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THE PROCEDURE FOR ULTRACYTOCHEMICAL DEMONSTRATION OF DEHYDROGENASES IN THE RAT ADRENAL CORTEX USING COPPER FERROCYANIDE

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This is a procedure for the ultracytochemical demonstration of 3β-hydroxysteroid dehydrogenase (3β-HSD) and glucose-6-phosphate dehydrogenase (G6PD) and the localization of these enzymes in the adrenocortical cell of rat is presented. The procedure involves pre-fixation of tissues, tissue sectioning and incubation of specimens. Brief fixation (for 30 min) in a mixture of glutaraldehyde and formaldehyde (0.25% : 1% and 2% : 2%) or 2.7% glutaraldehyde was excellent to preserve both the activity of 3β-HSD and G6PD and fine cellular structure. Unfrozen sections obtained by Vibratome (Oxford) were superior to frozen sections obtained by a cryostat for preservation of the ultrastructure and enzyme activity of the cell. Sections used were 40–100 μm in thickness. In the present method dehydroepiandrosterone (DHA), etiocholane-3β-17-one (etiocholane) and pregnenolone were utilized as substrate, potassium ferricyanide as a final electron acceptor instead of tetrazolium salt, and phenazine methosulfate (PMS) as an intermediate electron carrier.

As a result, the activity of 3β-HSD was localized in the cytoplasmic matrix and intracristal space of mitochondria. The activity of G6PD was visualized mainly in the cytoplasmic matrix near the plasma membrane. The reaction did not take place in any cells incubated either in the substrate-free medium or in the medium markedly inhibited by respiratory chain inhibitors such as Rotenone and Antimycin A. The findings mentioned so far were evidently those of the specific ultracytochemical reaction. Several problems will be discussed in this paper concerning the procedure for the ultracytochemical demonstration.

It is generally considered to be difficult to visualize the precise ultracytochemical localization of NAD(P)-dependent dehydrogenase because of its soluble nature. Furthermore, the ultracytochemical method for demonstrating dehydrogenase concerned in steroidogenesis has not yet been established basically. There are many problems of the basic method including the prefixation of tissues, tissue sectioning and incubation of specimens.

Bradbury and Steward (7) first reported diformazan deposits as the final reaction product of 3β-HSD in adrenocortical cells of rat using tatrazolium salt. Although they used 12.5% hydroxyadipaldehyde as a fixative, the fine cellular structure was not satisfactorily preserved nor could the precise localization be confirmed. Okamura (12), Laffargue (11), Bara and Anderson (3) and Benköel (4) also demonstrated the enzyme activity in poor ultrastructural cells; formazan deposits as the final

reaction products were of low contrast and ill-defined. Smooth-surfaced endoplasmic reticulum (SER) was markedly distended and the internal structure of mitochondria was not well-preserved.

Recently, Berchtold (5, 6) succeeded in visualizing discrete ultracytochemical localization of 3β-HSD and G6PD by utilizing potassium ferricyanide as a final electron acceptor and PMS as an intermediate electron carrier. A mixture of 1% formaldehyde and 0.25% glutaraldehyde permitted the showing of both the cytochemically detectable activity of the enzyme and the sufficient ultrastructural conservation in adrenocortical cells of the rat. In the present study the authors attempted to establish the best basic method for pre-fixation of tissue, tissue sectioning and incubation of specimens, for disclosing the localization of 3β-HSD and G6PD in adrenocortical cells of rat.

MATERIALS AND METHODS

1. Animals: Adult male Wistar rats weighing 150–350 g were used for the present study and both adrenal glands were cut into four pieces each.
2. Dehydrogenases examined: 3β-hydroxysteroid dehydrogenase (3β-HSD) and glucose-6-phosphate dehydrogenase (G6PD) were ultracytochemically examined.
3. Pre-fixation of tissue: The experiment was carried out using both fixed and unfixed tissues, the former being fixed in 0.1 M phosphate buffer, pH 7.4, mixed with the following fixatives individually, at 0–4°C for 30 min.
   a) Glutaraldehyde: 4%, 2.7%, 1.5% and 0.25%
   b) Paraformaldehyde: 2% and 1%
   c) A mixture of glutaraldehyde and formaldehyde: 2% : 2% and 0.25 : 1%, respectively.

   After fixation the tissues were washed for 30 min in the same buffer.
4. Tissue sectioning: In order to prepare unfrozen sections, both fixed and unfixed tissues were cut by a Vibratome (Oxford) into 40–100 μm in thickness, or by a razor blade into small blocks of 0.5–1 mm^3, and for frozen sections, fixed and unfixed tissues were also cut into 20–40 μm in thickness by a cryostat. The sections were washed immediately in the phosphate buffer at pH 7.4 for 30 min before incubation.
5. Incubations of specimens: Copper ferrocyanide was used to demonstrate the activity of 3β-HSD and G6PD. Sections were incubated in the freshly prepared medium (Table 1) which was essentially the same as that of Berchtold except for the use of tris-buffer instead of PVA, for the G6PD incubation medium in our study. The incubation was carried out at 37°C in the darkness with shaking. To obtain the best condition of the incubation in the demonstration of the enzyme activity, the incubations were attempted under the various conditions as follows;
   a) Substrates
      i) Substrate for 3β-HSD: Dehydroepiandrosterone (DHA), etiocholane-3β-ol-17-one (eitocholane) and pregnenolone were used.
      ii) Substrate for G6PD: Monosodium salt glucose-6-phosphate (monosodium G6P) and disodium salt glucose-6-phosphate (disodium G6P) were used.
   b) Medium containing phenazine methosulfate (PMS): PMS was tested at various final concentrations ranging from 0.5 mM to 3.0 mM (0.5 mM, 0.8 mM,

TABLE 1. *Composition of incubating media*

1. Medium for 3β-hydroxysteroid dehydrogenase (3β-HSD)...(5)	
Dehydroepiandrosterone (DHA) or Etiocholane 3β-ol-17-one (etiocholane) or Pregnenolone (in DMSO 0.6 ml, or in a mixture of Propylene glycol 0.3 ml and PMF 0.3 ml)	2.0 mg.
NAD (in 6.4 ml. of 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. (0.5 mM)
Total (Utilized)	10.0 ml.
2. Medium for glucose-6-phosphate monosodium salt (G6PD)...(6)	
D-glucose-6-phosphate monosodium salt or D-glucose-6-phosphate disodium salt	31.9 mg.
NADP	10.1 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. (0.5 mM)
Total (Utilized)	10.0 ml.

