model MPS-50L.

Purity of the Mitochondrial Fraction with Respect to Contamination with Other Cellular Components

For examination of purity of the mitochondrial fraction obtained from the zona glomerulosa, the following marker enzymes were assayed: cytochrome *c* oxydase (cytochrome *c* : 0_2 oxidoreductase, EC 1.9.3.1), a marker for the inner mitochondrial membranes, was tested at 30° C polarographically with a Clark-type electrode. Activity was measured by the oxygen consumption of mitochondria before and after the addition of ascorbate to the reaction mixture. The reaction medium contained 0.25 M sucrose, 2 mM ascorbate, 10 mM Tris-Cl, pH 7.4, and cytochrome *c* (0.5 mg/ml) (Type IV, Sigma Chemical Co.). Lysolecithin (1.0–1.2 mg/mg of mitochondrial protein) was added to the reaction mixture as a solubilizing agent in order to obtain full activity. Glucose-6-phosphatase (EC 3.1.3.9), a marker for microsomes, was assayed essentially according to the method of Appelmans et al. [43]. The reaction mixture contained 8 mM glucose-6-phosphate, 50 mM cacodylate buffer, pH 6.5, and appropriate amounts of samples (total of about 2 mg of protein). The reaction mixture was incubated for 10 min at 37° C, and the reaction was stopped by the addition of perchloric acid. The precipitated protein was removed by centrifugation, and inorganic phosphate in the supernatant was determined by the method of Fiske and Subbarow [44]. Acid phosphatase (EC 3.1.3.2), a marker for lysosomes was measured as described by Appelmans et al. [43]. Inorganic phosphate after liberation was determined as in the case of glucose-6-phosphatase. Catalase (EC 1.11.1.6), a marker for microbodies, was measured essentially according to the method of Baudhuin et al. [45]. The reaction mixture was incubated at 0° C for 5 min in a total volume of 4.5 ml containing 10 mM imidazole HCl buffer, pH 7.2, bovine serum albumin (0.1%), and hydrogen peroxide (0.008%). The reaction was stopped by the addition of 2 ml of a threefold dilution of a saturated solution of titanium sulfate (titanium(IV) oxysulfate, Sigma Chemical Co.) in $2NH_2SO_4$, and the remaining hydrogen was determined spectrophotometrically.

All the samples had been frozen–thawed 10 times before assays were carried out to release the enzymes specified above, except for cytochrome oxidase in which freshly prepared samples were used.

Morphological Analysis

For light microscopic examination the adrenal gland was fixed in formalin and stained with hematoxylin–eosin. For electron microscopic examination samples were fixed with glutaraldehyde which was 0.25 M sucrose ane 0.05 M cacodylate (potasium salt) at a final pH of 7.4. A sample was mixed with an equal volume of glutaraldehyde solution to a

final concentration of 0.1%. The mixture was kept for 10 min at 25° C. The sample was then sedimented in a clinical centrifuge for 30 min, the pellets then being washed in a medium, 0.25 M sucrose and 0.05 M K cacodylate (at pH 7.4). After interaction with glutaraldehyde, the pellets were then exposed to 0.1% osmium tetroxide. After postfixation with osmium tetroxide, samples were first exposed to 25% ethanol containing 1% uranyl acetate before dehydration with ethanol solutions of graduallly increasing ethanol concentration. The exposure to 100% ethanol was repeated three times. Dehydrated samples were then exposed twice to absolute propylene oxide for 10 min. For embedding, the dehydrated samples were exposed first to a mixture of equal parts by volume of propylene oxide and Epon (for 4 hr). Specimens were sectioned with a diamond knife or glass knives and were examined in a Hitachi 12 or 11D electron microscope operated at 75 kV.

Results

Identification and the isolation procedures for the zona glomerulosa of the bovine adrenal cortex were described in full in a previous communication [33]. The yield of mitochondria obtained from the zona glomerulosa is shown in Table I. Yields of mitochondria of the zona glomerulosa obtained from scraped tissues and homogenates are higher than those from mitochondria of the zona fasciculoreticularis. This may be mainly due to the fact that the zona fasciculoreticularis contains a larger amount of blood than the zona glomerulosa.

Ultrastructure of Mitochondria in Situ in the Zona Glomerulosa

Figure 1 shows an electron micrograph in which the capsule (+ a portion of the zona glomerulosa) thus obtained was fixed in glutaraldehyde and postfixed in osmium tetroxide. A cell of the zona glomerulosa is seen beneath the capsule. At higher magnifications mitochondria in the zona glomerulosa are clearly discernible (Fig. 2A). Mitochondria in the zone have tubular or tubulovesicular cristae compared with mitochondria in the zona fasciculoreticularis (Fig. 2B) which have been scraped away from the capsule as reported previously [33]. Mitochondria in the fasciculoreticularis have vesicular cristae which are characteristic of mitochondria in steroid-producing organs.

Configuration of Mitochondria of the Zona Glomerulosa Isolated in Various Media

When mitochondria are isolated from the zona glomerulosa either in a sucrose–Tris (ST) medium (Fig. 3A) or in a sucrose–Tris–EDTA (STE)

