Intracellular Distributions of Glucose 6-phosphate Dehydrogenase and Hexose 6-phosphate Dehydrogenase in Rat Testicular Interstitial Cells

By

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(*With 4 Tables*)

The microsomal fraction of vertebrate livers contains a glucose 6-phosphate dehydrogenase which has properties quite distinct from the cytosol glucose 6-phosphate dehydrogenase (G6PD), thus being designated as hexose 6-phosphate dehydrogenase (H6PD) or type III G6PD (Ohno *et al.*, 1966; Beutler and Morrison, 1967; Kamada and Hori, 1970). Despite the striking difference with respect to several parameters, it has been recently demonstrated that they share at least one antigenic determinant in common, thus suggesting their divergence from a common ancestral molecule (Matsuoka and Hori, 1979). The time of the divergence might be near the echinoderm evolution, since echinoderms are the only invertebrates that possess both G6PD and H6PD (Mochizuki and Hori, 1973, 1976; Ohnishi and Hori, 1977; Kamada *et al.*, 1978).

The physiological role of H6PD is still unknown, but it might be possible that the enzyme is involved in drug metabolism. This has been suggested by our previous finding that phenobarbital causes an increase of the activity of H6PD bound to smooth-surfaced endoplasmic reticulum without affecting the activities of H6PD bound to rough-surfaced endoplasmic reticulum and cytosol G6PD (Hori and Takahashi, 1974). If this hold, it is probable that organs involved in steroid metabolism might be relatively rich in H6PD as in the liver and that the H6PD activity might be increased when the metabolism is stimulated, since steroids are predicted to be natural substrates of the drug metabolizing systems (Kuntzman *et al.*, 1965, 1966; Conney and Klutch, 1963). The organ-specific distribution of H6PD examined electrophoretically appears to support this prediction; the enzyme is relatively rich in the liver, adrenal and ovary. In order to further substantiate the above prediction, the specific activity of H6PD in the microsomal fractions of

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various rat organs, the intracellular distribution of H6PD in testicular interstitial cells which rank next to the liver with respect to the content of H6PD and effects of human chorionic gonadotrophin (hCG) treatment on the H6PD were examined in the present study.

Materials and Methods

Chemicals were obtained from the following commercial sources; glucose 6-phosphate, galactose 6-phosphate and soybean trypsin inhibitor from Sigma, NADP from Kyowa Hakko Kogyo Co., collagenase from Worthington and hCG from Teikokuzoki Co. All other chemicals were commercial products of the highest grade available.

Rats used were males of Wistar strain aged 50 and 80 days at sacrifice. HCG was injected intraperitoneally at a dose of 100 U per day for two weeks.

Testes, six at a time, were decapsulated in 10 ml of Krebs-Ringer-bicarbonate (KRB) buffer containing glucose (1 mg/ml) and collagenase (0.3 mg/ml) in a 50 ml Erlenmeyer's flask and incubated at 37°C for 10 min with constant shaking. After incubation, the flask was cooled and 20 ml of KRB buffer containing glucose (1 mg/ml) and soybean trypsin inhibitor (0.15 mg/ml) were added. Partially dissociated testes were then transferred into 20 ml of KRB buffer in a Petri dish cooled with iced water for manual separation of interstitial tissues and seminiferous tubules by the method of Christensen and Mason (1965).

Light microscopic observation of the two fractions thus obtained showed that the interstitial tissue fraction contained interstitial cells, blood vessels, other connective tissue elements and a small amount of sperms, while the tubular fraction consisted mostly of seminiferous tubules with a small amount of interstitial tissues. The above method was the combination of the methods of Moyle and Ramachandran (1973) and of Christensen and Mason (1965) which resulted in a better separation than either one of the two methods alone.

The interstitial cell and seminiferous tubule fractions were separately washed with KRB buffer, collected by centrifuging at 100 × g for 10 min, homogenized in 6 ml of 0.25 M sucrose using a Potter-Elvehjem homogenizer and centrifuged for 10 min at 950 × g in order to remove nuclei and tissue debris.

In the first series of experiments, the supernatant was centrifuged at 144,000 × g for 1 hr to obtain particulate and cytosol fractions, while in the second series of experiments, it was centrifuged successively at 10,000 × g for 30 min and at 144,000 × g for 1 hr to obtain mitochondrial, microsomal and cytosol fractions.

