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ULTRACYTOCHEMICAL LOCALIZATION OF DEHYDROGENASE IN THE RAT ADRENAL CORTEX

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The ultracytochemical localization of 3β-hydroxysteroid dehydrogenase (3β-HSD) and glucose-6-phosphate dehydrogenase (G6PD) was investigated in cells of the rat adrenal cortex using copper ferrocyanide. After incubation in a 3β-HSD medium, final reaction products of copper ferrocyanide were observed in the cytoplasmic matrix near the smooth endoplasmic reticulum (SER) and in the intracristal spaces of mitochondria of the fascicular zone and reticular zone. No deposits appeared in the Golgi apparatus or nuclei. Cells of the glomerular zone were devoid of enzyme activity. The final reaction products of copper ferrocyanide of the G6PD activity were found mainly in the cytoplasmic matrix in the vicinity of the plasma membrane of the fascicular zone. Some cells of the glomerular zone demonstrated the same reaction products in the cytoplasmic matrix. This finding was consistent with the fact that G6PD is a cytoplasmic enzyme. The result of our ultracytochemical study on the localization of 3β-HSD activity has not always been in accord with the biochemical data in cell fraction reporting that most of the 3β-HSD activity is associated with the microsomal fraction.

3β-HSD is a very important enzyme as a catalyzer when oxidizing the 3β-hydroxysteroid to a 3-ketosteroid and regulates the biosynthesis of steroid hormones. It is, therefore, attractive to study and discuss the function of steroidogenesis in the adrenal cortex. Most of the reports on the localization of 3β-HSD in cell fraction are inconsistent; the enzyme is localized either in the microsomal fraction (5, 9) or in the mitochondrial fraction (12), or in both fractions (8, 11). The precise ultracytochemical localization of the 3β-HSD activity is even more controversial. According to the ultracytochemical studies reported, reaction products of steroid producing cells are noted in mitochondria (6) or in SER (2, 7), or both organelles (1, 3, 10).

G6PD is widely distributed in living cells, plays a key role in controlling pentose sugar synthesis and is quite important in corticosteroid synthesis because reduced nicotinamide-adenine dinucleotide phosphate (NADPH) is utilized by hydroxylase in forming all the classes of adrenal steroids. The zonal distribution of glucose-6-phosphate dehydrogenase corresponds with the hydroxylation in the adrenal cortex. Although it has been reported that the accurate ultrastructural demonstration of the G6PD activity is difficult for its soluble nature, we succeeded in demonstrating the ultracytochemical localization of G6PD activity in well preserved rat adrenal with the method of Berchtold (4).

MATERIALS AND METHODS

Both adrenals of male Wistar rats cut into four pieces were fixed in a mixture of 1% formaldehyde and 0.25% glutaraldehyde or that of 2% formaldehyde and 2% glutaraldehyde in a 0.1 M phosphate buffer at pH 7.4, for 20–30 min at 0–4°C. After fixation the tissue was washed in the same buffer for 30 min and cut by Vibratome (Oxford) into sections of 40–100 μm or by a razor blade into small blocks of 0.5–1 mm^3. The sections were washed again in the phosphate buffer at pH 7.4 for 30 min before incubation.

The sections were then incubated in the following media (Table 1) which were essentially those of Berchtold. The incubation were carried out for 1 hr in the G6PD medium and for 90 min or 2 hr in the 3β-HSD medium at 37°C under shaking in darkness.

After incubation the sections were quickly washed three times in the phosphate buffer at pH 7.4. They were stored for one night in the same buffer at 0–4°C, postfixed in a 1% osmium tetroxide solution for 1 hr, dehydrated in acetone and carefully embedded in Epon 812. Ultra-thin sections stained with lead citrate were examined in the JEOL 100-B electron microscope. Control sections were incubated in a substrate-free medium, in a medium containing Antimycin A (30 μg/ml) and in that of Rotenone (33 μg/ml).