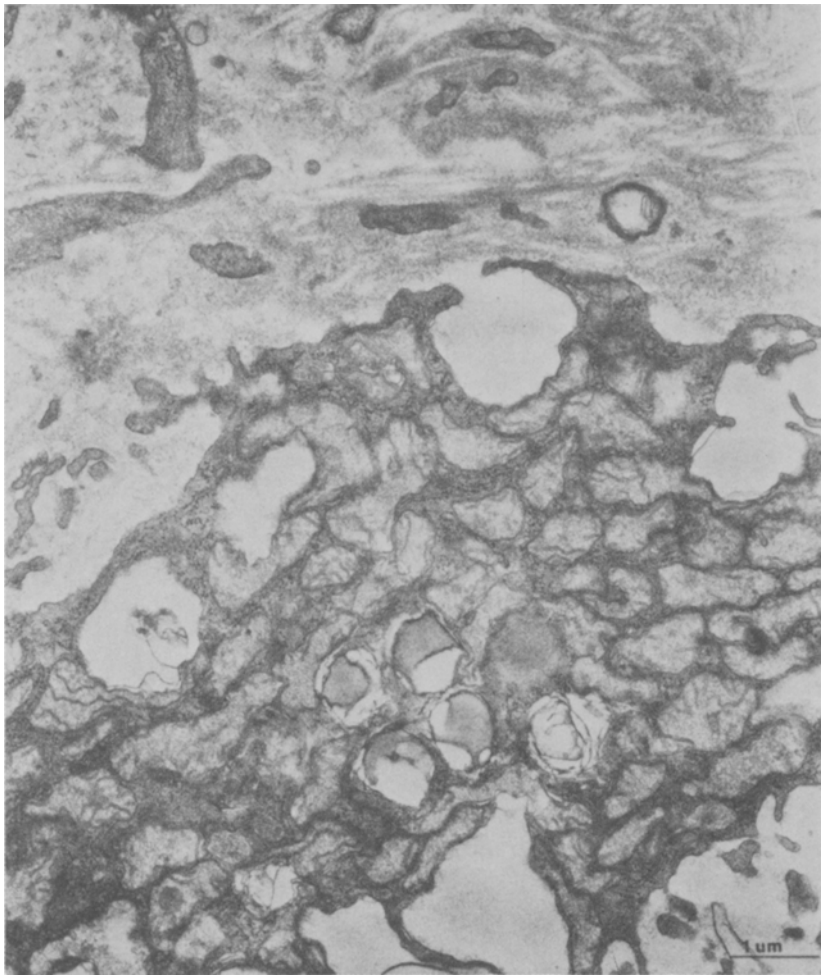
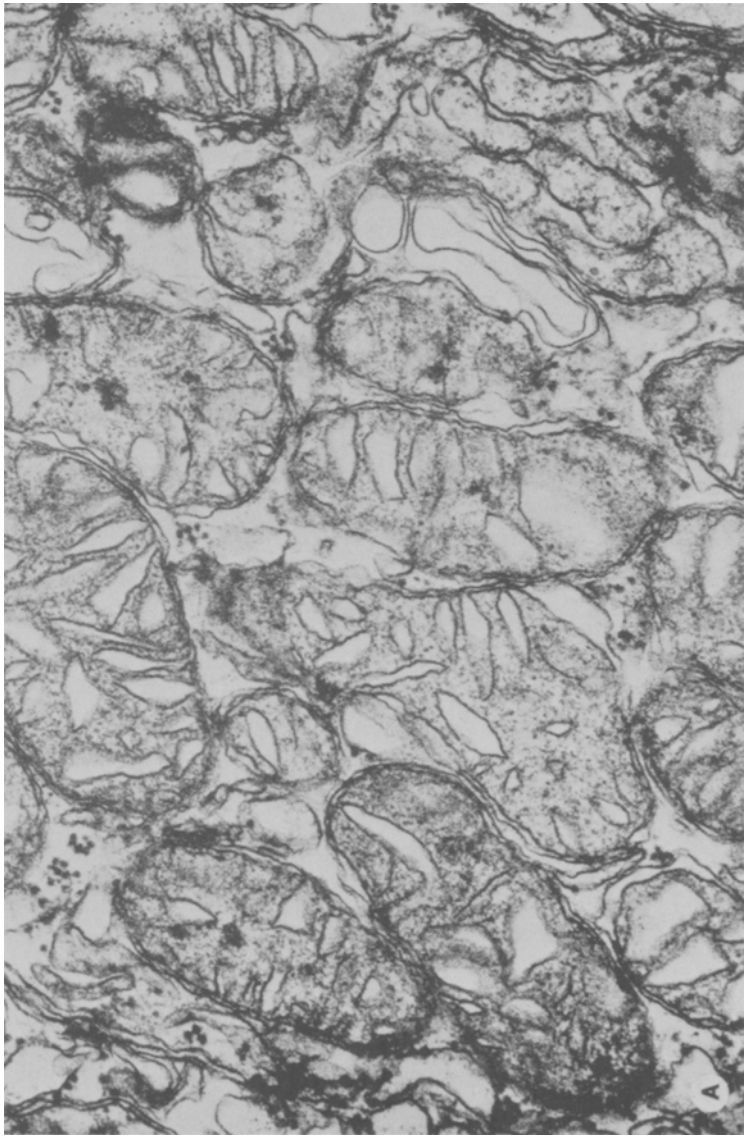


Figure 1. A low-magnification electron micrograph of a zona glomerulosa cell. A capsule from which both the medulla and the cortical tissue has been scraped away was fixed in glutaraldehyde and postfixed in osmium tetroxide. Underneath the capsule a zona glomerulosa cell is seen (22,500. Reduced 45% for reproduction).

medium (Fig. 3B), they invariably show the condensed (aggregated) configuration in which the matrix space is highly condensed and the intracristal space is expanded as reported previously [33]. Mitochondria in the zona fasciculoreticularis isolated in a STE medium show the condensed configuration (Fig. 3C), whereas they show the orthodox



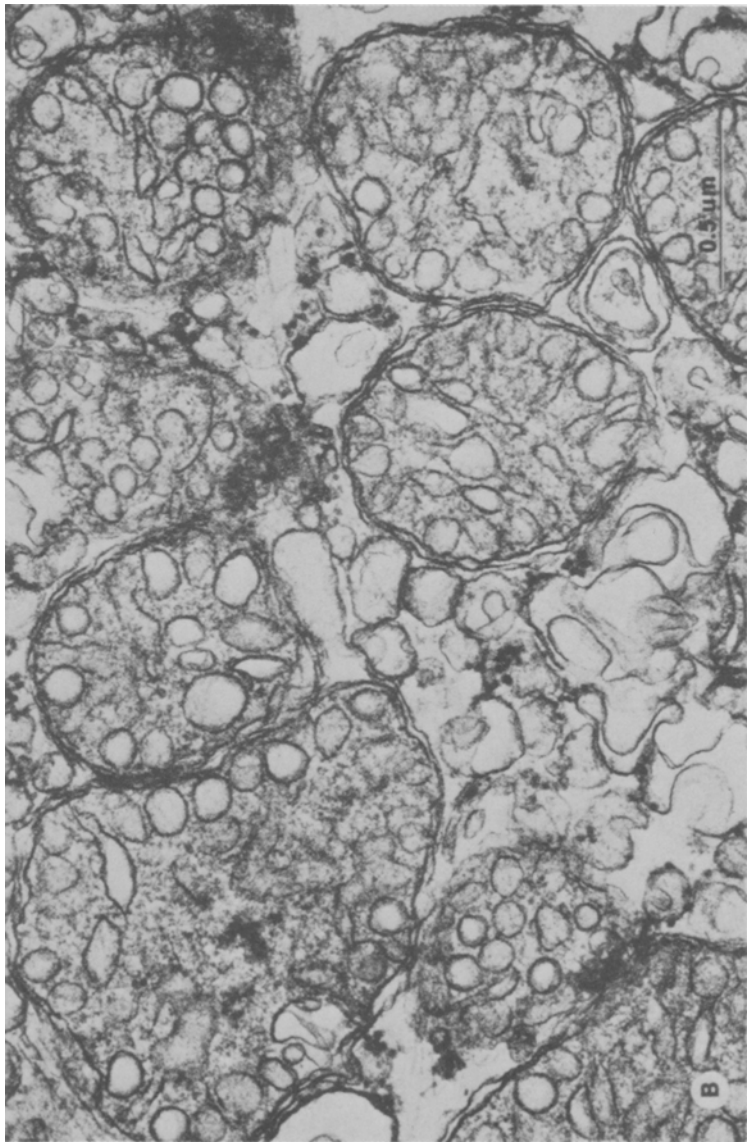
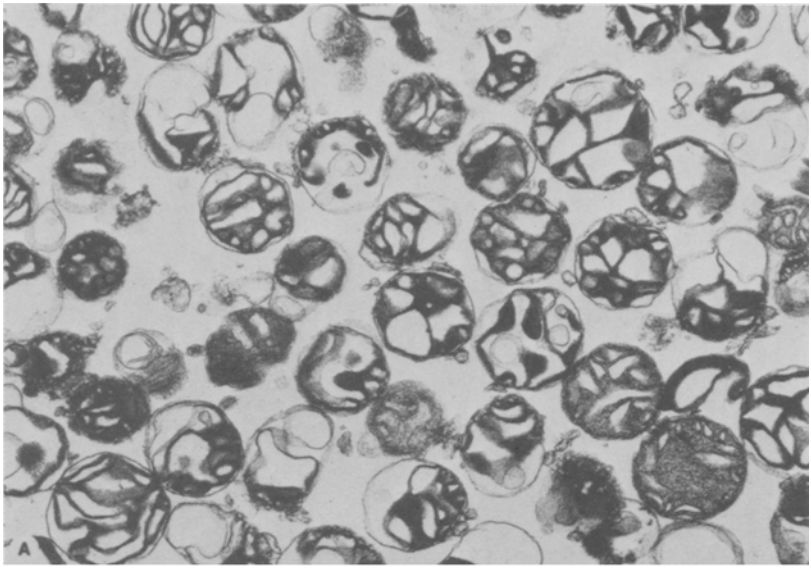


