LOCALIZATION OF ENERGY METABOLISM-RELATED OXIDOREDUCTASES IN THE TESTIS

YASUHIRO SAKAI, KOUSUKE CHIDA AND KENJIRO YASUDA*

*Department of Anatomy, School of Medicine, Kitasato University, Kanagawa 228 and
Department of Anatomy*, School of Medicine, Keio University, Tokyo 160*

Received for publication May 18, 1981 and in revised form June 22, 1981

It is well known that mammalian spermatozoa utilize fructose as an energy source, and glycerol can also be metabolized for this purpose. Further, oxidoreductases related to energy metabolism, such as sorbitol dehydrogenase and α -glycerophosphate dehydrogenase, are contained in the testis. These enzymes contribute fructose synthesis and glycerol metabolism, respectively.

In this paper, the precise localization of these energy metabolism-related oxidoreductases was studied by electron microscopy. The results indicated that sorbitol dehydrogenase located in the cytoplasm of Sertoli cells adjacent to the late stage spermatids. On the other hand, α -glycerophosphate dehydrogenase was recognized in the mitochondria of late stage spermatids and spermatozoa. Therefore, fructose is presumably synthesized in the cytoplasm of the Sertoli cells, and glycerol is metabolized at the mitochondria of the sperm.

It is well known that mammalian spermatozoa utilize fructose instead of glucose as an energy source (Mann, 1949). In the mouse, fructose is mainly produced at the seminal vesicles and coagulating gland by two types of oxidoreductases. Aldose reductase (EC 1.1.1.21) converts glucose to sorbitol, and sorbitol dehydrogenase (EC 1.1.1.14) converts sorbitol to fructose (Hers, 1960).

These two oxidoreductases are known to be present in the testis. Since these reactions require NADP and NAD, respectively, they should be detectable by the TNBT method. Using this histochemical method, Micucci *et al.* (1971) tried to detect these oxidoreductases in the bovine testis and showed that aldose reductase and sorbitol dehydrogenase were localized in late stage spermatids and Sertoli cells at the light microscopic level. However, since late stage spermatids are completely surrounded by Sertoli cells, it is impossible to distinguish the reaction products in spermatid from those in Sertoli cell at the light microscopic level. Therefore, the localization of this enzyme must be determined at the electron microscopic level.

On the other hand, it is also well known that spermatozoa can metabolize glycerol as an energy source. Spermatozoa given glycerol consumed oxygen and accumulated lactate (Mann and White, 1957). Mohri and Masaki (1967) stated that the glycerol was converted to α -glycerophosphate and then to dihydroxyacetone phosphate by glycerokinase and α -glycerophosphate dehydrogenase respectively. These enzymes, which are linked to glycerol metabolism, are also present in the

testis (Mohri and Masaki, 1967). By histochemical analysis at the light microscopic level, Mietkiewski and Tukaszyk (1968) suggested that α -glycerophosphate dehydrogenase was present in mitochondria of late stage spermatids.

In this paper, in order to identify more precisely the sites of the fructose synthesis and glycerol metabolism, the localizations of sorbitol dehydrogenase and α -glycerophosphate dehydrogenase were analyzed by electron microscopy, using the TNBT method.

MATERIALS AND METHODS

Analysis of the enzyme reactions:

Mouse testes were homogenized with 3 volumes of cold PBS (0.01 M phosphate buffer, pH 7.2, 0.9% NaCl). The homogenate was centrifuged for 10 min at 10,000 g and 0.1 ml of the supernatant was mixed with 0.9 ml of reaction mixture followed by incubation for 3 hr at 37°C. The reaction mixture for sorbitol dehydrogenase was the same medium as was used in the histochemical study described later. Following the suggestion of Micucci *et al.* (1971), the reaction medium for aldose reductase was the same as that for sorbitol dehydrogenase except that NAD was replaced with NADP. After incubation, 9 ml of acetone was added to the reaction mixture and was centrifuged. One ml of supernatant was dried with 0.4 μ moles of fucose. The dried samples were analyzed by gas chromatography.

Preparation of specimens:

Under Nembutal anesthesia, a mouse was perfused from the heart with PBS for 5 min then fixed with 2% glutaraldehyde buffered with 0.05 M cacodylate buffer, pH 7.4, for 30 min at room temperature. The testes were removed, washed overnight, frozen, sliced in a cryostat and subjected to histochemical staining. The reaction for sorbitol dehydrogenase was carried out at room temperature for 30 min by the method of Chida *et al.* (1975) with slight modification. The reaction mixture was composed of 5 mg sorbitol, 2 mg nicotinamide adenine dinucleotide (NAD), 0.1 mg phenazine methosulfate, 0.5 mg tetranitroblue tetrazolium (TNBT) (dissolved in 0.1 ml of dimethyl sulfoxide) and of 2 mg bovine serum albumin, in 1 ml of Tris-buffer pH 8.4 at a final concentration of 100 mM.

