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On the Glucose 6-Phosphate Dehydrogenases in Rat Livers, with Special Regard to Sex Difference^{1),2)}

By

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(With 1 Text-figure)

It is well accepted that sex determination in mammals is governed by the XX-XY mechanism. Generally, female individuals have two sets of X-linked genes, while one such set occurs in males. The protein synthesis controlled by a certain X-linked gene would, therefore, take place twice as much in females as in males, if two X chromosomes are equally active. This quantitative relationship between the number of gene and the amount of its product is called "dose effect".

It has been known that some X-linked genes do not show dose effect. Stern (1960) has explained the lack of dose effect by assuming the presence of the compensator genes.

Recently, another important and interesting explanation has been proposed for the lack of dose effect by Russell (1961, 1963, 1964) and Lyon (1961), independently. The hypothesis called "The Single-Active-X hypothesis" is based on the assumption that one of the two X's in females is genetically inert, that it is a matter of chance which X, maternal or paternal, becomes inert, and that when inertness of the single X has once been determined during early embryogenesis, the inertness never changes afterwards. In other words, the cell having the active maternal X does not yield the cell having the active paternal X in its offspring.

The hypothesis was originally based on the genetical findings with mice, but it soon gained supports from some cytologists who had confirmed the following facts from the morphological standpoint of view: Firstly, sex chromatin which is observed in resting nuclei of female cells, but not in male cells, is of an X chromosome origin. Secondly, it is a matter of chance which X becomes sex chromatin. Thirdly, the condensed status of an X chromosome in resting nuclei does not seem to favor the synthesis and release of messenger RNA, so that a possibility exists of its genetical inertness (Ohno *et al.*, 1959; Ohno and Hauschka,

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1960; Ohno and Makino, 1961; Ohno and Weiler, 1961; Taylor, 1960; Hsu, 1962).

A very strong support to this single-active-X hypothesis has been provided by biochemists who had been working on the glucose 6-phosphate dehydrogenase (G6PD) deficiency in human red blood cells (RBC) (Childs *et al.*, 1958; Gross *et al.*, 1958; Motulsky *et al.*, 1959; Beutler *et al.*, 1962; Porter *et al.*, 1962; Tarlov *et al.*, 1962; Tönz and Rossi, 1964). Their findings were; (1) that the G6PD in human RBC is under the control of an X-linked gene, but the enzyme activity is at the same level in males and females; (2) that there are some people whose RBC G6PD is deficient; and (3) that, in heterozygous female carriers of the gene for G6PD deficiency, RBC consists of two populations, one normal and one deficient for G6PD. These findings are easily understood if the single-active-X hypothesis is taken into consideration. Namely, if one of the two X's in females be genetically inert, both male and female cells possess one active X. It is then evident that the G6PD controlled by an X-linked gene shows the same activity in both sexes. Likewise, females heterozygous for G6PD deficiency would possess two sorts of cells, one being inert, and one being normal with regard to G6PD activity.

The present report deals with our recent findings on the sex difference in both the total activity and zymograms of hepatic G6PD in rats, and on the hormonal control of hepatic G6PD activity.

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Materials and Methods: Animals used were normal suckling and adult rats of Wistar-King A strain. For induction of G6PD, rats were fed with a diet containing 30% casein and 60% glucose according to the method of Tepperman and Tepperman (1958). Some rats were given 0.06% 3'-methyl-4-dimethylaminoazobenzene for 20 days.

Adult rats castrated 2 weeks previously were used for the study of effects of sex hormones on hepatic G6PD. Some ovariectomized rats were daily injected subcutaneously with 1.5 mg dehydroepiandrosterone (Teikoku zoki) for 5 days. Similarly, orchietomized rats received five daily injections of 0.2 mg estradiol benzoate (Estradin B).

All the rats were killed at 4:00 to 5:00 p.m., in order to avoid the diurnal variation of the G6PD activity. Livers were removed, rinsed in saline, frozen on dry ice, homogenized with 2 volumes of water, and centrifuged at $15,000 \times g$ for 20 minutes. The supernatant was used for enzyme assay and electrophoresis.

Electrophoresis was carried out according to the method of Ornstein and Davis (1962). The supernatant was diluted appropriately with 3.9% large pore gel, and 0.35 to 0.45 ml of this mixture were layered over 0.3 ml of large pore gel.