Figure 2. Higher-magnification electron micrographs of the zona glomerulosa (A, 60,000) and the zona fasciculoreticularis (B, 60,000). (Illustrations reduced 25% for reproduction).


A


B
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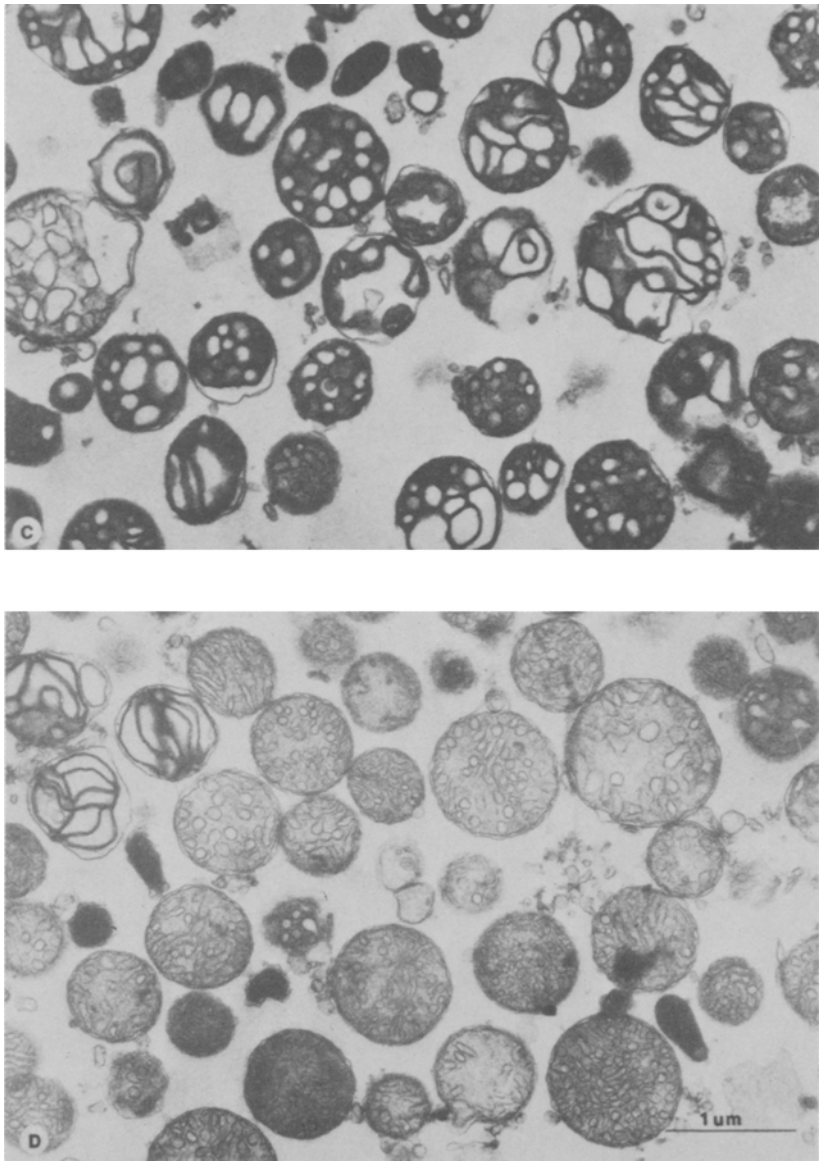


Figure 3. Configurations of adrenal cortex mitochondria isolated in various media. Mitochondria of the zona glomerulosa isolated in an ST medium (A, 30,000) or in an STE medium (B, 30,000) show the condensed configuration. Mitochondria of the zona fasciculoreticularis isolated in an STE medium (C, 30,000) show the condensed configuration; they show the orthodox configuration in an ST medium (D, 30,000). (All illustrations reduced 40% for reproduction).

(vesicular) configuration in more than 70% of the population when they are isolated in a ST medium (Fig. 3D). In the orthodox configuration, the volume of the intracristal space is minimal and the volume of the matrix space in maximal.

When mitochondria are isolated from the zona glomerulosa in a Ca^{2+}-free sucrose–Tris [ST(Ca^{2+}-free)] medium or in a mannitol–sucrose–Tris–EDTA (MSTE) medium, they again show the condensed configuration, whereas mitochondria isolated from the zona fasciculoreticularis in a ST(Ca^{2+}-free) medium show a mixed population of both the condensed and the orthodox configuration, and they show the condensed configuration in a MSTE medium.

Thus, mitochondria isolated from the zona glomerulosa invariably showed the condensed configuration regardless of isolation media, whereas the configuration of mitochondria isolated from the zona fasciculoreticularis varied depending on isolation media.

Effect of Ca^{2+} on the Configuration of Mitochondria Isolated from the Zona Glomerulosa

In the previous section it has been shown that mitochondria from the zona glomerulosa are isolated in the condensed configuration regardless of isolation media. To study the effect of Ca^{2+}, mitochondria of the zona glomerulosa or the zona fasciculoreticularis isolated in a STE medium were used since commercial sucrose is contaminated with Ca^{2+} [34].