The reaction medium for α -glycerophosphate dehydrogenase was the same as that of sorbitol dehydrogenase except that the substrate was α -glycerophosphate in place of sorbitol. For light microscopic observation, the reacted specimens were mounted with glycerol. For electron microscopic observation, the reacted specimens were post-fixed in 1% osmium tetroxide for 1 hr, then dehydrated and mounted with epoxy resin. Ultrathin sections were cut and observed without any staining.

RESULTS

1. Gas chromatography:

Though Micucci *et al.* (1971) considered that aldose reductase and sorbitol dehydrogenase could be detected by the TNBT method, Johnson (1966) insisted that the reaction of aldose reductase could not be distinguished from that of sorbitol dehydrogenase. Thus, we analyzed the reaction products produced in the histochemical reaction mixture by gas chromatography. A mixture of glucose, sorbitol, fructose and fucose showed independent peaks (Fig. 1a). The gas chromatogram

FIG. 1. Gas chromatographic analysis of reaction products. a. Fructose, glucose, sorbitol and fucose ($0.4 \mu\text{moles}$ each) were mixed and analyzed by gas chromatography. Fucose, fructose, glucose (α -form) sorbitol and glucose (β -form) appeared in that order as independent peaks. b. Homogenate of testis was added to the reaction mixture of sorbitol dehydrogenase. After incubation for 3 hr, the reaction mixture was analyzed by gas chromatography. The peak of fructose (arrow) was seen in addition to those of fucose and sorbitol. c. The reaction mixture of aldose reductase was analyzed as described for 1b. The peak of fructose (arrow) was seen, but no peak of glucose was recognized.

FIG. 3. The localization of α -glycerophosphate dehydrogenase. Mouse testis was prepared by the same technique as described in Fig. 2 and incubated in the medium for α -glycerophosphate dehydrogenase. a. The seminiferous tubules which contained late stage spermatids showed a strong positive reaction, but those with early stage spermatids never showed a positive reaction. $\times 75$ b. The seminiferous tubules were observed at high magnification. The positive reaction showed a sharp linear form. $\times 300$

FIG. 2. The localization of sorbitol dehydrogenase. Mouse testis was fixed for 30 min with 2% glutaraldehyde by the perfusion method. The preparation was washed, then frozen sections were made and incubated with the reaction medium for sorbitol dehydrogenase. a. The seminiferous tubules which contained late stage spermatids showed intense positive reaction, while those of early stage spermatids never showed a positive reaction. $\times 75$ b. The seminiferous tubules which contained late stage spermatids were observed at high magnification. Positive reactions were recognized around the spermatids in diffuse form. $\times 300$ c. The specimen was incubated with substrate-free reaction mixture. No positive reaction was seen. $\times 75$

FIG. 4. Localization of sorbitol dehydrogenase. The specimens were stained by the procedure described in Fig. 2 and mounted in Epon. a. Positive reaction was recognized at the cytoplasm of the Sertoli cells, but not in the spermatids. $\times 12,000$ b. The control specimens reacted with substrate-free mixture showed no reaction. $\times 12,000$

FIG. 5. Localization of α -glycerophosphate dehydrogenase. a. Positive reactions were recognized in the mitochondria of the late stage spermatids, but the Sertoli cells never showed any reaction. $\times 12,000$ b. The epididymal spermatozoa showed a positive reaction in the mitochondria. $\times 17,000$

of the reaction mixture for sorbitol dehydrogenase showed the peak of fructose in addition to sorbitol and fucose (Fig. 1b). This result indicates that sorbitol is converted to fructose by the action of sorbitol dehydrogenase. The pattern for aldose reductase was similar and no peak of glucose appeared (Fig. 1c).

The fact that sorbitol was converted not to glucose but to fructose indicated that no reaction of aldose reductase had occurred and that the sorbitol dehydrogenase reaction proceeds even with NADP. Therefore, the positive reaction obtained by the TNBT method is considered to be due to the action of sorbitol dehydrogenase in spite of the type of coenzyme used.

2. Light microscopic observation:

The reaction pattern of sorbitol dehydrogenase in the mouse testis depended on the stage of spermatogenesis. The seminiferous tubules which contain late stage spermatids showed an intense reaction, while those of early stage spermatids showed no positive reaction (Fig. 2a). The positive reactions of seminiferous tubules were restricted to around the late stage spermatids and no reaction was seen at the basal side of seminiferous tubules (Fig. 2b). The control specimens which were reacted with the substrate-free reaction mixture gave no reaction product (Fig. 2c).

The reaction pattern of α -glycerophosphate dehydrogenase also depended on the stage of spermatogenesis; the seminiferous tubules which contained late stage spermatids showed a strong reaction but those containing early stage spermatids showed no reaction (Fig. 3a). The localization pattern of α -glycerophosphate dehydrogenase was similar to that of sorbitol dehydrogenase when observed at low magnification. However, observation at high magnification showed that the reaction of the former was different from that of the latter. The reaction products of α -glycerophosphate dehydrogenase activity gave linear shapes (Fig. 3b). Since the conditions of the histochemical reaction were the same except for the substrate, the difference seems to be due to a difference of enzyme localization and not due to diffusion or non-specific adsorption.