After electrophoresis for about 2.5 hours at 20°C in 0.05 M tris-glycine buffer, pH 8.3, with a constant current of 3 mA per gel column ($12 \times 3 \times 100$ mm), the gel was rinsed in water, and stained in the incubating medium consisting of nitro blue tetrazolium (NBT) (0.3 mM), or iodinitro tetrazolium (INT) (0.5 mM), tris-maleate buffer (pH 7.2, 50 mM), KCN (10 mM), nicotinamide adenine dinucleotide phosphate (NADP) (0.25 mM), glucose 6-phosphate (G6P) (1.5 mM) and phenazine methosulfate (PMS) (0.07 mM).

Incubating media in which G6P was replaced by 6-phosphogluconate (6PG), and those which were devoid of either G6P or NADP or PMS were also used.

For the measurement of G6PD activity, the supernatant was diluted 30 times with water, and 0.05 ml of this dilution were added to 0.5 ml of the incubating medium containing 3 mM each of G6P and 6PG, or 3 mM 6PG alone in addition to INT, NADP, KCN, PMS and buffer at the same concentrations as those described above.

Following incubation for 10 minutes at 37°C, formazan was extracted by vigorously

shaking with 5 ml of ethyl acetate. The optical density was read at 490 $m\mu$. The activity of G6PD was taken as the difference between the amounts of formazan reduced in the presence of both G6P and 6PG, and 6PG alone.

The results are given in Figure 1, where the value for adult males is taken as 100 per cent.

Observations

In livers of adult rats the sex difference in the G6PD activity was striking as shown in Figure 1, the activity of females being about twice as high as that of males in agreement with the data of Glock and McLean (1953, 1954). Though not shown in the figure, no such sex difference occurred in the kidney, lung, adrenal, spleen and RBC.

The sex difference was also evident between zymograms obtained from the livers of both sexes; the G6PDs were separated into 6 molecular forms, which were designated as A-F in an increasing order of electrophoretic mobility. Bands D and E were stained similarly in males, while in females band D was apparently intenser than band E.

In suckling rats, the enzyme activity was approximately at the same level in both sexes, showing roughly 75 per cent of the activity of adult males. No sex difference was found in their zymograms. However, the zymograms of suckling rats differed from those of adult rats in that the former did not exhibit band D.

The above findings suggested that the hepatic G6PD activity greatly increased during maturation only in females, and that band D would probably be differentiated from band E during maturation more abundantly in females than in males. A speculation was also possible that band D enzyme might be in a repressed condition in males.

Attempt was therefore made to enhance the total enzyme activity of males in order to learn whether band D enzyme would increase specifically whenever the total enzyme activity increases.

As a result, the enzyme induction by dietary carbohydrates and by azo dye feeding were found to be effective on the specific induction of band D enzyme. In other words, male rats subjected to these experiments exhibited an intensely stained band D, the feature being similar to that of females (Fig. 1).

Based on the fact that the G6PD activity and zymograms shifted during maturation of animals, effects of hormones and castrations were examined in the following.

In male rats, the enzyme activity and zymogram were not affected by both castration alone and castration followed by injections of estradiol.

In female rats, castration alone exerted no effect on the enzyme, while the injection of dehydroepiandrosterone into ovariectomized rats resulted in a drastic decrease of the total enzyme activity. Interestingly, band D enzyme of the hormone-treated, ovariectomized rats was no longer intenser than band E.

Omission of G6P or NADP from the incubating medium caused no staining of gels, while the replacement of G6P by 6PG resulted in a staining of different pattern. This indicates the absence of 6PG dehydrogenase in electrophoretically separated G6PDs.