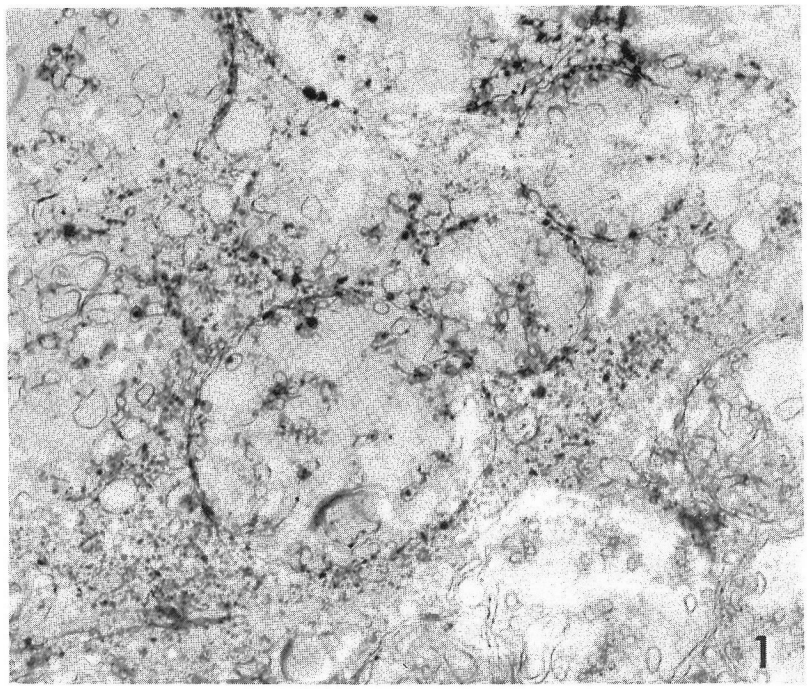
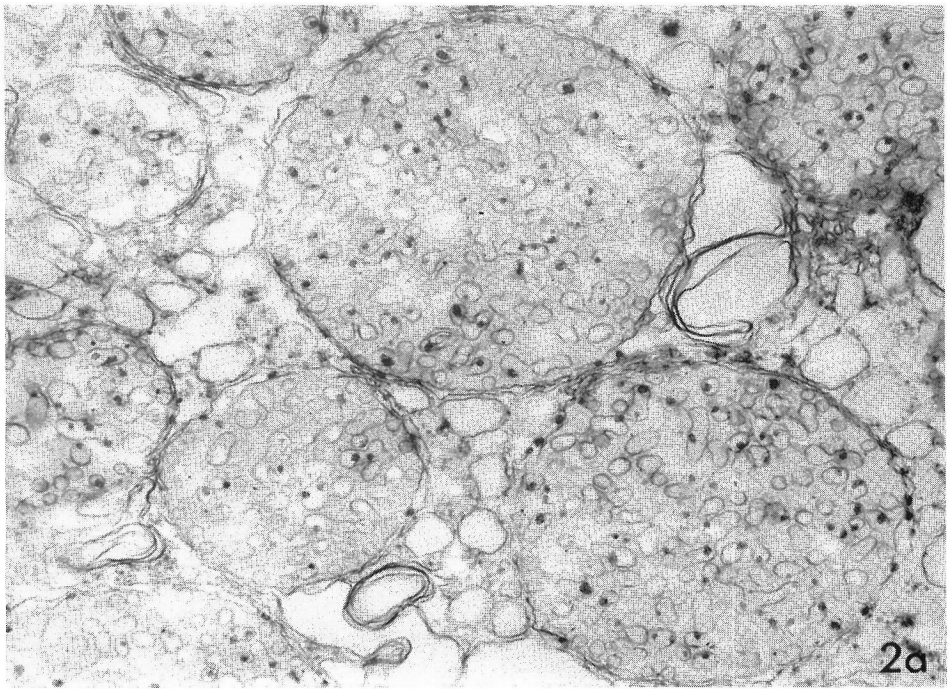


FIG. 1. 3β-HSD in unfixed section: Mitochondria are swollen with disruption. Some final reaction products are observed in the outer membrane of mitochondria and are diffused in the cytoplasmic matrix. ×30,000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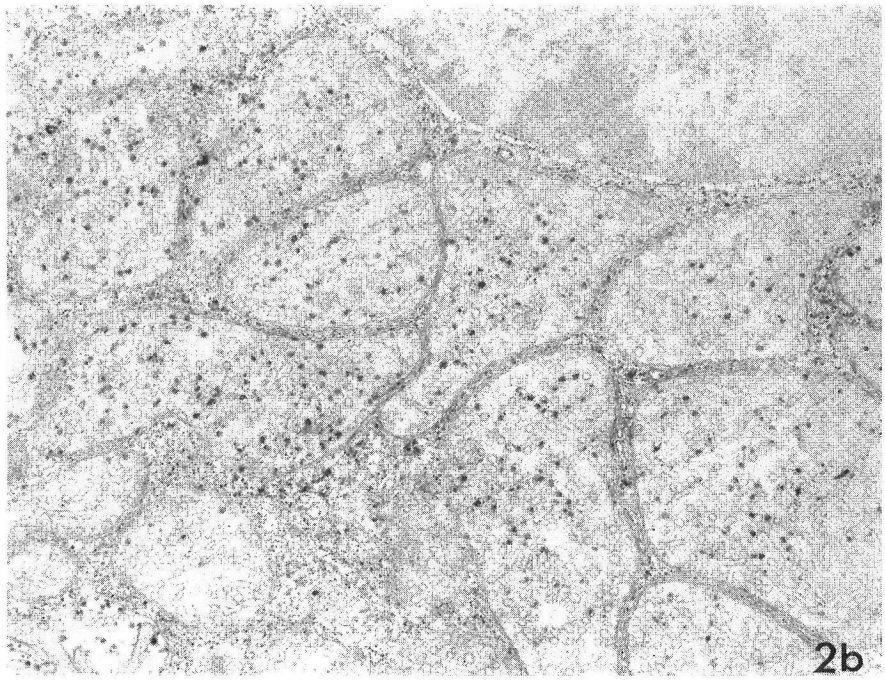
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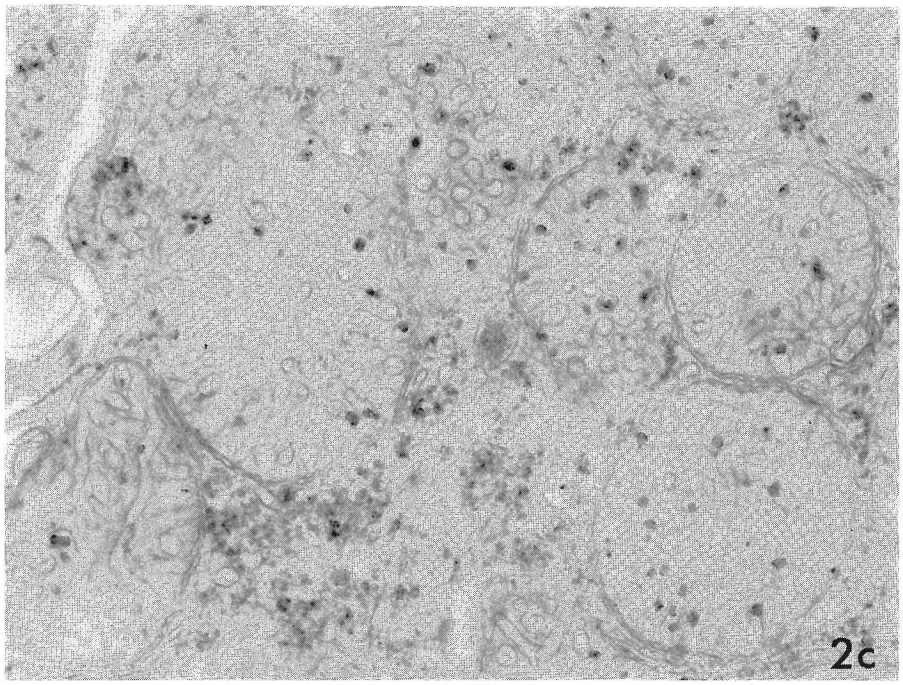


FIG. 2. a) 3β-HSD in section fixed in a mixture of 0.25% glutaraldehyde and 2% formaldehyde: Discrete localization of the enzyme activity appeared. The internal structure of mitochondria are well-preserved. ×54,000 b) 3β-HSD in section fixed in a mixture of 2% glutaraldehyde and 2% formaldehyde: Same as Fig. 2-a). ×26,000 c) 3β-HSD in section fixed in 4% glutaraldehyde: Though weaker, the enzyme activity can be detected. ×56,000

1.0 mM, 2.0 mM and 3.0 mM) and was added to the medium just before incubation in the dark.

c) Control media: The following control media were prepared:
   i) Substrate-free medium (Medium without substrate)
   ii) NAD-free medium or NADP-free medium (Medium without NAD or NADP)
   iii) Substrate-free medium without NAD or NADP: A combination of i) and ii)
   iv) Medium containing a respiratory chain inhibitor such as Rotenone and Antimycin A.

d) Incubation times: For satisfactory demonstration of the activity of 3β-HSD and G6PD, the incubations were attempted for 30, 60, 90 and 120 min, individually.