When mitochondria isolated either from the zona glomerulosa or the zona fasciculoreticularis in a STE medium are incubated with Ca^{2+}, the transition from the condensed to the orthodox configuration takes place. In Fig. 4 mitochondria isolated from the zona glomerulosa (Fig. 4A) or from the zona fasciculoreticularis (Fig. 4B) in an STE medium were incubated with Ca^{2+} for 5 min at 25° C at a concentration of 200 nmol/mg of mitochondrial protein. Although both mitochondrial preparations isolated from the different zones showed the orthodox configuration after incubation with Ca^{2+}, mitochondria isolated from the zona glomerulosa have tubular or tubulovesicular cristae, whereas mitochondria isolated from the zona fasciculoreticularis have vesicular cristae. The correlation between the isolation medium and the configuration of adrenal cortex mitochondria and the effect of Ca^{2+} on their configuration are summarized in Table II and Fig. 5. The amount of Ca^{2+} required for the transition of the cristal membrane from the condensed to the orthodox (tubular or tubulovesicular) configuration in mitochondria of the zona glomerulosa ranged from 200–400 nmol/mg of protein depending on isolation media. The amounts of Ca^{2+} required to induce the orthodox configuration were essentially the same in mitochondria of the zona fasciculoreticularis. As was described before, mitochondria isolated either from the zona glomerulosa or the zona

fasciculoreticularis in a STE medium showed the condensed configuration. However, it must be stressed here that the details in ultrastructure are different for these two types of mitochondria, as is shown in Fig. 5. The inner membranes of mitochondria isolated from the zona fasciculoreticularis in the condensed configuration are somewhat vesicular, whereas those of mitochondria isolated from the zona glomerulosa in the condensed configuration are rather straight and parallel.

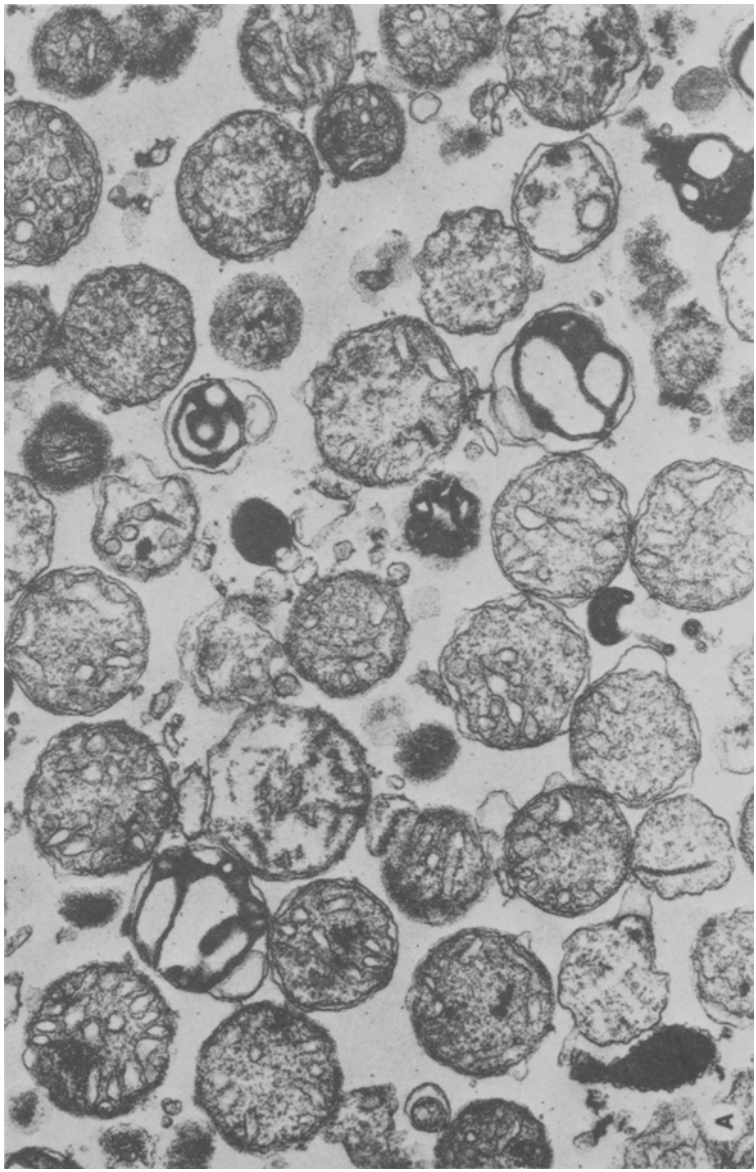
TABLE II. Correlation between the isolation medium and the configuration of adrenal cortex mitochondria and the effect of Ca^{2+} on their configuration

Isolation medium	Sources of mitochondria	Configuration	
		Initial	Final[a]
Sucros-Tris	Z. gl.	Condensed	Tubular or tubulovesicular
	Z. fas.ret.	Vesicular	Vesicular
Sucrose-Tris (Ca^{2+}-free)	Z. gl.	Condensed	Tubular or tubulovesicular
	Z. fas.-ret.	Condensed + vesicular	Vesicular
Sucrose-Tris-EDTA	Z. gl.	Condensed	Tubular or tubulovesicular
	Z. fas.-ret.	Condensed	Vesicular
Mannitol-sucrose-Tris-EDTA	Z. gl.	Condensed	Tubular or tubulovesicular
	Z. fas.-ret.	Condensed	Vesicular

[a] Mitochondria isolated either from the zona glomerulosa or from the zona fasciculoreticularis in various media specified in the Table were incubated with Ca^{2+} at concentrations ranging from 200 to 400 nmol/mg protein for 5 min at 25°C.

Purity of Mitochondria Isolated from the Zona Glomerulosa with Respect to Reference Enzyme Activities

In the previous section, it was shown that mitochondria isolated from the zona glomerulosa are distinguished electron microscopically from those isolated from the zona fasciculoreticularis. Thus, various reference enzymes have been assayed in mitochondrial fractions obtained from the zona glomerulosa in order to examine contaminations with other cellular organelles (Table III). As is readily seen in the table, the specific activity of glucose-6-phosphatase in the zona glomerulosa is much lower than that in the liver. In the liver, the specific activity of the enzyme in three times washed mitochondrial fraction (R_2''') is significantly decreased