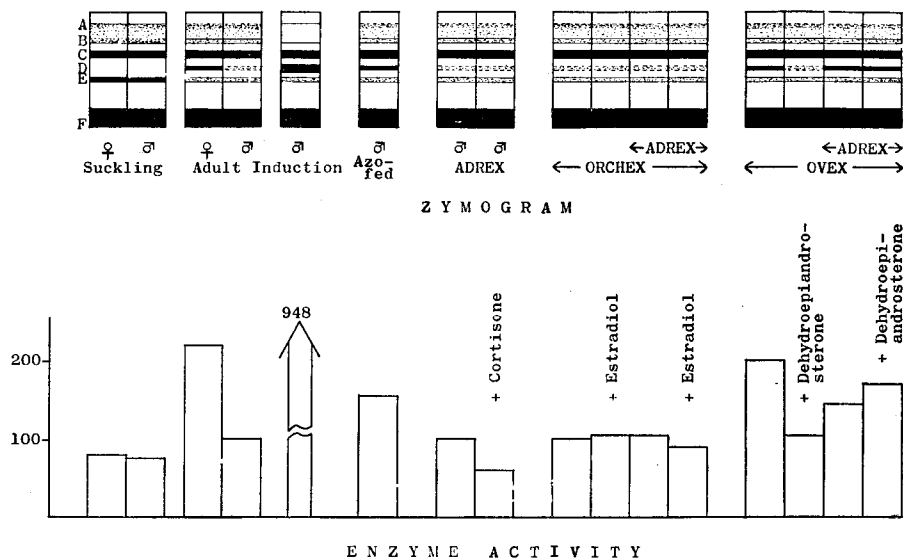


Fig. 1. Graphical representation showing relative activities and zymograms of hepatic G6PD in normal and experimental rats.

Omission of PMS from the medium resulted in weak and diffuse stainings of bands C and F indicating the location of diaphorase in these bands. Zymograms of a good quality was never obtained without PMS. This is against Tsao's finding (1960) that sufficient diaphorase migrates with enzymes.

Both NBT and INT gave comparable results in every respect. An attempt to assay the amounts of INT reduced by each isozyme on acrylamide gel column was unsuccessful on account of the insolubility of gel-bound formazan in ethyl acetate.

Addition of NADP to the sample mixture prior to electrophoresis did not affect the electrophoretic pattern of enzymes.

Discussion

The present study has shown that 6 electrophoretically distinct bands of G6PD activity can be recognized in water extracts of adult rat livers. Since it is possible that a single macromolecule interacts with the solvent medium to show two well defined bands on gels (Cann and Goad, 1964), the 6 bands demonstrated

might not necessarily represent the existence of 6 isozymes in rat livers. Although further investigations are required for this possibility, bands C, D and F seem to represent 3 different isozymes so far as the present study has gone. This assumption is made on the basis of the distance between and distinctness of bands C and F, and of the differential response of band D to various stimulations.

The possibility that some of the bands may result from contamination of plasma and erythrocytes was ruled out by the finding that all the bands were demonstrated clearly with various dilutions of the samples.

Sex-determined differences in electrophoretic patterns of certain enzymes have been reported by Allen and Hunter (1960), Friedman and Lapman (1961), Shaw and Koen (1963), Allen *et al.* (1965), and Ruddle (1966). The present study has revealed that the hepatic G6PD is another example which is under the influence of sex hormone. Our preliminary data showed that the *in vivo* inhibitory effect of dehydroepiandrosterone was confined only to hepatic G6PD, but not to kidney and lung enzymes.

Potter and Ono (1961) reported that induction of hepatic G6PD by dietary carbohydrates was interfered with X-irradiation, adrenalectomy, and puromycin injection, and was restored in adrenalectomized rats by cortisone injections. Their findings suggest that the hepatic G6PD activity might be under the control of the adrenal cortex, though its action might be indirect.

It is interesting that whatever the cause of the activity increase may be, band D enzyme is always activated at a greater rate than the other electrophoretically separated enzymes. If such a differential activation is associated with an increase in the net synthesis of the enzyme protein, it appears that the process involved in the synthesis of band D enzyme is specifically sensitive to stimulations. Further investigation on the hormonal control of hepatic G6PD is now being in progress.

Summary

The glucose 6-phosphate dehydrogenases of the rat liver were separated into 6 molecular forms by means of acrylamide gel electrophoresis. They were referred to as A to F in the order of increasing anodal mobility. The enzyme activity decreased in the order: C > F ≫ B ≥ A > D ≥ E. It was found that band D enzyme, though it is less intense in normal adult males, exhibited higher levels of activity than band E enzyme in normal females, while males fed high glucose diets or azo dye showed a rather intense band D. Such a sex-difference was confined to adult rats.

Dehydroepiandrosterone exerted a strikingly inhibitory effect on female enzymes, while estradiol did not affect the enzyme activity in males.

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Addendum in proof

Our recent data indicated that estradiol benzoate (N.B.C.) had a strikingly stimulative effect on the hepatic G6PD of orchietomized rats, this being in disagreement with the present data. The cause of the disagreement is now being under study.