After incubation the sections were quickly washed in the cold (0–4°C) 0.1 M phosphate buffer at pH 7.2 three times. They were stored for one night in the same buffer at 0–4°C and post-fixed in 1% osmium tetroxide solution for 30 min or one hr. Then, the sections were dehydrated in graded acetone and embedded in Epon 812. Ultra-thin sections were examined after staining with lead citrate.

In addition to the ultracytochemical observation of the enzyme activity, X-ray

TABLE 2. *The effects of various fixatives on the cell structure and the enzyme localization*

		Preservation of cellular ultrastructure	Precise localization of enzyme activity (3β-HSD)
Unfixed tissue		Not good	Not good
Fixed tissue by Glutaraldehyde	4%	Good	Not good
	2.7%	Good	Good
	1.5%	Not good	Not good
	0.25%	Not good	Not good
Fixed tissue by Paraformaldehyde	2%	Not good	Not good
	1%	Not good	Not good
Fixed tissue by Mixture of glutaraldehyde and formaldehyde	2% : 2%	Good	Good
	0.25% : 1%	Good	Good

microanalysis was made of the final reaction products in the ultra-thin section incubated for 3β-HSD to examine the chemical nature of them. Gold meshes were used.

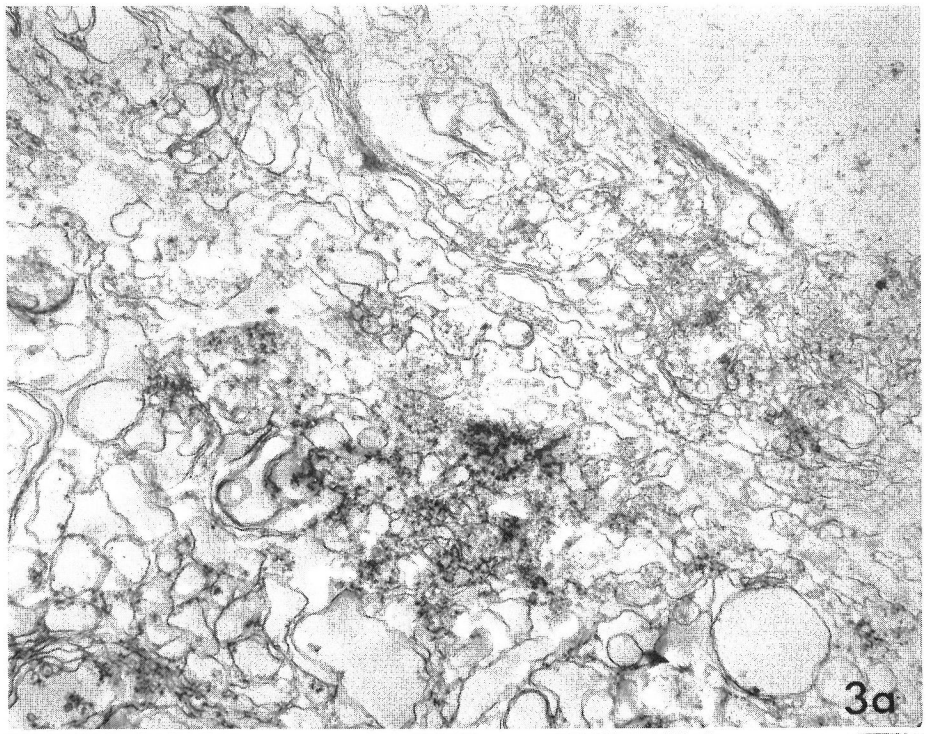
RESULTS

1. Pre-fixation of tissues: The results obtained from using various fixatives are summarized in Table 2. The enzyme activity was demonstrated as being more prominent in the fixed sections than in the unfixed sections. In the unfixed sections cell organelles were markedly destroyed and mitochondria were swollen with disruption. Some final reaction products were localized in the outer membrane of mitochondria and were diffused in the cytoplasmic matrix (Fig. 1). The sections fixed in a mixture of glutaraldehyde and formaldehyde showed a discrete localization of the enzyme activity with an excellent preservation of the cellular structure (Figs. 2a, 2b). The internal structure of mitochondria was well-preserved. The final reaction deposits were localized in the intracristal space of mitochondria and in the cytoplasmic matrix near smooth-surfaced endoplasmic reticulum (SER). The enzyme activity could be detected even in the sections fixed in 4% glutaraldehyde though its activity was weaker (Fig. 2-c).

   The best fixative for demonstrating the discrete localization of the enzyme activity (3β-HSD and G6PD) was, therefore, the mixture of glutaraldehyde and paraformaldehyde, 2% : 2% and 0.25% : 1% respectively, and 2.7% glutaraldehyde.

2. Tissue sectioning: Thin frozen sections of 20–40 μm could easily be obtained with the cryostat, but when the tissue was not fixed enough, cells became destroyed and the cellular structure was blurred. Besides, diffusion of the reaction products