The particulate fractions thus prepared were suspended in 1–4 ml of 20 mM phosphate buffer, pH 6.4, treated with 1.0% Triton X–100, and assayed for enzyme and protein. The experiments were repeated three times in each series.

The purity of the mitochondrial and microsomal fractions of interstitial cells was examined electronmicroscopically after fixation in 2% paraformaldehyde–2.5% glutaraldehyde (Karnovsky, 1965) followed by 1% OsO_4 in 0.1 M cacodylate buffer, pH 7.4.

Microsomal fractions were also isolated from several organs other than the testis by the method described above, and assayed for protein and H6PD activity.

G6PD and H6PD activities were assayed spectrophotometrically. Enzyme solutions were first incubated with anti-H6PD antiserum in the case of G6PD assay and with anti-G6PD antiserum in the case of H6PD assay in the presence of 0.6 μmol NADP in a total volume of 0.25 ml for 20 min at 21°C. After adding 0.7 ml of glycine-NaOH buffer (I=0.2, pH 10.0 for G6PD and 10.8 for H6PD), the reaction was started by the addition of 6 μmol of G6P, and extinction changes at 340 nm were recorded at 21°C for 5 min. One unit of activity was defined as the amount of enzyme that reduced 1 μmol of NADP per minute. The amounts of antibody used were so determined as to inhibit the activity of co-existing H6PD or G6PD completely. The anti-H6PD antiserum used was the same lot used in a previous study (Takahashi and Hori, 1978), while anti-G6PD antiserum was supplied by Mr. K. Ohnishi. The methods for purification of rat liver G6PD and for preparation of the specific antibody against it will be described elsewhere.

Protein was assayed according to the method of Lowry *et al.* (1951).

Results

The specific activities of H6PD in the microsomes from various organs were assayed in the presence of 1% Triton X-100 and are listed in Table 1. As predicted, the enzyme activity was high in the liver, testicular interstitial cells, ovary and adrenal which are all actively involved in steroid metabolism. The activity would be even greater in the ovary than is shown in Table 1, if non-steroid metabolizing cells are excluded.

Quantitative distributions of G6PD and H6PD in seminiferous tubules and interstitial cells of normal and hCG-treated, young and adult rats are shown in Table 2. The results demonstrate that the two enzymes are much more con-

Table 1. Specific activities of H6PD (*m*units/mg protein) in the microsomal fractions from various rat organs.

Tissues	H6PD activity
Liver	11.9
Testicular interstitial cells	8.3
Ovary	6.0
Adrenal	4.8
Kidney	3.2
Spleen	1.7
Lung	1.3
Brain	1.2
Intestine	0.6

centrated in interstitial cells than in seminiferous tubules, except H6PD of young rats, and markedly increase in activity during maturation and after hCG treatment in interstitial cells. In seminiferous tubules, a 40–60% increase was observed only after hCG treatment. Generally, the effect of hCG was much

Table 2. Intratesticular distribution of G6PD and H6PD in normal and hCG-treated rats.

		Protein mg/testis	H6PD *m*units/testis	G6PD *m*units/testis
Seminiferous tubules				
Particle	50d normal	11.1±1.0	22.9±1.0	21.4± 0.5
	hCG	12.8±0.6	23.9±2.1	32.1± 3.6*
	80d normal	10.2±1.1	26.0±3.1	16.3± 5.6
	hCG	10.7±0.1	20.8±3.1	26.0± 5.6
Cytosol	50d normal	13.1±1.0	9.8±0.9	107.8± 7.7
	hCG	12.8±0.8	13.4±0.9	149.6± 6.6*
	80d normal	15.3±0.8	10.7±0	106.7± 6.6
	hCG	14.9±2.0	18.7±4.5	172.7±46.2
Interstitial cells				
Particle	50d normal	1.4±0.1	16.0±1.5	59.6± 4.0
	hCG	2.9±0.4*	43.5±2.9**	187.9±32.3*
	80d normal	2.2±0**	31.9±0**	85.9± 3.0**
	hCG	1.9±0.1	34.8±4.5*	88.9± 7.1*
Cytosol	50d normal	1.8±0.8	17.1±0.6	378.3±18.4
	hCG	2.6±0.3	43.9±3.4**	895.3±85.4**
	80d normal	2.1±0.3	33.1±1.1**	548.1±10.7**
	hCG	2.5±0.3	37.6±1.1**	602.4±17.5**

Each value is the mean (± S.E.) of three experiments. * $P<0.05$; ** $P<0.01$

Table 3. Intracellular distribution of G6PD and H6PD in the interstitial cells of normal and hCG-treated rats.