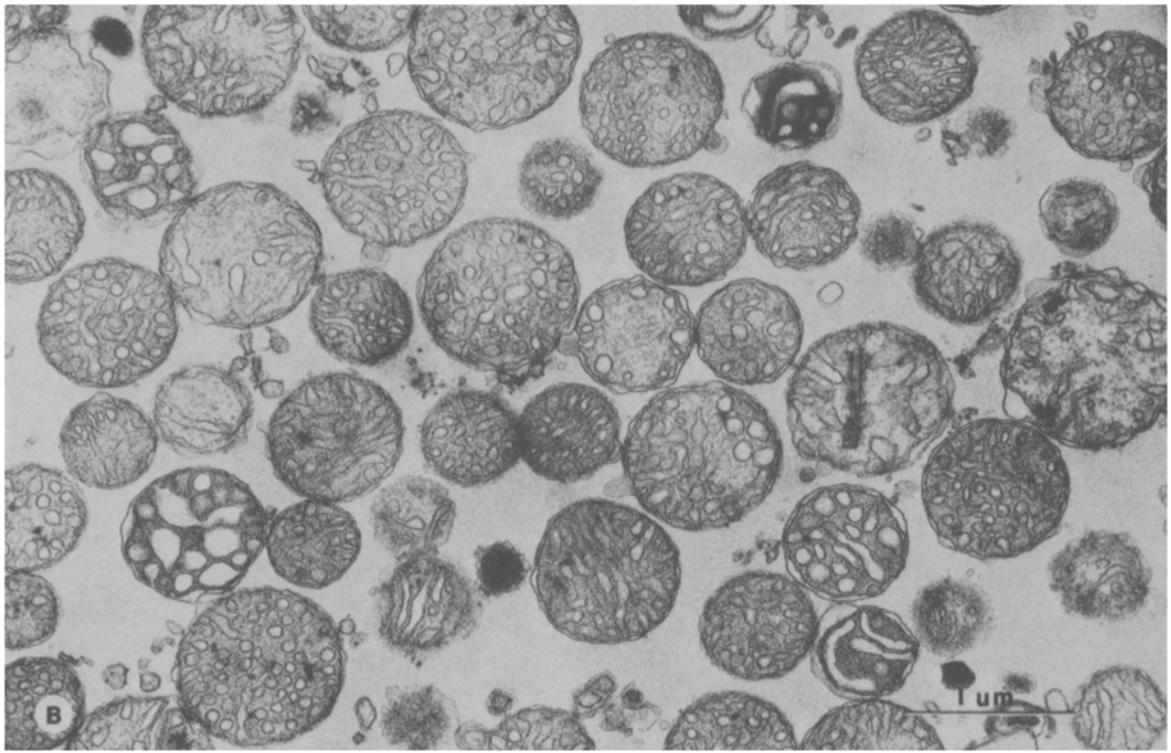


Figure 4. Induction of the orthodox configuration in adrenal cortex mitochondria by Ca^{2+}. Mitochondria isolated from the zona glomerulosa (A, 30,000) or from the zona fasciculoreticularis (B, 30,000) in an STE medium were incubated with Ca^{2+} at a concentration of 200 nmol/mg of protein for 5 min at 25° C. (Illustrations reduced 25% for reproduction).

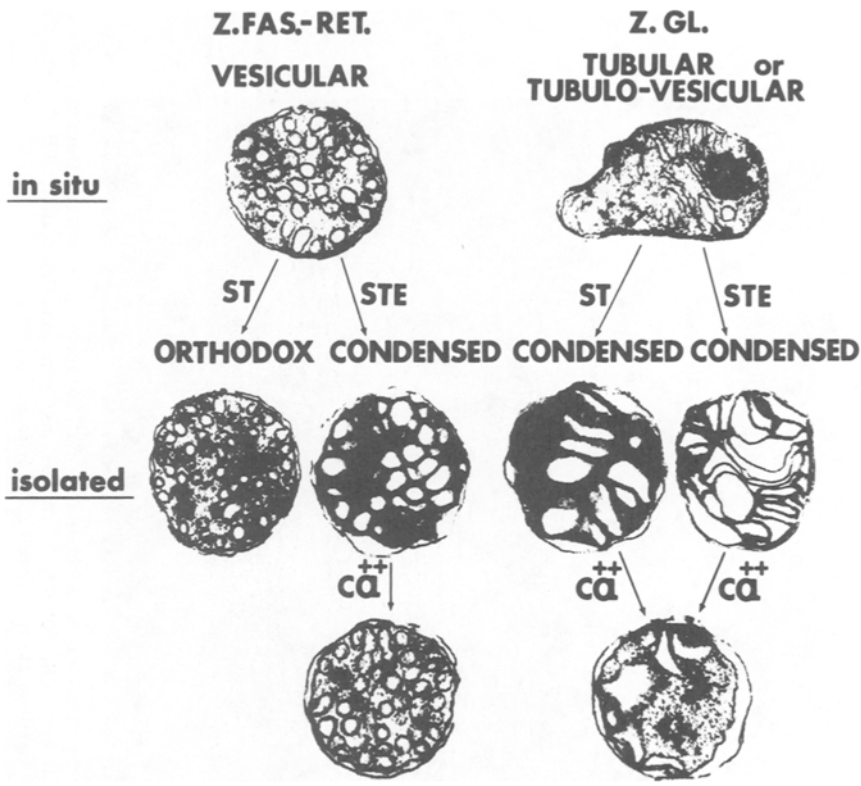


Figure 5. Demonstration of typical mitochondria isolated either from the zona glomerulosa or the zona fasciculoreticularis. Note mitochondria of the zona glomerulosa differ in arrangement of the cristal membranes from those of the zona fasciculoreticularis both in the condensed and orthodox configurations.

compared with that in a crude mitochondrial fraction (R_2), whereas the specific activity of the enzyme in a crude mitochondrial fraction of the zona glomerulosa stayed almost at the same level after three washings. Catalase activity in the zona glomerulosa was again quite low compared with that in the liver. Details of the distribution of the two enzymes described above will be discussed in a later paper.

Isolation Medium and the Coupling Efficiency of Mitochondria Isolated from the Zona Glomerulosa

Mitochondria isolated from the zona glomerulosa were poorly coupled regardless of whether the isolation medium was ST, ST(Ca^{2+}-free) or STE medium (Table IV and Fig. 6). When succinate was used as the oxidizable substrate (in the presence of rotenone), the respiratory

TABLE III. Purity of mitochondria isolated from the zona glomerulosa with respect to reference enzymes[a,b]

	Glucose-6-phosphatase			Acid phosphatase		
	Z. gl. S.A (T.A)	Z. fas.-ret. S.A. (T.A)	Liver S.A (T.I)	Z. gl. S.A (T.A)	Z. fas.-ret. S.A (T.A)	Liver S.A (T.A)
Homogenate	10.8 (100.0)	8.3 (100.0)	37.0 (100.0)	18.2 (100.0)	13.8 (100.0)	8.4 (100.0)
R_2	6.4 (10.0)	5.7 (8.3)	26.7 (3.7)	15.1 (14.0)	10.8 (9.7)	16.6 (14.6)
R_2'''	6.2 (3.3)	3.6 (1.9)	8.7 (0.7)	16.2 (5.0)	9.9 (3.2)	20.3 (7.0)
	Cytochrome oxidase			Catalase		
	Z. gl. S.A (T.A)	Z. fas.-ret. S.A (T.A)	Liver S.A (T.A)	Z. gl. S.A (T.A)	Z. fas.-ret. S.A (T.A)	Liver S.A (T.A)
Homogenate	65.4 (100.0)	65.2 (100.0)	92.7 (100.0)	4.7 (100.0)	4.4 (100.0)	179.1 (100.0)
R_2	250.0 (35.0)	244.8 (28.8)	141.1 (1.3)	1.3 (1.9)	1.6 (1.6)	499.2 (30.2)
R_2'''	307.0 (12.0)	285.6 (10.7)	340.0 (15.6)	0.3 (0.6)	0.4 (0.5)	500.9 (13.4)

[a] Specific activities (S.A.) are expressed in nmol P_i/mg/min for glucose-6-phosphatase and acid phosphatase, n-atoms oxygen/mg/min for cytochrome oxidase, and $-A_{410} \times 10$/mg/min for actalase.