FIG. 3. a) 3β-HSD in unfixed and frozen section cut by cryostat: The cellular structure is blurred and diffusion of the reaction product has occurred in the cytoplasmic matrix. ×23,000 b) 3β-HSD in frozen section fixed in 4% glutaraldehyde cut by cryostat: The cellular structure is well-preserved, but the reaction of the final products is weaker. ×33,000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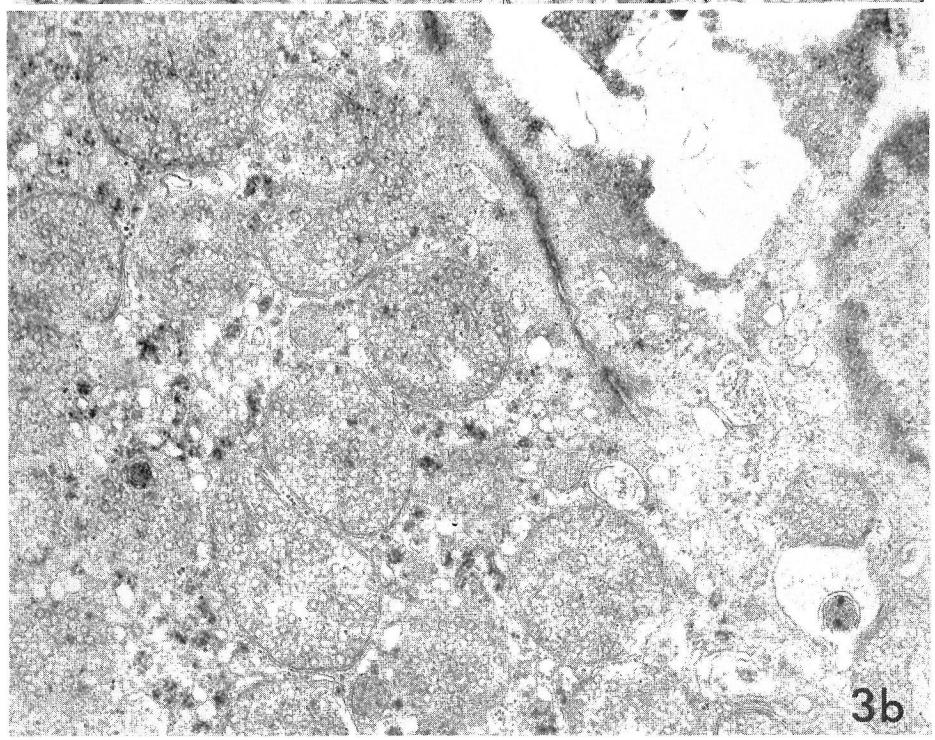
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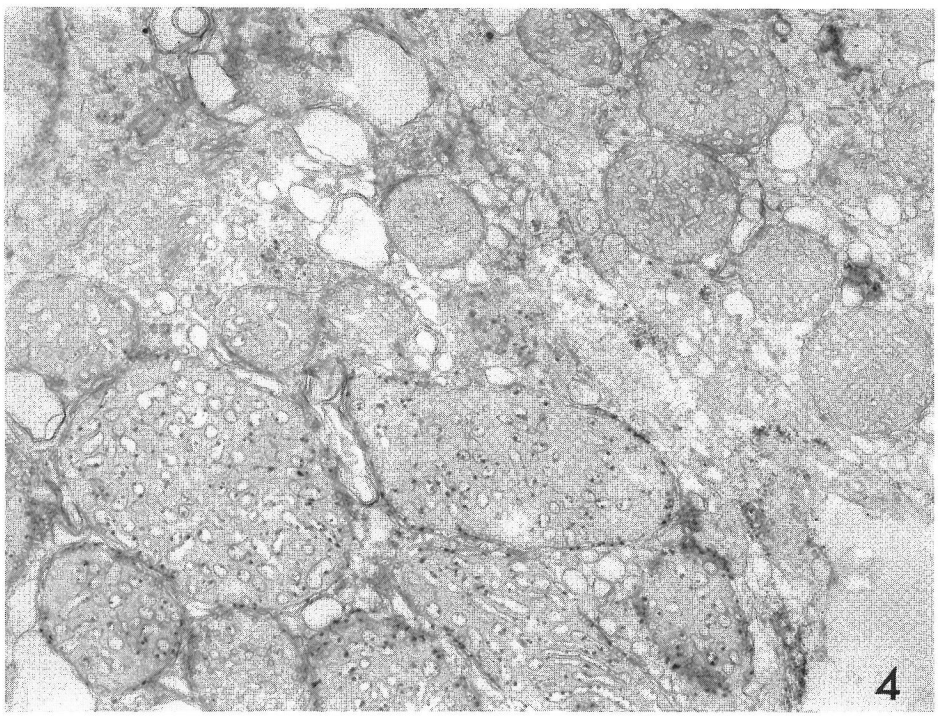


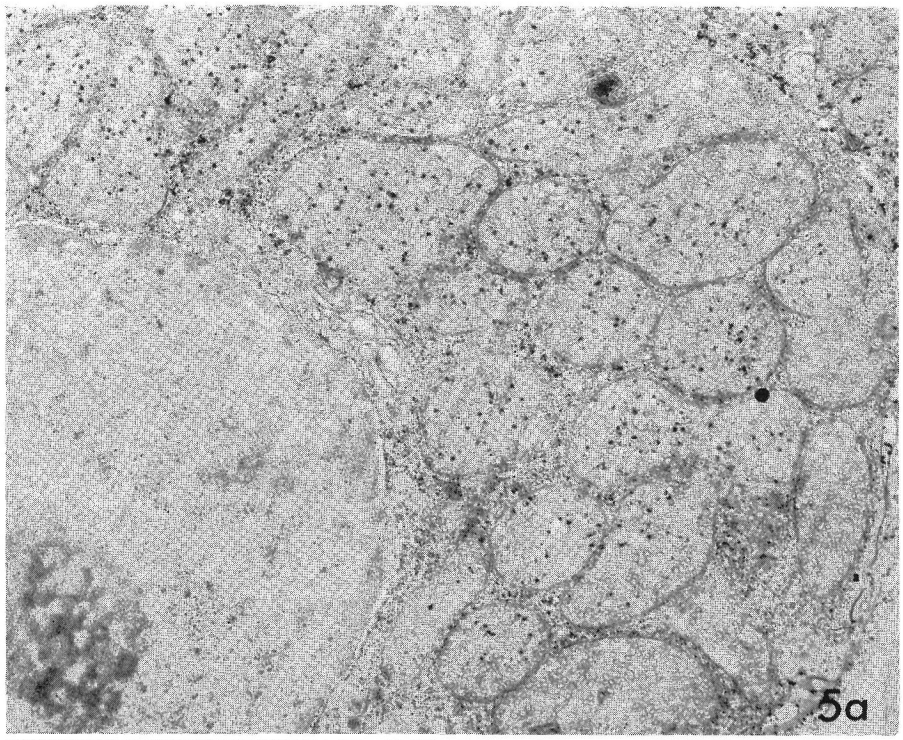
FIG. 4. 3β-HSD in section fixed in a mixture of 2% glutaraldehyde and 2% formaldehyde cut by a razor blade: The enzyme activity shows the same localization as that observed in the section cut by Vibratome (Fig. 2 a, b, c). ×32,000

occurred into the cytoplasmic matrix and the enzyme activity was weaker than in the unfrozen section cut by Vibratome (Fig. 3a). The enzyme activity was also noted in the well-fixed section of 4% glutaraldehyde (Fig. 3b).

It is better to use the unfrozen section of 40–100 μm cut by Vibratome in order to preserve the ultrastructure of cells and to obtain the specific reaction products. The sections cut with a razor blade showed the same localization of the enzyme activity as that observed in the sections cut by Vibratome although the intensity of the reaction product was somewhat variable (Fig. 4).

3. Incubation
   a) Substrate
      i) Substrate for 3β-HSD: The intensity and the localization of the enzyme activity did not significantly vary with the substrate such as DHA, etiocholane or pregnenolone (Fig. 5a, 5b).
      ii) Substrate for G6PD: The localization and the intensity of the enzyme activity were not changed after incubation in the reaction medium containing disodium G6P and monosodium G6P (Fig. 6). The reason for adopting monosodium G6P was its rather stable nature compared with disodium salt