	Protein mg/testis	H6P dehydrogenase		G6P dehydrogenase	
		*m*units/testis	*m*units/mg protein	*m*units/testis	*m*units/mg protein
Mitochondria					
50d normal	0.80±0.05	11.2±1.2	14.0±1.2	34.3±1.7	42.9±2.7
hCG	1.32±0.07**	21.6±1.8**	16.4±0.8	89.8±5.0**	68.0±7.5*
80d normal	1.68±0.09**	18.5±0.7**	11.0±0.4	78.3±1.9**	46.6±3.5
Microsome					
50d normal	0.28±0.04	3.3±0.2	11.8±1.5	1.6±0.1	5.7±0.9
hCG	0.56±0.02**	10.8±0.2**	19.3±0.8**	4.4±0.2**	7.8±0.7
80d normal	0.64±0.02**	8.7±0.8**	13.6±1.4	3.2±0.2**	5.0±0.5
Cytosol					
50d normal	1.38±0.14	13.7±1.1	9.9±1.2	302.2±19.2	219.0±24.3
hCG	2.10±0.10**	31.3±7.7*	14.9±3.4	657.1±12.1**	312.9±23.1*
80d normal	2.78±0.15**	22.5±0.6**	8.1±0.5	580.2±12.2**	208.7±10.4

Each value is the mean (± S.E.) of five experiments. * $P<0.05$; ** $P<0.01$

less prominent in adult rats than in young rats. Further detailed examinations of the intracellular distribution of G6PD and H6PD were therefore performed only with interstitial cells, the results of which are shown in Table 3.

The activities per testis of both G6PD and H6PD increase 1.6～3.3-fold in the three subcellular fractions after hCG treatment and during maturation. The specific activity also increased in microsomal H6PD (64%) and in mitochondrial (59%) and cytosol (43%) G6PD after hCG treatment, but not during maturation.

The intracellular distribution of H6PD in interstitial cells is quite different from that in the liver reported previously (Hori and Takahashi, 1974). The ratio of H6PD activity among the mitochondrial, microsomal and cytosol fractions of normal adult rats is 37:18:45 in interstitial cells on a per testis basis in contrast to 7:73:20 in the liver on a per g liver basis. In the case of interstitial cells, however, correction must be made for the above values, since the electron microscopic examination revealed that the mitochondrial fraction contains considerable amounts of rough-surfaced endoplasmic reticulum in addition to a small amount of smooth-surfaced endoplasmic reticulum, while the microsomal fraction consists exclusively of smooth-surfaced endoplasmic reticulum. This is in striking contrast to the previous finding on the liver that the mitochondrial and microsomal fractions prepared by the same procedure are almost free of contamination. In any case, the present data clearly indicate that about a half of H6PD molecules exist not as a membrane-bound form in interstitial cells. Implication of this finding is obscure at present. On the other hand, G6PD is predominantly located in the cytosol fraction of interstitial cells, as in the liver.

Increases of the activities of H6PD and G6PD during maturation and after hCG treatment have been proved to be due to the increases in the amounts of enzymes by immunological techniques; i.e., given amounts of antibodies to H6PD and G6PD precipitated the same levels of activities of the respective antigens in control and experimental samples.

Table 4. Weights of body, testes, seminal vesicles and prostates.

	Body weight (g)	Testes (mg)	Seminal Vesicles (mg)	Prostates (mg)
50d normal	163±5	746±41	220±17	108± 7
hCG	193±3**	970±74*	544±25**	232±14**
80d normal	283±3**	1420±19**	1140±60**	326±12**

Each value is the mean (± S.E.) of five experiments. * $P<0.05$; ** $P<0.01$

That the hCG treatment did stimulate the steroidogenesis under the present experimental conditions was proved by the results given in Table 4; the weights of male accessory organs increased significantly after hCG treatment as well as during maturation.