[b] Total activity (T.A. = S.A. × total protein) of homogenates is expressed as 100%.

TABLE IV. Effect of isolation medium on coupling efficiency of mitochondria isolated from the adrenal cortex[a]

Preparation medium	Oxygen uptake											
	Z. glomerulosa				Z. fasciculoreticularis				Whole cortex			
	State 4[b]	State 3	RCI	P/O	State 4[b]	State 3	RCI	P/O	State 4[b]	State 3	RCI	P/O
Sucrose-Tris	47.7	62.8	1.3	0.6	40.6	58.2	1.4	0.7				
Sucrose-Tris (Ca^{2+}-free)	45.0	53.1	1.2	0.6	50.1	64.3	1.3	0.5				
Sucrose-Tris-EDTA	57.9	67.0	1.2	0.5	47.3	50.0	1.1	0.5	52.0	62.4	1.2	0.4
Mannitol-sucrose-Tris-EDTA									51.9	61.1	1.1	0.4

[a] The basic experimental conditions are as described in the legend of Fig. 6A. The oxidizable substrate was succinate at a final concentration of 5 mM.

[b] State 4 shows a slow respiration due to absence of phosphate acceptor and state 3 shows a rapid respiration by virtue of the presence of the phosphoryl acceptor, ADP. The numbers in the columns show rates of oxygen consumption in n-atoms/min/mg protein.

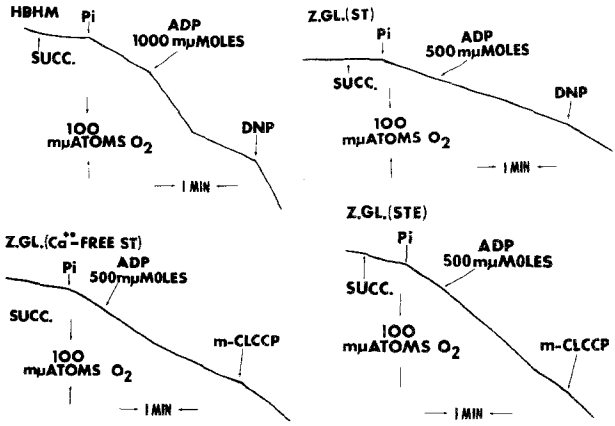


Figure 6A. Correlation between the nature of the isolation medium and the oxygen utilization of mitochondria isolated from the zona glomerulosa. The reaction mixture was 0.25 M sucrose (Ca^{2+}-free), 10 mM Tris-Cl, pH 7.4, and 3 mM $MgCl_2$, and contained rotenone (2 μg/mg of mitochondrial protein); and 1–2 mg protein/ml (5-ml system). Succinate (5 mM), P_i (10 mM), and ADP (500–1000 nmol) were added where indicated by arrows. The temperature of the reaction cell was 25° C. The uncouplers DNP and mCl-CCP were added at final concentrations of 20 μM and 1 μM, respectively.

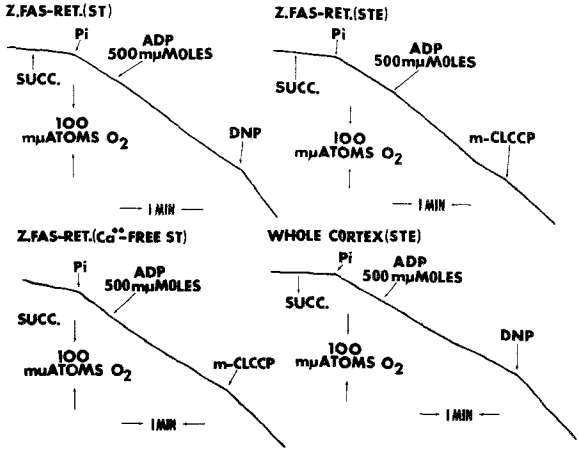


Figure 6B. Correlation between the nature of the isolation medium and the oxygen utilization of mitochondria isolated from the zona fasciculoreticularis. The experimental conditions are described for Fig. 6A. Preparation of a mitochondrial fraction from the whole adrenal cortes is described in the text. The oxidizable substrate was succinate (5 mM).

control index (RCI) ranged from 1.2 to 1.5 and the P/O ratio from 0.4 to 0.6. 2,4-Dinitrophenol or mCl-CCP enhanced the respiration 1.5–2.0 times compared with the state 4 respiration. A typical polarographic trace obtained from heavy beef heart mitochondria isolated in a STE medium is shown in Fig. 6A as a control. Mitochondria isolated from the zona fasciculoreticularis also showed a poorly coupled state regardless of isolation media (Fig. 6B). In these cases, adrenal glands were buried in a rather large amount of fat when they were brought to the laboratory in ice.

It is well known that the whole procedure must be carried out as soon as possible to obtain a tightly coupled mitochondrial preparation. Since it takes a long time to scrape the zona glomerulosa from the capsule; it requires 6–7 hr to go through the entire procedure in order to obtain mitochondrial preparations from the zona glomerulosa and the zona fasciculoreticularis. The reason for obtaining a poorly coupled mitochondrial preparation from the adrenal cortex was first ascribed to such long preparation times. However, even when adrenal cortical tissues were scraped from the capsule, neglecting zonal differences (the whole cortex free from the medulla) to shorten the preparation time, it was still not possible to obtain well-coupled mitochondria from such a preparation (see Table IV). Moreover, the respiratory control was not improved when a mitochondrial fraction was prepared from the whole cortex in a STE medium which is often employed to obtain tightly coupled mitochondria from the liver.

When adrenal glands buried in a large amount of fat were brought to the laboratory without chilling on ice and the scrapings of the zona glomerulosa and the zona fasciculoreticularis were carried out at room temperature, mitochondrial preparations obtained from such tissues were as poorly couples as those mitochondrial preparations shown in Table IV regardless of isolation media.