FIG. 5. a) 3β-HSD in medium containing etiocholane as substrate. ×23,000 b) 3β-HSD in medium containing pregnenolone as substrate: The intensity and the localization of the enzyme activity do not significantly vary with the substrate such as etiocholane and pregnenolone. ×24,000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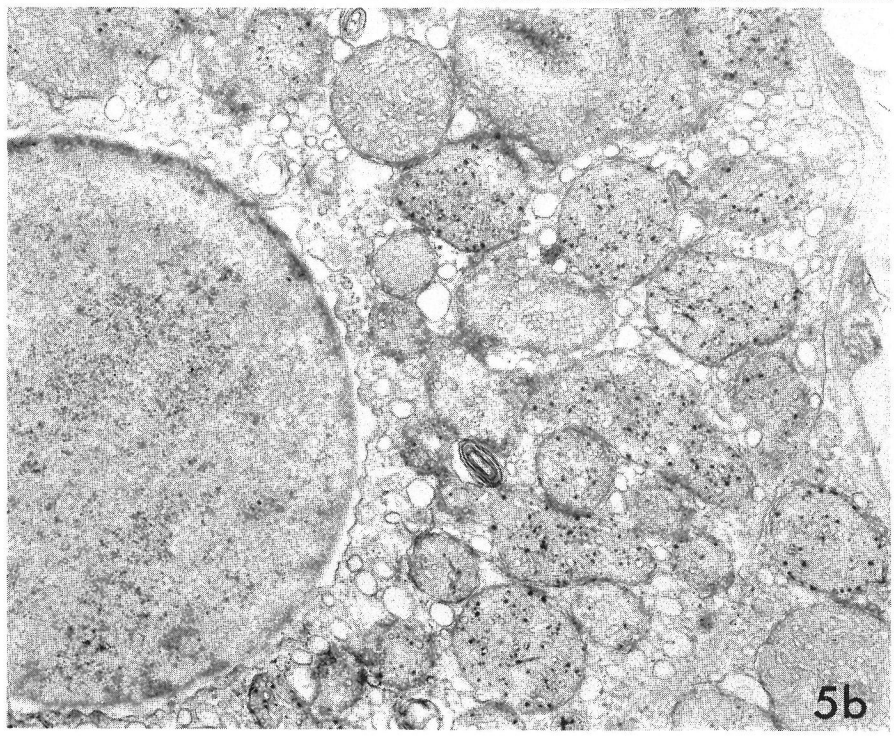
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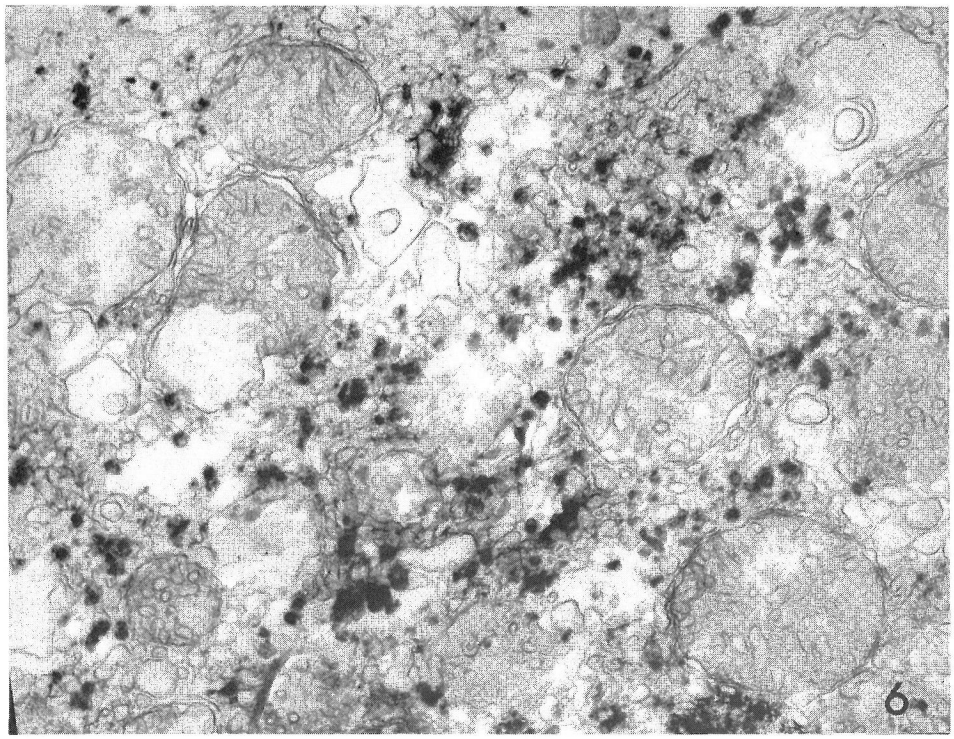


FIG. 6. G6PD in section from medium containing monosodium G6P as substrate: The final reaction products are localized in the cytoplasmic matrix.

when it would be exposed to atmospheric moisture in the room.

b) Medium containing PMS: Adding PMS into the medium increased the electron density and the reaction product (Fig. 7a, 7b). Higher concentration of PMS in the incubation medium, 2.0 mM or 3.0 mM, produced non-specific reaction products in the intercellular spaces and in mitochondria (Fig. 8b). The best concentration of PMS was 0.5 mM or 0.8 mM (Fig. 8a).

c) Control media: No reaction took place in most cells incubated in the substrate-free medium, or in the medium without NAD or NADP, or in the substrate-free medium without NAD or NADP or in the medium containing the respiratory chain inhibitor such as Rotenone and Antimycin A. Sometimes reaction products appeared in the intercellular spaces, or in a few mitochondria incubated in the substrate-free medium (Fig. 9a) or in the medium with Antimycin A (Fig. 9b) or Rotenone.

d) Incubation time: The results from various times of the incubation are summarized in Table 3. No reaction products of 3β-HSD or of G6PD were found 30 min after incubation (Fig. 10a). The reaction products of G6PD were observed 60 min after incubation (Fig. 10b) and those of 3β-HSD appeared 90 min after incubation (Fig. 11).

X-ray microanalysis of the reaction products is shown in Fig. 12. The X-ray spectrum of the final reaction products in the intracristal spaces of mitochondria