Discussion

The present data clearly indicate that steroid metabolizing tissues show higher specific activities of the microsomal H6PD than other tissues, and that the H6PD activity in interstitial cells increases significantly after stimulation of steroidogenesis by the hCG treatment. At first sight, this appears to suggest that H6PD might be involved in steroid metabolism as predicted. Since the Km's for G6P and NADP are of the same order of magnitude in H6PD and G6PD (Hori and Sado, 1974), it is possible that H6PD may be able to function as a NADPH supplier for the microsomal system as efficiently as G6PD may be. It must be noted, however, that the cytosol G6PD has also been increased after stimulation with hCG. The increase is only 1.4-fold, but the activity in hCG-treated rats expressed on a per testis basis is more than 30 times as great in the cytosol G6PD as in the microsomal H6PD. Even in untreated, adult rats, the cytosol G6PD activity is about 12 times as great as that of total H6PD.

A question thus arises as to why the membrane of endoplasmic reticulum has to have its own built-in NADPH generator, which increases in the specific activity upon stimulation.

It has been known that G6PD is inhibited by the steroids possessing an oxo group at the C–17 or C–20 position and an OH group at the 3 position, and that the K_i values are 6.2 μM for dehydroepiandrosterone, 0.6 μM for dehydroepiandrosterone sulfatide, 7.1 μM for pregnenolone and 12 μM for 17α-hydroxyprogesterone, being small enough to be physiologically significant (Marks and Banks, 1960; Benes *et al.*, 1970; Oertel and Rebelein, 1970). In contrast, H6PD is not affected by dehydroepiandrosterone at all (Hori and Sado 1974; Sado and Hori 1976; Mochizuki and Hori 1976; Matsuoka *et al.*, 1977). It seems, therefore, to be advantageous for the interstitial cells to have H6PD as the NADPH generator for steroidogenesis. If the NADPH generator actually functioning in steroidogenic cells is H6PD, but not G6PD, another question arises why the interstitial cells possess G6PD and elaborate more G6PD when stimulated by hCG. Would the role of G6PD in steroidogenic cells be to provide NADPH for biosynthetic pathways other than steroidogenesis only when intracellular concentrations of steroids are relatively low ?

There is still another question about the physiological role of H6PD; i.e., in the case of liver, there is an abundance of NADPH in the cytosol. It is so abundant as to inhibit G6PD almost completely (Krebs and Engglestone 1974). There is no reason to assume, however, that microsomes are unable to utilize NADPH present in the cytosol, because NADPH-cytochrome c reductase is present on the cytoplasmic surface of the microsomal membrane (Omura *et al.*, 1967; Kuriyama et al., 1969; DePierre and Dallner 1975) and isolated microsomes are capable of hydroxylate drugs and steroids *in vitro* in the presence of oxygen and NADPH (Conney *et al.*, 1957a, b; Conney and Klutch, 1963; Remmer and Merker, 1963).

The above discussions are all focused on the role of H6PD as a NADPH generator. However, H6PD has an affinity to NAD as well as to NADP, and this

property has been conserved during evolution; i.e., the Km for NAD is 1 μM in all H6PD so far examined (rat, Hori and Sado, 1974; toad and crucian carp, Sado and Hori, 1976; starfish, Matsuoka *et al.*, 1977). Possibility can not be ruled out, therefore, that H6PD is functioning *in vivo* as a NADH generator rather than a NADPH generator, although the maximal velocity attained with NAD is much lower than that with NADP.

The wide occurrence of H6PD in echinoderms and vertebrates and the strict conservatism in its properties strongly suggest its physiological importance, but its role can not be precisely defined at this stage of knowledge.

Summary

The specific activities of microsomal H6PD in various organs and intracellular distributions of G6PD and H6PD in testicular interstitial cells before and after hCG treatment were examined in order to obtain information on the physiological significance of H6PD.

The results indicate that the microsomal H6PD activity is higher in the liver, ovary, adrenal and interstitial cells than in the kidney, spleen, lung, brain and intestine, and that the microsomal H6PD as well as the cytosol G6PD increase in the specific activity after hCG treatment. The specific activity of microsomal H6PD in interstitial cells of hCG-treated rats is greater than that in hepatocytes of phenobarbital-treated rats.

The present data are not inconsistent with a supposition that H6PD might be involved in drug or steroid metabolism, but further studies are needed to prove this supposition.

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