Effect of temperature on Isolation Procedures of Mitochondria from the Zona Glomerulosa

If adrenal glands buried in thick fat were brought in ice to the laboratory, only the surface of the adrenal gland was cold when fat was removed. Mitochondria prepared from such adrenal glands were, as described above, uncoupled.

When adrenal glands were trimmed to remove fat immediately after the glands were obtained, and kept in ice for 1–2 hr, not only the surface but also inner parts of the gland were chilled. Mitochondrial preparations from such adrenal glands in an STE medium now revealed improved respiratory control and ADP/O ratios when succinate was oxidizable substrate (Table V). However, ADP/O ratios were not much improved when malate was the oxidizable substrate. Pyruvate, β-hydroxybutyrate,

and glutamate were ineffective as substrates for oxygen utilization. Moreover, such mitochondrial preparations obtained from the adrenal glands which had been chilled enough in ice did not show improved ADP/O ratios when they were isolated in an ST medium.

TABLE V. Effect of albumin on rates of oxygen utilization by mitochondria isolated from zona glomerulosa by addition of various substrates[a]

Substrate		Oxygen uptake[b]			
		State 4	State 3	RCI	ADP/O
Succinate	−BSA	13.1	50.0	3.8	1.7
	+BSA	8.7	67.9	7.8	1.8
Malate	−BSA	11.1	40.5	3.6	1.9
	+BSA	2.9	30.1	10.4	2.9
Glutamate					
Glutamate + malate	−BSA	5.8	40.1	6.9	2.2
	+BSA	5.8	31.9	5.5	2.7
Pyruvate					
Pyruvate + malate	−BSA	6.4	33.1	5.2	2.1
	+BSA	3.4	21.1	6.1	2.7

[a] Mitochondria of the zona glomerulosa were isolated in an STE medium from the adrenal gland which had been thoroughly chilled (see text for explanation).
[b] The rate of consumption of oxygen is expressed in nmol/mg protein.

Effect of Bovine Serum Albumin on Coupling Efficiency of Mitochondria of the Zona Glomerulosa

Allman et al. [36] have shown that mitochondria isolated from the zona fasciculata (+ a portion of the zona reticularis) in an ST medium are uncoupled, but they restore normal P/O ratios in the presence of bovine serum albumin. Thus, the effect of bovine serum albumin on the coupling state of mitochondria of the zona glomerulosa was also examined.

First, mitochondria of the zona glomerulosa or the zona fasciculoreticularis were prepared from the adrenal glands which had not been chilled enough: the exicised adrenal gland was buried in a large amount of retroperitoneal fat, placed on ice, and the scraping of the zona glomerulosa and the zona fasciculoreticularis was carried out within 1 hr. Mitochondria thus obtained from either zone were uncoupled when succinate was oxidizable substrate as described before. However, as shown in Fig. 7, the ADP/O ratios increased after the addition of albumin. In Fig. 7 mitochondria of the zona glomerulosa or the zona

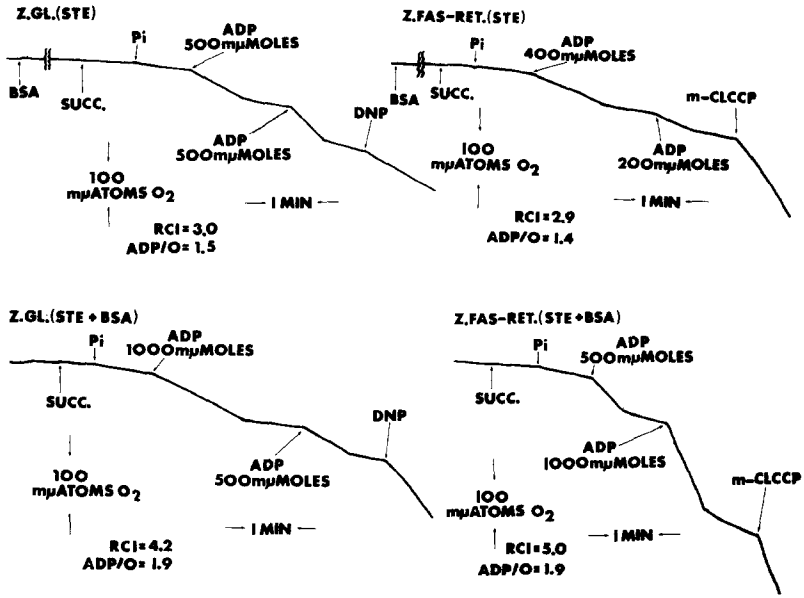


Figure 7. Effect of albumin on the coupling efficiency of mitochondria isolated from the zona glomerulosa. Mitochondria were isolated from the zona glomerulosa of the bovine adrenal cortex which had not been thoroughly chilled (see text for explanation) in an STE medium in the presence or absence of albumin (in the former case, the isolation medium contained albumin at a concentration of 0.1%). The reaction mixture was 0.25 M sucrose (Ca^{2+}-free), 10 mM Tris-Cl, pH 7.4, 3 mM $MgCl_2$, and rotenone (2 μg/mg protein). Mitochondria were incubated with albumin at a final concentration of 0.1% for 3 min at 25° C, and then succinate (5 mM), P_i (10 mM), and ADP were added to the reaction mixture.

fasciculoreticularis obtained from the adrenal glands under the conditions specified above were incubated with albumin at a final concentration of 0.1% (5 ml system) for 3 min at 25° C, and then succinate, P_i, and ADP were added to the reaction mixture. Both respiratory control and ADP/O ratios were considerably improved. Moreover, when mitochondria were isolated from such adrenal glands in the presence of albumin, respiratory control was further improved and ADP/O ratios were significantly restored. Such effects of albumin on the respiratory states were noted on mitochondria isolated either from the zona glomerulosa or from the zona fasciculoreticularis regardless of isolation media.

Second, the effect of albumin was examined on mitochondria of the zona glomerulosa or the zona fasiculoreticularis isolated from adrenal glands which had been thoroughly chilled: the adrenal gland was

trimmed to remove fat immediately after it was excised from the animal, and placed in ice for 3 hr. Mitochondria of the zona glomerulosa obtained from such adrenal glands revealed a well-coupled state when succinate was used as the substrate as described before (see Table V). However, mitochondria were not well coupled when substrates other than succinate are used as the oxidizable substrates. In Table V, an experiment is described in which albumin was added to the reaction mixture at a final concentration of 0.1%, and mitochondria of the zona glomerulosa isolated in an STE medium from the adrenal glands specified above were incubated with the reaction mixture for 3 min (1 mg mitochondria/1 ml of the reaction mixture; 5-ml system). Respiratory ratios and ADP/O ratios thus obtained were now improved not only when succinate but also malate was used as oxidizable substrate. These ratios were not improved, however, when glutamate, pyruvate, and β-hydroxybutyrate were used as substrates.