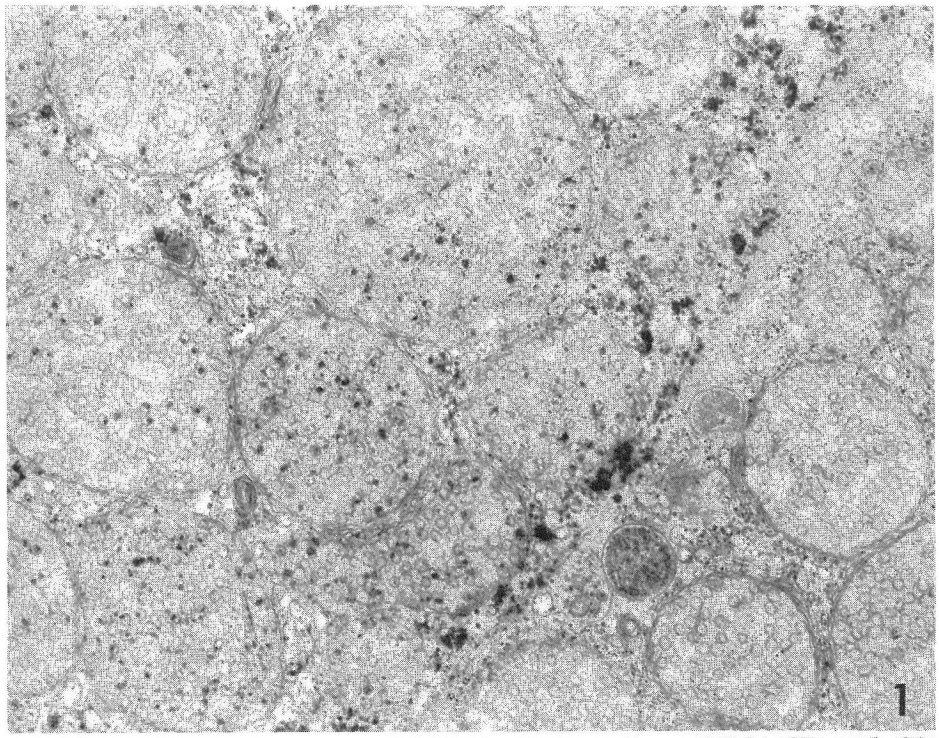
RESULTS

1. *Incubation in the 3β-HSD medium*

Final reaction products of copper ferrocyanide appeared in cells of the inner portion of the fascicular zone and the reticular zone. The final reaction products consisted of electron dense granular deposits measuring up to 300Å in diameter (Fig. 2)

TABLE 1.