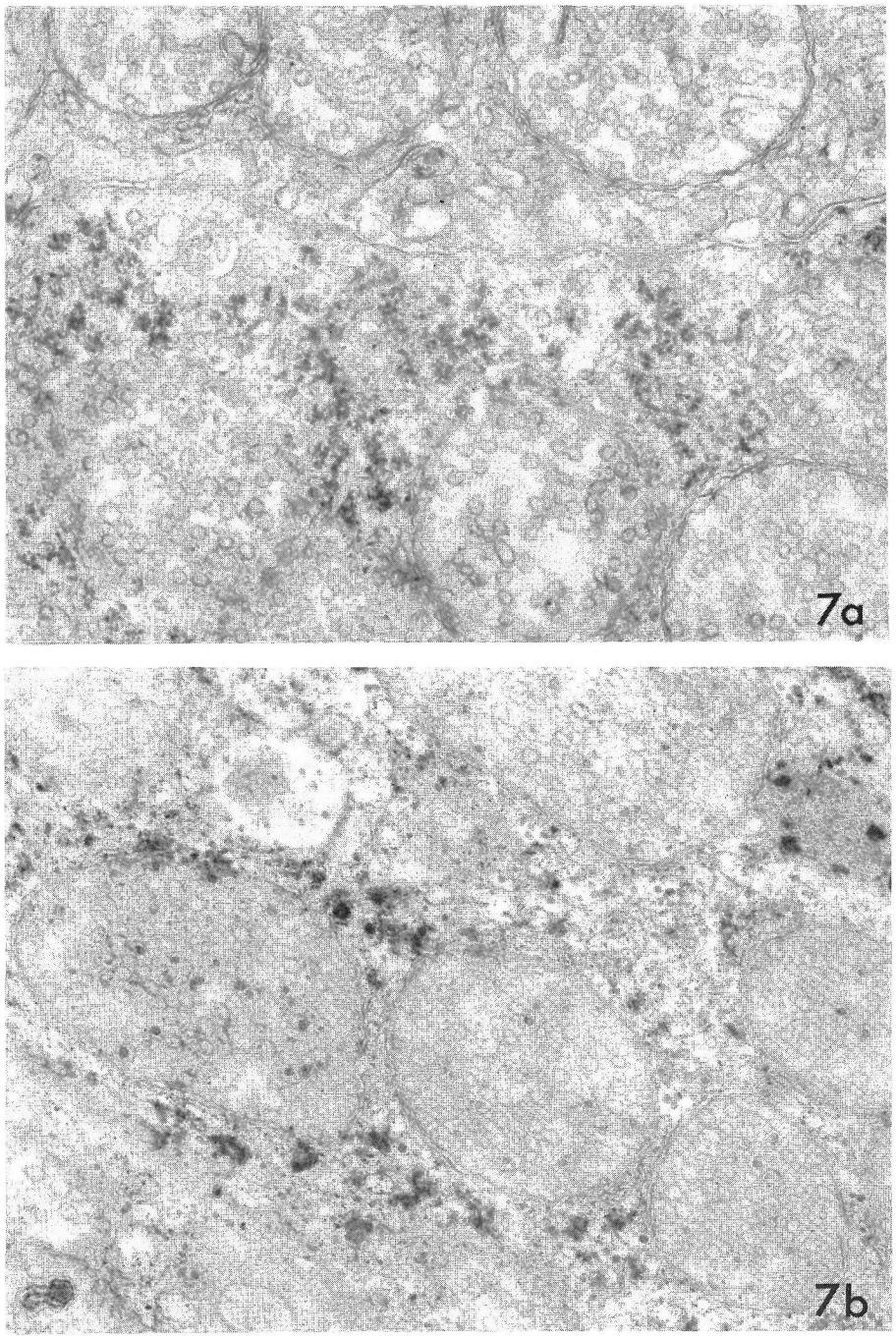
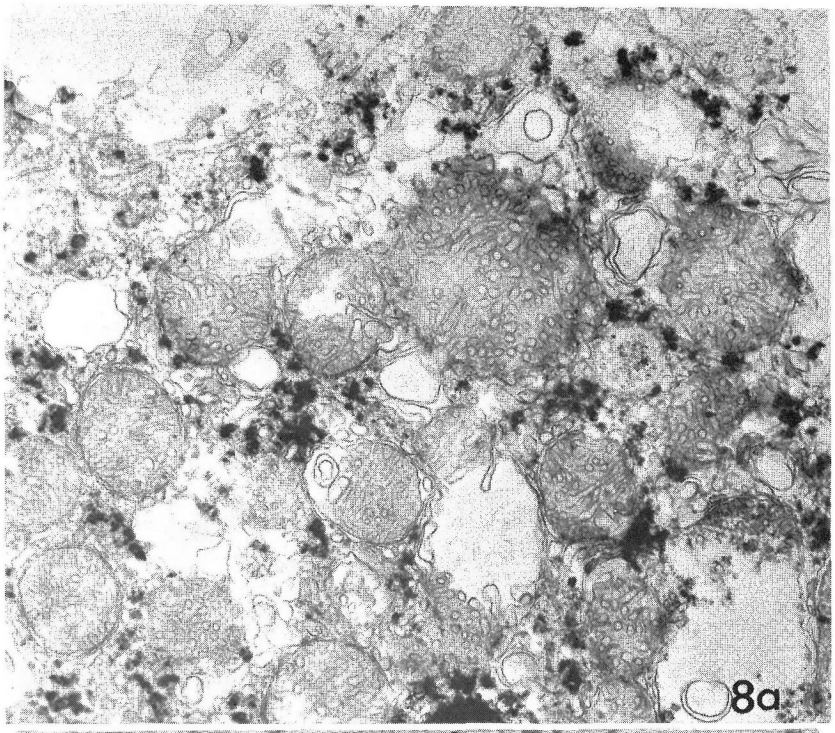