Cytochromes of Mitochondria Isolated from the Zona Glomerulosa

Table VI summarizes the cytochrome content of mitochondria isolated from the zona glomerulosa. As is seen in the table, the cytochrome content in mitochondria either from the zona glomerulosa or the zona fasciculoreticularis are much lower than those in mitochondria from the heart. This might be correlated with the fact that the cristae of mitochondria of the zona glomerulosa or the zona fasciculoreticularis are not well developed compared with those of mitochondria of the the heart. Cytochrome content in mitochondria of the zona glomerulosa or the zona fasciculoreticularis is higher than in mitochondria of the liver. This, however, does not seem to parallel in structural appearance of these mitochondria. Cristae of mitochondria of the liver are poorly developed

TABLE VI. Cytochrome content and ATPase activity in mitochondria isolated from zona glomerulosa[a]

Sources of mitochondria	Component (nmol/mg protein)				ATPase[b]	
	$a + a_3$	b	$c + c_1$		−DNP	+DNP
Zona gl.	0.51	0.42	0.23	Z. gl.	99.2	119.8
Zona fas.-ret.	0.63	0.52	0.26	Z. fas.-ret.	84.1	96.6
Rat heart[c]	0.98[c]	0.76[c]	0.83[c]	Beef heart	522.4	573.8
Rat liver[c]	0.27[c]	0.28[c]	0.38[c]	Mouse liver	115.9	133.9

[a] Respiratory-chain cytochromes were determined from absolute and difference spectra using the extinction coefficient indicated (42).
[b] Specific activity is expressed in nmol P_i/mg/min.
[c] Data from J.N. Williams, Jr., *Biochim. Biophys. Acta*, 162 (1968), 175.

compared with those of adrenal cortex mitochondria either from the zona glomerulosa or the zona fasciculoreticularis. Moreover, it is interesting to note that both cytochrome oxidase (see Table III) and ATPase activities in adrenal cortex mitochondria from either zone are lower than those in the liver mitochondria.

Contents of cytochrome $c + c_1$ obtained in the present study were significantly lower ($a + a_3 : b : c + c_1 = 1 : 0.85 : 0.45$ for mitochondria of the zona glomerulosa, and $1 : 0.83 : 0.42$ for mitochondria of the zona fasciculoreticularis) than those of mitochondria isolated from the rat heart or liver ($a + a_3 : b : c + c_1 = 1 : 0.87 : 0.85$ and $1 : 1.04 : 1.41$, respectively). It takes rather a long time to prepare mitochondria from the adrenal cortex and, more likely, repeated washing of mitochondrial fractions might have resulted in the loss of some cytochrome *c*.

Discussion

Identification of Mitochondria Isolated from the Zona Glomerulosa

In contrast to other types of mitochondria from mammalian tissues, mitochondria from the zona fasciculoreticularis of the adrenal cortex have vesicular types of cristae in the orthodox configuration in situ. Moreover, they can be isolated in the orthodox configuration in an ST medium. It has been stressed by several workers [7, 21–22] that mitochondria isolated from the adrenal cortex are polymorphous; some have condensed cristae and some have vesicular cristae. However, Allmann et al. have clearly demonstrated that the configuration of adrenal cortex mitochondria is regulated by the nature of the isolation media [34, 35]. This was established also in the present study. Other types of mitochondria from mammalian tissues, e.g., heart or liver, invariably show the condensed configuration regardless of isolation media.

Thus, the zona fasciculoreticularis of the adrenal cortex is the only case where mitochondria can be isolated in the same configuration as they appear in situ. Mitochondria in the zona glomerulosa, on the other hand, have tubular or tubulovesicular cristae in the orthodox configuration in situ. When they are isolated, they invariably show the condensed configuration regardless of isolation media. Their ultrastructure differs from mitochondria isolated from the zona fasciculoreticularis in the condensed configuration. Thus, mitochondria isolated from the zona glomerulosa are distinguished from those isolated from the zona fasciculoreticularis either in the orthodox or in the condensed configuration.

Effect of Chilling the Adrenal Gland on Coupling Efficiency of Mitochondria

In the previous communication we have shown that mitochondria isolated from the zona glomerulosa in an STE medium are well-coupled when succinate was the oxidizable substrate and that those in an ST medium are uncoupled [33]. However, it is clear from the data presented in this study that mitochondria from the zona glomerulosa isolated in an STE medium are uncoupled if the adrenal gland is not chilled enough before isolation procedures for mitochondria start. Even when the adrenal gland is chilled in ice for several hours immediately after the animal is slaughtered, only the surface of the adrenal gland beneath the thick adipose tissue is cold. Cammer and Estabrook have extensively studied the coupling efficiency of mitochondria isolated from the adrenal cortex; they have claimed that respiratory control ratios greater than 3 have not been observed and that the ADP/O ratios for malate were constantly in excess of 1 but never greater that 2 [12]. However, it has been shown in the present study that respiratory control ratios can exceed even 4, and the ADP/O ratios for malate are greater than 2 if the adrenal gland is thoroughly chilled in ice and proper isolation media for mitochondria are employed.

Ca^{2+} as a Mediator for the Orthodox–Condensed Transition in Mitochondria Isolated from the Zona Glomerulosa

It has been shown that mitochondrial cristal membranes isolated from the zona glomerulosa undergo the transition from the condensed to the orthodox configuration by Ca^{2+}.

Several other reagents are known to be modulators for the condensed (aggregated) to the orthodox configurational transition. Harris et al. [46] have shown that endotoxin of *Bordetella bronchioseptica* induces the orthodox configuration in heart mitochondria. Silicomolybdate [36] is another such reagent in the case of adrenal cortex mitochondria.

Ca^{2+}, as described before, is a modulator for the condensed–orthodox configurational transition in adrenal cortex mitochondria (isolated either from the zona glomerulosa or from the zona fasciculoreticularis), but the reagent could not induce the orthodox configuration in beef heart or mouse liver mitochondria if the same conditions as have been employed for the adrenal cortex mitochondria were employed (200 nmol Ca^{2+}/mg protein for 5 min at 25° C). Since mitochondria isolated from the zona glomerulosa in an ST medium do not show the orthodox configuration, the order of the sensitivity of mitochondria to Ca^{2+}, as far as the configuration is concerned, is as follows: mitochondria of the zona fasciculoreticularis, mitochondria of the zona glomerulosa, and mitochondria of the heart or liver. Considering the fact that Ca^{2+} is required

to stimulate steroidogenesis in the adrenal cortical gland [20, 23–25, 47–50], it seems important that adrenal cortical mitochondria are sensitive to Ca^{2+}. It is interesting that the cristae of mitochondria of the zona glomerulosa in the orthodox configuration in situ or after addition of Ca^{2+} are mainly tubular and not vesicular, and yet those mitochondria have cytochrome P-450 [51]. Distribution of cytochrome P-450 in the zona glomerulosa and several biochemical and structural characteristics of microsomal fraction obtained from the zone will be reported in a later paper.

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