Incubation medium for 3β-hydroxysteroid dehydrogenase (3)		
Dehydroepiandrosterone (or Etiocholane 3β-ol-17one)	(in 0.6 ml DMSO)	2.0 mg
NAD	(in 6.4 ml phosphate buffer pH 7.2)	10.0 mg
0.1 M Sodium citrate solution		1.0 ml
15 mM Copper sulfate solution		1.0 ml
5 mM Potassium ferricyanide solution		1.0 ml
Phenazine methosulfate (PMS)		1.5 mg
Total		10.0 ml
Incubation medium for glucose-6-phosphate dehydrogenase (4)		
Disodium (or Monosodium) salt D-glucose-6-phosphate		31.9 mg
NADP		9.9 mg
0.1 M Sodium citrate solution		1.0 ml
15 mM Copper sulfate solution		1.0 ml
5 mM Potassium ferricyanide solution		1.0 ml
0.1 M tris buffer (pH 7.2)		7.0 ml
Phenazine methosulfate (PMS)		1.5 mg
Total		10.0 ml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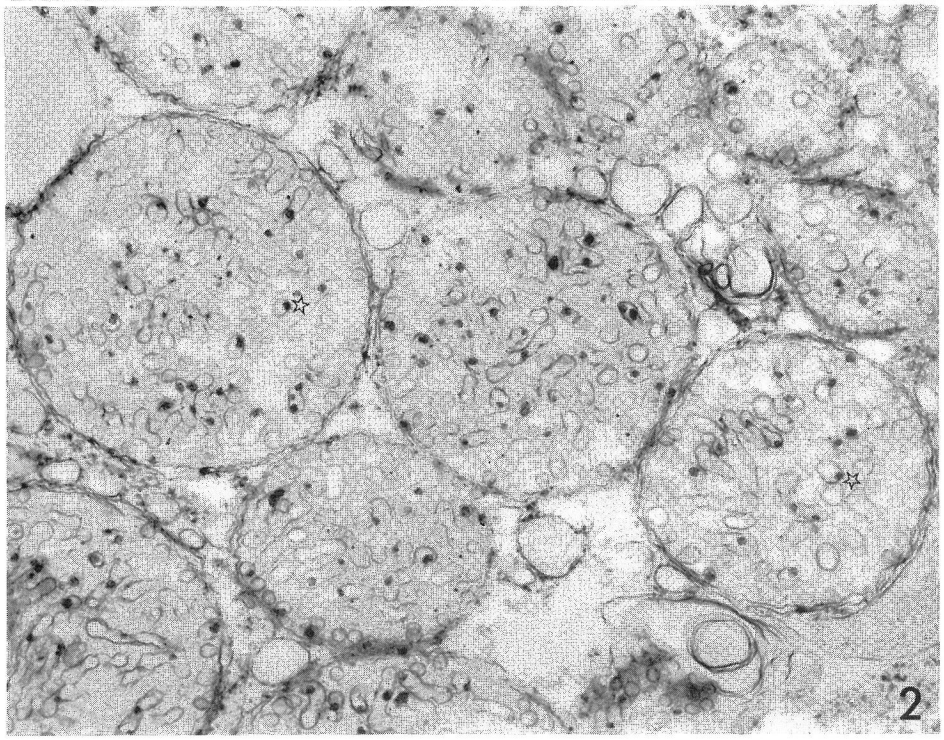
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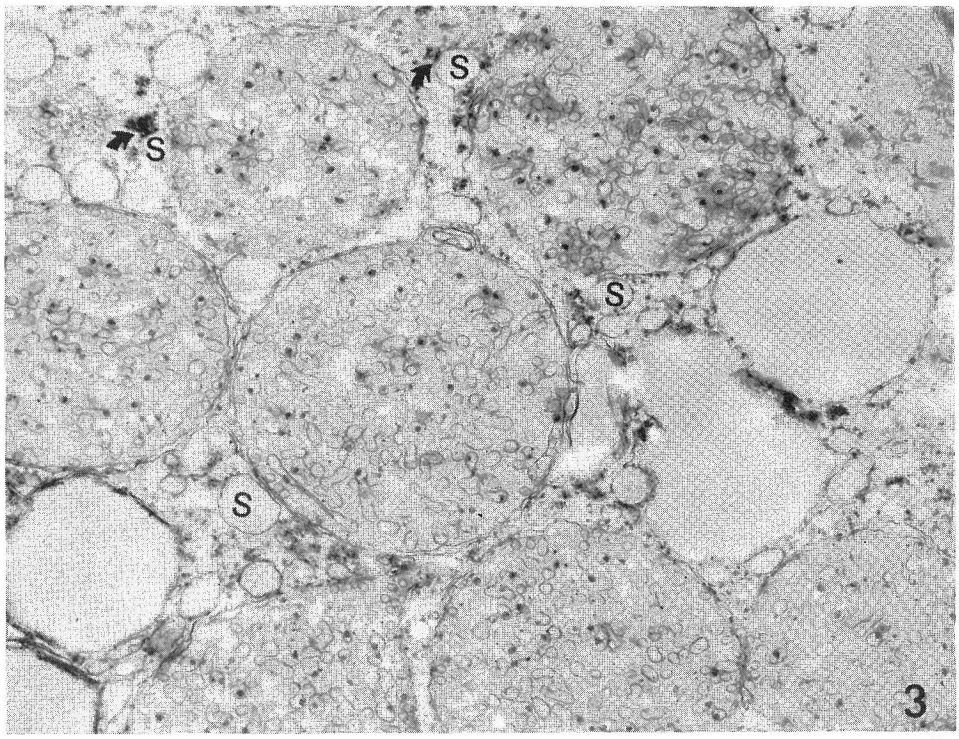
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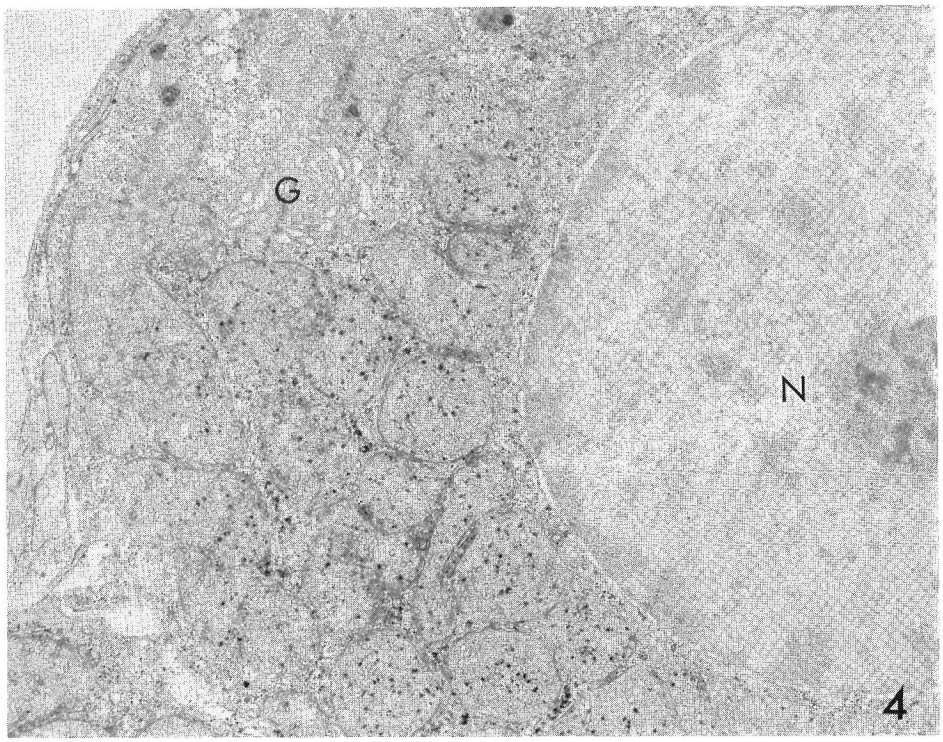
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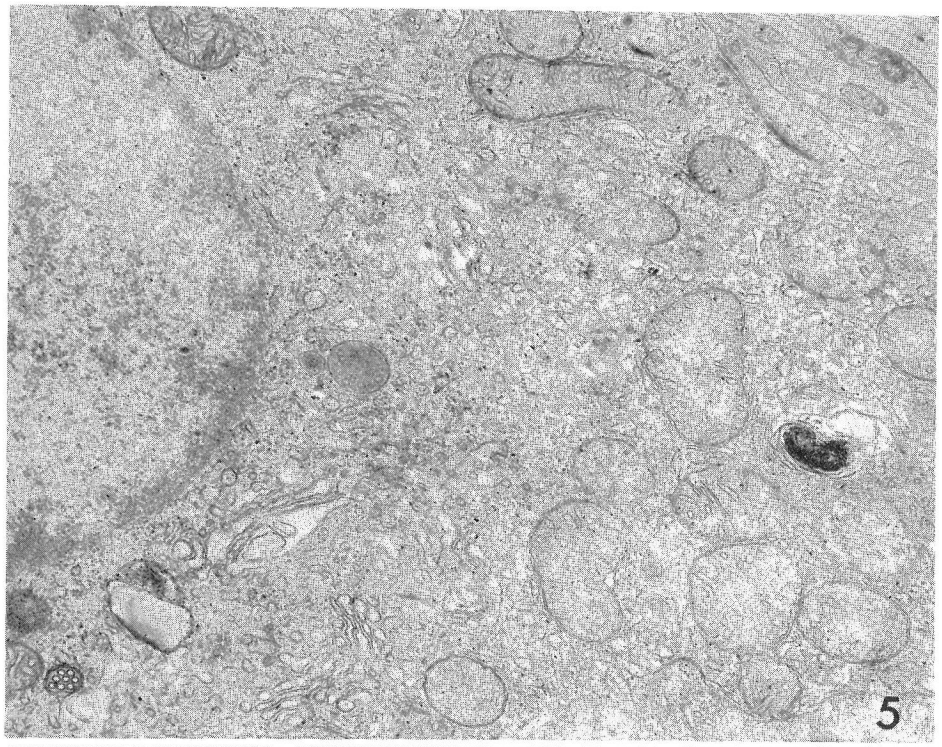
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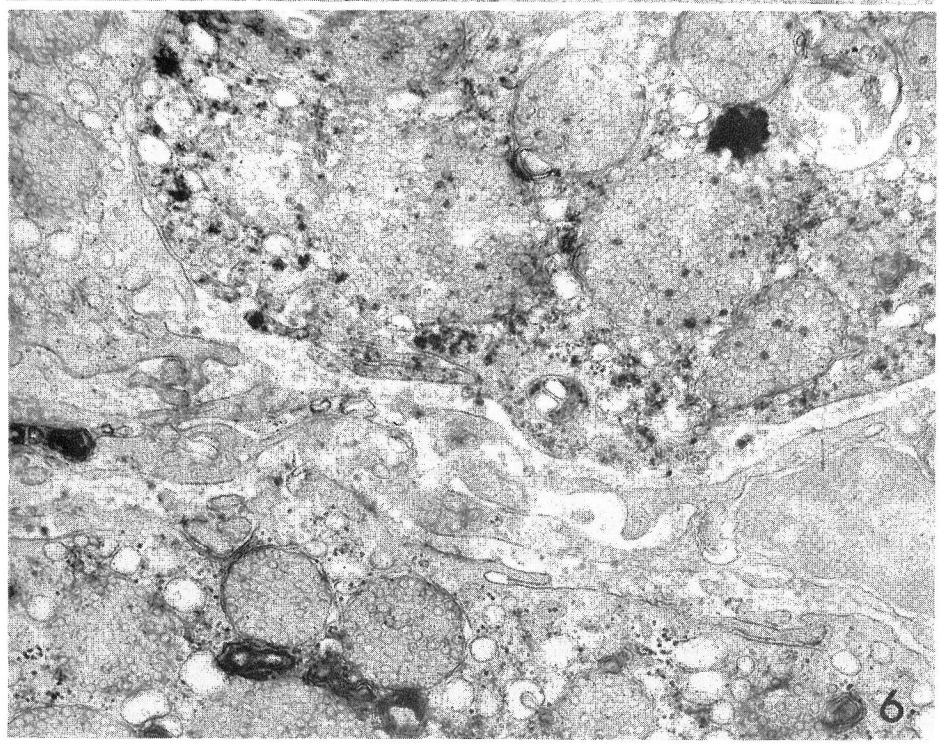
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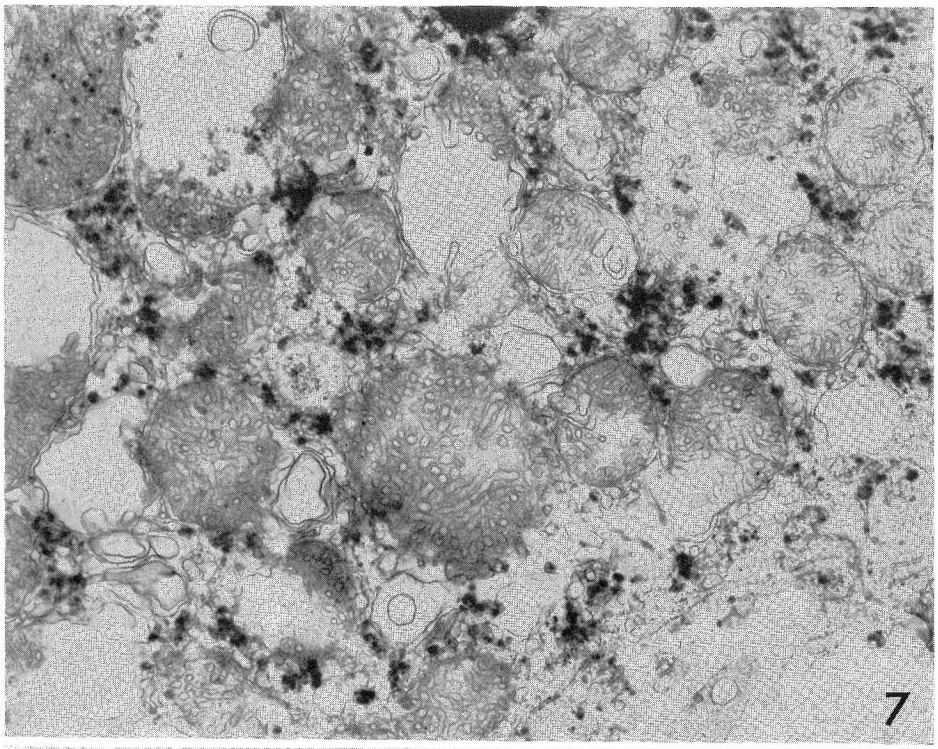