3. Electron microscopic observation:

Electron microscopic observation of sorbitol dehydrogenase showed that the reaction product was localized in the cytoplasm of the Sertoli cells and was not present in the spermatids (Fig. 4a). The positive reaction was restricted to the region surrounding the spermatids and the basal side of Sertoli cells showed no reaction. The control specimens which were reacted in the substrate-free reaction medium showed no reaction (Fig. 4b).

The electron microscopic observation of α -glycerophosphate dehydrogenase indicated that the reaction products were present only at the mitochondria of late stage spermatids (Fig. 5a). Sertoli cells showed no reaction. These results coincided with the light microscopic observation that the reaction of sorbitol dehydrogenase seemed very diffuse but that of α -glycerophosphate dehydrogenase seemed very sharp. In the epididymal spermatozoa, there was no apparent positive reaction of sorbitol dehydrogenase, but positive reactions of α -glycerophosphate dehydrogenase were seen at the mitochondria of the spermatozoa (Fig. 5b).

DISCUSSION

During spermatogenesis, the germinal cells change their energy source and spermatozoa utilize fructose as an energy source instead of glucose (Mann, 1949). In the mouse testis, a positive reaction of sorbitol dehydrogenase was seen at the cytoplasm of Sertoli cells which enclosed late stage spermatids, and was not associated with early stage spermatids. These results indicated that fructose is supplied by Sertoli cells after the late stage of spermatogenesis. Therefore, it is considered that in the early stage germinal cells utilize glucose, while late stage spermatids utilize fructose. The site of fructose synthesis was restricted to the cytoplasm of Sertoli cells adjacent to the late stage spermatids. These findings suggest that local specialization occurs in the Sertoli-cell cytoplasm. Such local production of fructose would be advantageous in that the newly synthesized fructose could be easily transported to spermatids for utilization. On the other hand, sperm could metabolize glycerol as an energy source (Mann and White, 1957). α -Glycerophosphate dehydrogenase, an enzyme of the glycerol pathway, was detected in mitochondria of late stage spermatids and spermatozoa. These results are consistent with the histochemical study by Meitkiwski and Lukaszyk (1968) as well as with the results of biochemical analysis of sperm (Mohri and Madaki, 1967). Moreover, it was suggested that glycerokinase was localized at the midpiece of the spermatozoa. Therefore, the enzymes related to glycerol metabolism are considered to be localized at the mitochondria of the spermatozoa. In the mouse testis, sorbitol dehydrogenase and α -glycerophosphate dehydrogenase appeared at about the same time, so the glycerol metabolic pathway may be established at the same time as fructose is supplied. These considerations suggest that glycerol metabolism may be intimately related to the fructose metabolism. White (1957) observed that sperm which were incubated with glycerol produced fructose in addition to lactate. In the liver, fructose metabolism is considered to involve a split of F-1-P by aldolase and, as a result, a formation of glyceraldehyde and dihydroxyacetone phosphate. Though the latter is metabolized by the same pathway as glucose, the former is considered to be metabolized by three different pathways. At high concentrations of glyceraldehyde, it is metabolized to lactate through the glycerol pathway (Sillero, 1969). Though the relation is not established between fructose metabolism and glycerol metabolism, it is possible that both are related to fructose utilization.

ACKNOWLEDGMENT

The authors wish to thank Dr. N. Kato for his help in gas chromatographic analysis.

REFERENCES

1. Chida, K., Yamamoto, N. and Yasuda, K.: Histochemical study of polyol dehydrogenase. The localization of sorbitol dehydrogenase in mouse liver and kidney. *Acta histochem. cytochem.* 8; 234-246, 1975.
2. Hers, H. G.: Le mécanisme de la formation du fructose séminal et du fructose foetal. *Biochem. Biophys. Acta* 37; 127-135, 1960.
3. Johnson, A. B.: Inability to reliably demonstrate the activity of aldose reductase histochemically.

- J. Histochem. Cytochem.* 14; 826, 1966.
4. Mann, T.: Metabolism of semen. *Advan. Enzymol.* 9; 329–390, 1949.
 5. Mann, T. and White, I. G.: Glycerol metabolism by spermatozoa. *Biochem. J.* 65; 634–639, 1957.
 6. Micucci, M., Rama, F., Castellano, M. A. and Germino, N. I.: The histochemical distribution of fructose metabolism enzymes in bovine spermatogenesis. *J. Anat.* 109; 209–214, 1971.
 7. Mietkiewski, K. und Lukaszyk, A.: Der α -Glycerophosphate Dehydrogenase Aktivität während der Spermiogenese im Rattenhoden. *Histochemie* 7; 28–38, 1966.
 8. Mohri, H. and Masaki, J.: Glycerokinase and its possible role in glycerol metabolism of bull spermatozoa. *J. Reprod. Fertil.* 14; 179–194, 1967.
 9. Sillero, M. A. G., Sillero, A. and Sols, A.: Enzymes involved in fructose metabolism in liver and glyceroaldehyde metabolic crossroads. *Europ. J. Biochem.* 10; 345–350, 1969.
 10. White, I. G.: Metabolism of glycerol and similar compounds by bull spermatozoa. *Am. J. Physiol.* 189; 307–310, 1957.