FIG. 7. a) 3β-HSD in medium without PMS. $\times$46,000 b) 3β-HSD in medium with PMS (0.8 mM). Adding PMS into the medium increases the electron density and reaction products. $\times$46,000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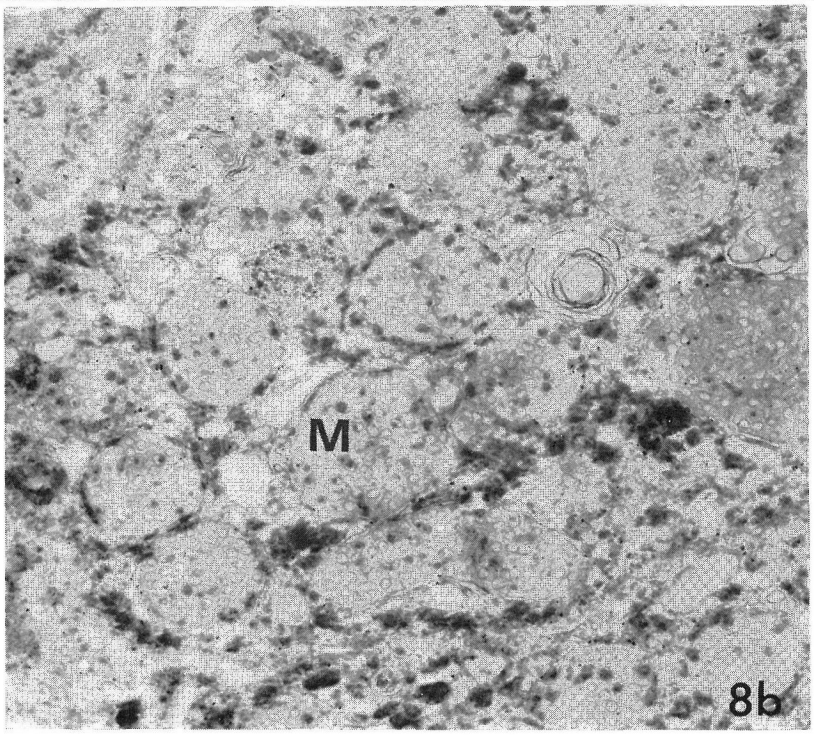
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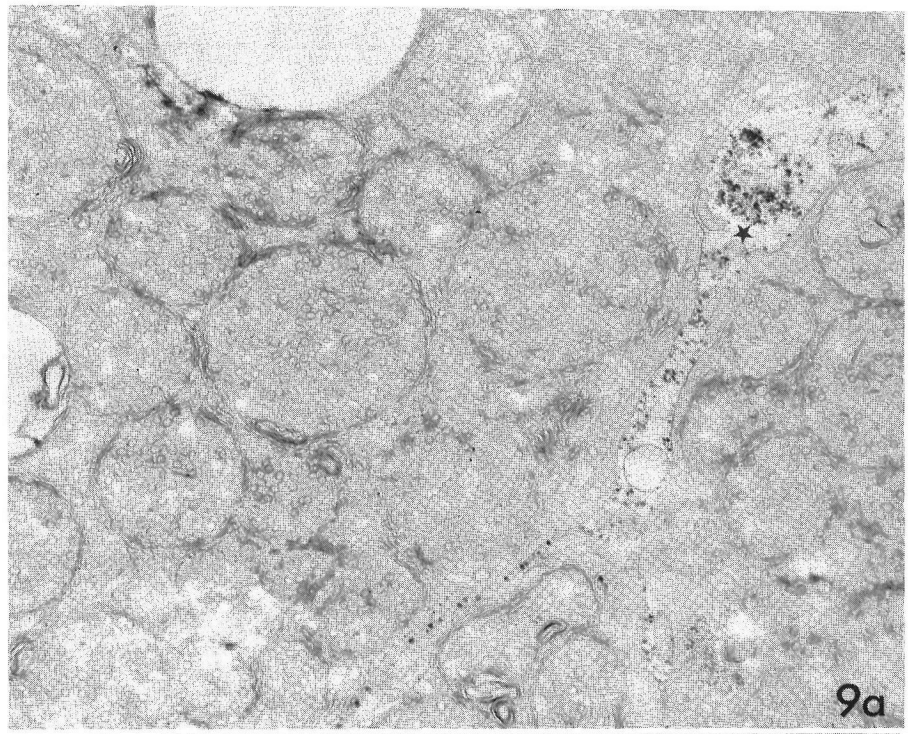
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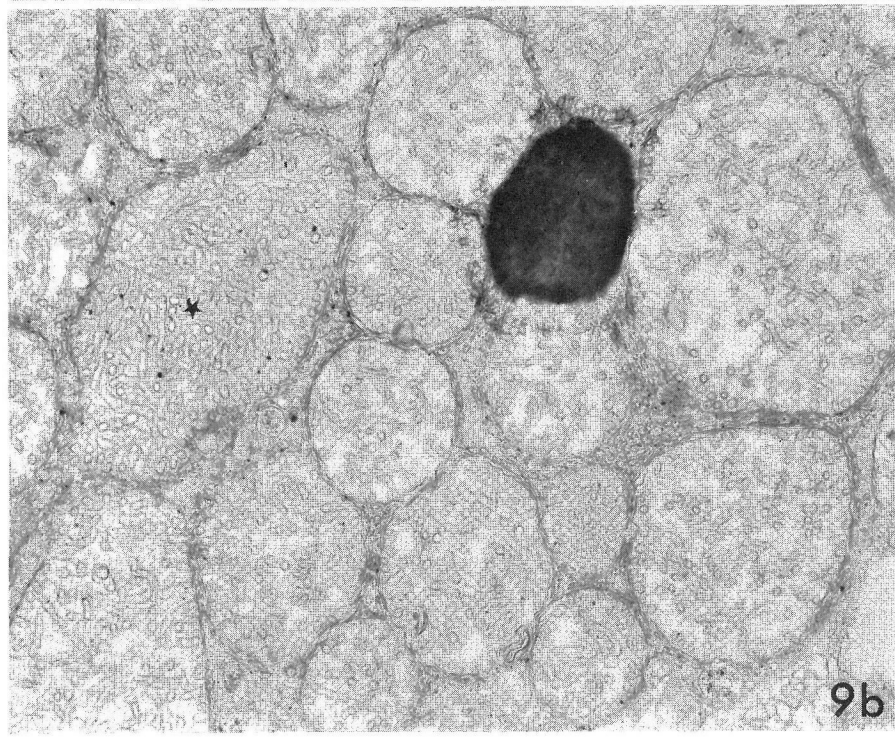
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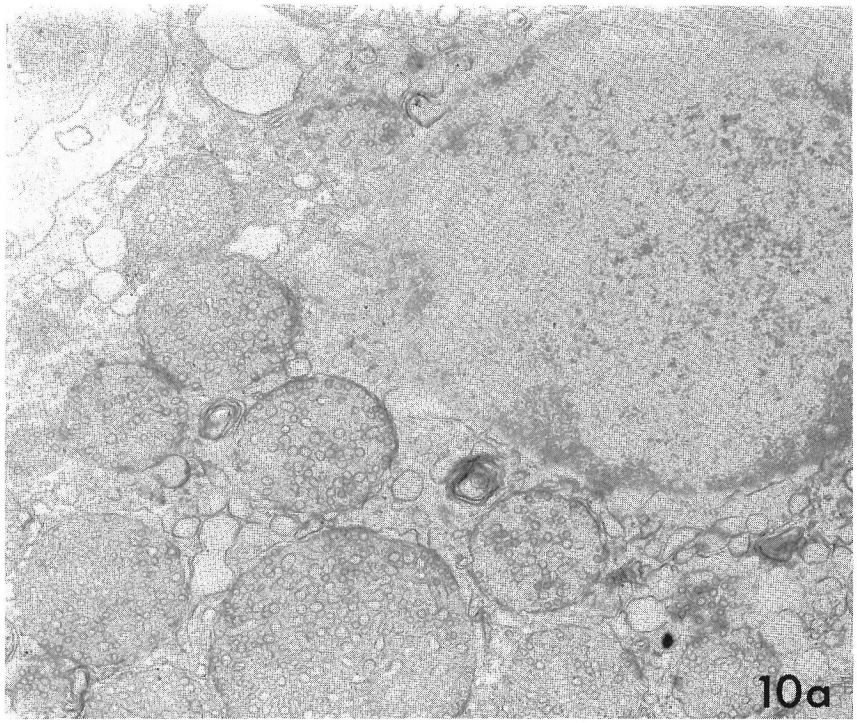
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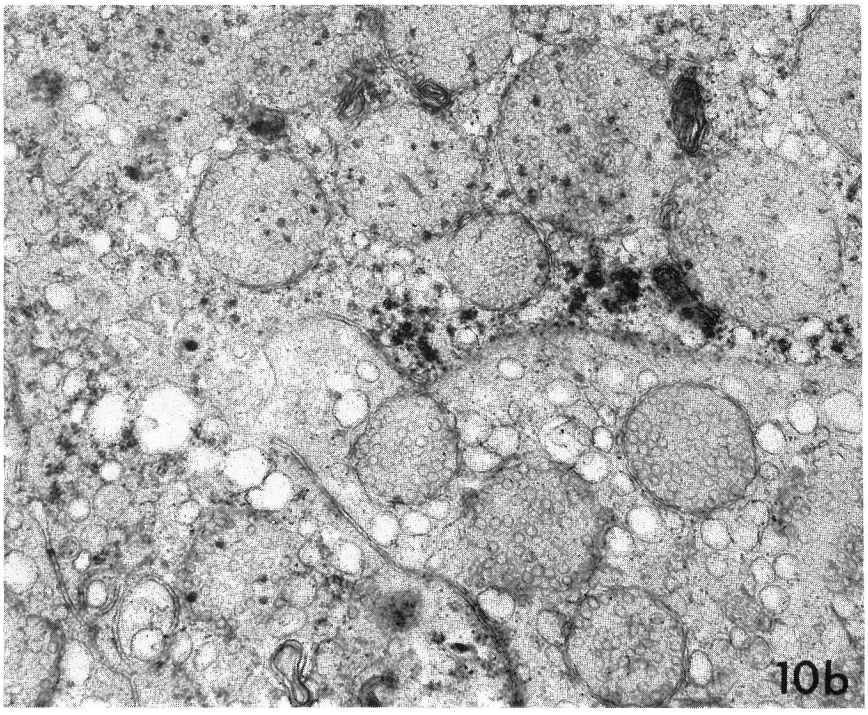
10b

and in the cytoplasmic matrix are represented by OsO_4(OS), Cl, Cu and Au. The reaction products contained Cu though small in amount.

TABLE 3. *The results of the incubating times attempted*

	30 minutes	60 minutes	90 minutes	120 minutes
3β-HSD	(−)	(−) or (+)	(+)	(+)
G6PD	(−)	(+)	(+)	(+)