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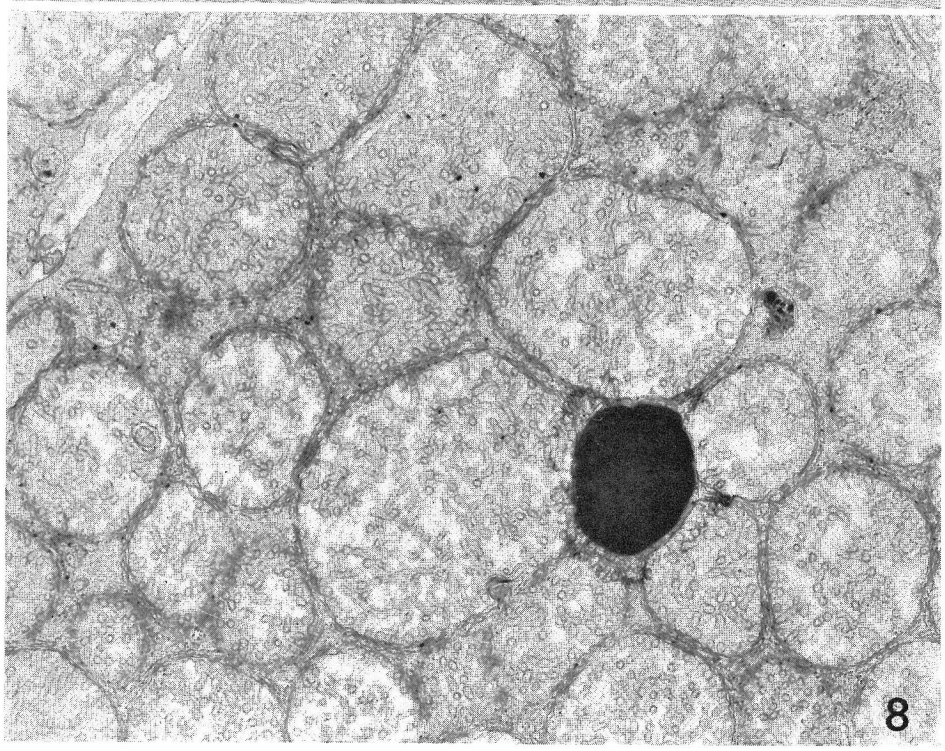

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and electron dense aggregations of fine granular deposits measuring up to 100 Å in diameter (Fig. 3). The reaction products varied from one cell to another. Cells devoid of reaction products were frequently found adjacent to the reaction positive cells (Fig. 1). The final reaction deposits were located; 1) in the cytoplasmic matrix near SER and some of them were directly connected with the outer surface of SER (Fig. 3); 2) in the intracristal spaces of both tubular and vesicular cristae of mitochondria, sometimes directly attached to the inner surface of cristae (Fig. 2). The intensity of this phenomenon was variable. Some cells had reaction products in the cytoplasmic matrix and mitochondria. Most cells showed reaction products in mitochondria and other cells in the cytoplasmic matrix near SER. The distribution of the reaction product in the cytoplasmic matrix closely resembled that seen in the free ribosome. No final reaction product was ever found in the Golgi apparatus or nuclei (Fig. 4). No reaction product was observed in cells of the glomerular zone (Fig. 5).

2. *Incubation in the G6PD medium*

The final reaction products of copper ferrocyanide appeared in cells of the fascicular zone (Fig. 6) and in some cells of the glomerular zone (Fig. 7). They were located in the cytoplasmic matrix in the vicinity of the cytoplasmic membrane, and were more abundant and much more electron-dense than those of the 3β-HSD medium and mostly consisted of aggregations of fine deposits measuring up to 100 Å in diameter. The reaction occasionally took place in the intracristal spaces of mitochondria, and the appearance and distribution were very similar to those which were observed in cells incubated in the 3β-HSD medium. No reaction deposits could be found in the Golgi apparatus, or nuclei or intercellular spaces.

3. *Incubation in the control medium*

No reaction products were noted in most cells incubated in the substrate-free medium or in the media containing Antimycin or Rotenone. The reaction products, however, appeared sometimes in the intercellular spaces and in a few mitochondria of the media containing Antimycin and Rotenone (Fig. 8) and of the substrate-free medium.