(−) : Negative (+) : Positive

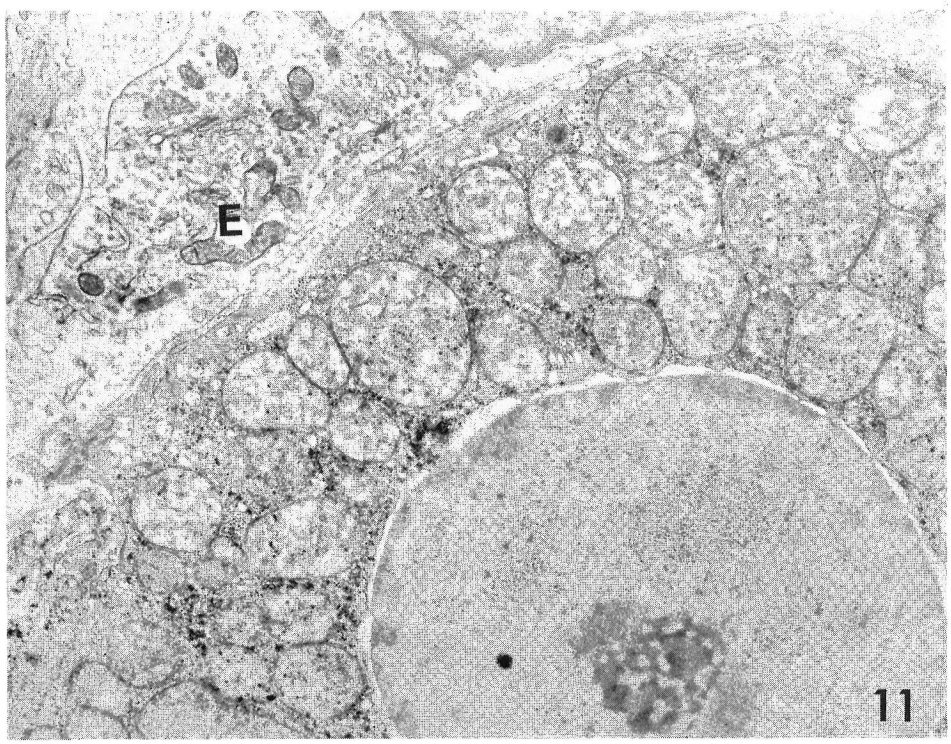

FIG. 11. 3β-HSD incubated for 90 min: The reaction products are seen but non-steroidogenic cell (endothelial cell (E)) give negative reaction. ×12,000

FIG. 8. a) G6PD in medium containing PMS (0.5 mM): The best concentration. ×23,000 b) G6PD in medium containing PMS (3.0 mM): The higher concentration produces non-specific reaction products in mitochondria (M) at the same time. ×23,000

FIG. 9. a) 3β-HSD in medium without substrate: The reaction products are noted in the intercellular space (*). ×26,000 b) 3β-HSD in medium containing Antimycin A: The reaction products are present in a few mitochondria (*). ×26,000

FIG. 10. G6PD reaction: a) Any reaction products are not found 30 min after incubation. ×23,000 b) The reaction products are observed 60 min after incubation. ×23,000

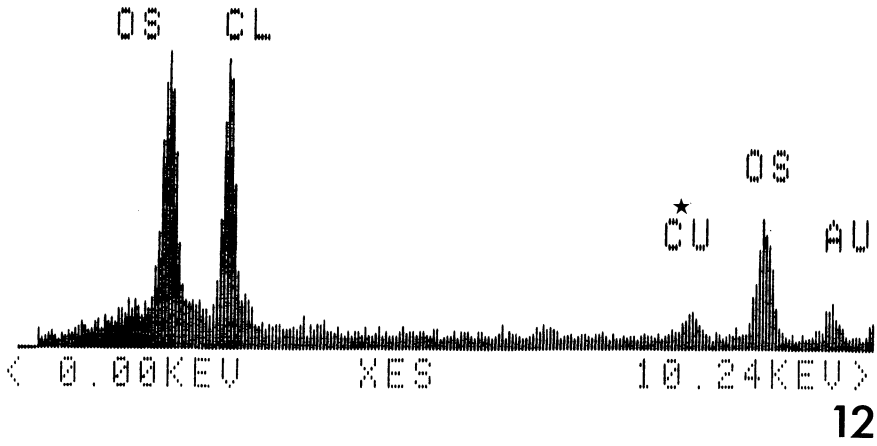

FIG. 12. X-ray microanalysis: The X-ray spectrum of the reaction products is represented by OsO_4 (OS), Cl, Cu(*) and Au. The reaction products contain Cu though small in amount.

COMMENT

1. Pre-fixation of tissues

Unfixed tissue has been utilized for the ultracytochemical study so that the fixatives may inhibit the enzyme activity (13, 17). The preservation of the cellular structure was, however, not satisfactory enough to demonstrate the precise localization of the enzyme activity. Preserving the fine structural details by fixation was necessary for achieving the discrete localization of products of the enzymic activity. We should be attentive, therefore, when choosing the proper fixatives to maintain the enzymic activity as much as possible. In the present study a brief fixation into either a mixture of glutaraldehyde and formaldehyde (0.25% : 1% and 2% : 2%) or 2.7% glutaraldehyde was excellent to obtain both the satisfactory preservation and the precise localization of the activity of 3β-HSD and G6PD. A brief fixation by glutaraldehyde showed no influence on the enzyme activity and was also helpful for good penetration of the components of the incubation medium into cells (2, 6), playing a role of immobilizing the enzyme (6).

2. Tissue sectioning

In order to detect the specific enzyme activities in the ultracytochemical sections, 20–40 μm might be the best thickness of the sections (1, 13, 15, 16) and frozen sections would not be preferable for it. Ogawa (16) emphasized the importance of the thickness of the sections used for incubation to demonstrate the exact ultracytochemical localization of the enzyme and the localization of alkaline phosphatase changed with the thickness of the sections. Attention should be paid to the thickness of the sections to reveal the specific enzyme activity.

3. Incubation

a) Substrate: Utilizing etiocholane was recommended by Bara and Anderson (3), and Berchtold (5). DHA, etiocholane and pregnenolone adopted in our study basically showed no differences in the localization of the enzyme and in the nature of the final reaction products.

b) Medium with PMS: PMS has been thought to be useful for the histochemi-

cal demonstration of NAD(P) dependent enzyme and to provoke the reaction of both 3β-HSD and G6PD to increase in the incubating medium as an intermediate electron acceptor (8, 9, 14) and this fact was ascertained in our experiment. The distribution of the NADH diaphorase activity was very similar to that of 3β-HSD. The localization of NAD(P) dependent enzyme is always interfered with by NAD(P)H_2 diaphorase, because the reduction of potassium ferricyanide takes place through the activity of an endogenous NAD(P)H_2 diaphorase (2, 8, 9, 14). Accordingly, the final reaction products are formed via the mediation of the NAD(P)H_2 diaphorase system in tissue (2, 8, 9, 14). PMS added into the medium served as the direct electron transfer and resulted in the reduction of potassium ferricyanide which prevented the false localization of the enzyme activity (5, 6, 8, 9, 14, 16). The higher concentration of PMS, however, produced non-specific reaction products at the same time, because of the important role of PMS when demonstrating the enzyme activity.

c) Control media: No reaction products were observed in any specimens from the substrate-free medium, or from the medium containing Antimycin A or Rotenone. Non-steroidogenic cells always gave a negative reaction. The results mentioned so far indicate that the enzyme activity seen in the present study was of specific dehydrogenase. The final reaction products observed in the intercellular spaces and in the mitochondria when lacking the substrate or adding the inhibitors might be attributed to the reduction of ferricyanide potassium by intrinsic factors of tissue (8). This has been accepted as a concept of "nothing dehydrogenase" (8).

Formazan deposits as the reaction products were low in contrast and ill-defined (3, 7, 11). On the contrary, the reaction products of copper ferrocyanide were high in contrast and well-demarcated (4, 5, 6, 15). Potassium ferricyanide might have penetrated more rapidly into tissues than tetrazolium salt and might have not adsorbed lipid (4, 13). Besides, unlike the formazan deposits, the copper ferrocyanide deposits were not soluble either into alcohol or into acetone (4). Consequently, the copper ferrocyanide method was superior to the tetrazolium method salt for identifying the discrete localization of 3β-HSD and G6PD in the rat adrenal. The present study proved the excellence of the copper ferrocyanide method, which would be quite available, for exploring the steroidogenic metabolism under various pathological conditions. The localization and the function of 3β-HSD and G6PD in the rat adrenal has been discussed (10).

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