FIG. 1. 3β-HSD; Final reaction products appear in the cell of the fascicular zone. The reaction varies from one cell to another. ×38,000

FIG. 2. 3β-HSD; The final reaction deposits which consist of electron dense granular deposits measuring up to 300 Å in diameter are located in the intracristal spaces of mitochondria. Sometimes it is directly attached to the inner surface of cristae (☆). ×46,000

FIG. 3. 3β-HSD; The final reaction deposits which consist of electron dense aggregations of fine granular deposits measuring up to 100 Å are located in the cytoplasmic matrix near SER (S) and some of them were directly connected with the outer surface of SER (↗). ×44,000

FIG. 4. 3β-HSD; No final reaction product is ever found in Golgi apparatus (G) and nucleus (N). ×20,000

FIG. 5. 3β-HSD; No reaction product is observed in the cell of the glomerular zone. ×39,000

FIG. 6. G6PD; Final reaction products are present in the cytoplasmic matrix of the cell of the fascicular zone. Occasionaly reaction takes place in the intracristal spaces of mitochondria. ×30,000

FIG. 7. G6PD; Final reaction products are found in the cytoplasmic matrix of the cell of the glomerular zone. ×44,000

FIG. 8. 3β-HSD; No reaction is seen in the cell incubated in the medium containing Rotenone. ×22,000

DISCUSSION

The results of the present study proved that the 3β-HSD activity of the rat adrenal was localized in the cytoplasmic matrix near SER and in the intracristal spaces of mitochondria in cells of the fascicular and reticular zones. The reported ultracytochemical findings of the 3β-HSD activity in steroidogenic cells were controversial in its localization (1, 2, 3, 6, 7, 10). The activity of 3β-HSD in the rat adrenal has been demonstrated in the mitochondrial matrix (6), mainly in the mitochondrial crista (10), and in the cytoplasmic area in the vicinity of SER and in the intracristal spaces of mitochondria in cells of the fascicular zone (3). On the other hand, the localization of the 3β-HSD activity in human or rat lutein cells were revealed mainly in SER (7, 10) although Bara and Anderson (1), and Benköel (2) visualized the 3β-HSD activity in the intracristal spaces of mitochondria in some of lutein cells.

We consider that this variety in localization of the 3β-HSD activity reflects the different steroidogenic activities in each steroid producing cell. In cells of the rat adrenal the 3β-HSD activity is dominantly localized in mitochondria which may play an integral role as well as SER in the conversion of pregnenolone into progesterone in steroidogenic pathway. It is very interesting that the distribution of the enzyme activity observed in the cytoplasmic matrix closely resembles that of free ribosome. This might indicate that the enzyme activity has a close relationship with free ribosome.

The biochemical analysis of the cell fractions has shown that the localization of 3β-HSD is present either in the microsomal fraction (5, 9) or in the mitochondrial fraction (12), or in both fractions (8, 11) in steroidogenic cells. It is generally accepted that in the rat adrenal a large amount of 3β-HSD is localized in the microsomal fraction. The probable reason for the discrepancy between the results of the histochemical and biochemical enzyme localization must be related with the state of tissue, unicellular or subcellular it may be, other than with the method utilized. Snoeijing (11) pointed out that the histochemical reaction must be carried out in the section of subcellular pellets.

Among numerous reports of the histochemical studies on G6PD, there have been only three reports (2, 4, 7) of ultracytochemical studies on G6PD. It is difficult to demonstrate the ultracytochemical localization of this enzyme because of its soluble nature which leads to the enzyme diffusion and results in false localization in tissue. The localization of diaphorase is easily mistaken for that of G6PD. Nevertheless, Berchtold (4) solved this problem recently by adding phenazine methosulface (PMS) in the incubating medium. He visualized a precise localization of G6PD using ferrocyanide copper in fixing the rat adrenal tissue with formaldehyde and glutaraldehyde. Berchtold's method also made it possible to demonstrate the accurate localization of the G6PD activity. Our results, however, were inconsistent with those of Berchtold (4), but were consistent with those of Benköel (2) and Laffargue (7). Berchtold (4) has found the G6PD activity in the hyaloplasm of rat adrenocortical cells, whereas, the G6PD activity was demonstrated in the cytoplasmic matrix in our experiments and was identical to the biochemical results indicating that G6PD is mainly localized in the cytoplasm. The localization of G6PD in mitochondria can be considered as an artifact due to the action of flavoproteins in electron transfer chain of mitochnodria (2).

The copper ferrocyanide method is superior to the tetrazolium method in identifying the precise localization of 3β-HSD and G6PD. The present study proved Berchtold's method (3, 4) is quite available for studying the steroidogenic metabolism in various pathologic conditions.

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