SUBCELLULAR DISTRIBUTION AND CIRCADIAN RHYTHM OF SOME GLYCOLYTIC ENZYMES IN RAT TESTIS

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The circadian variations in plasma testosterone levels in men (Saxena, Leyendercker, Chen, Gandy & Peterson, 1969) and the suggested circadian-based production of FSH and LH in birds (Murton, Bagshawe & Lofts, 1969), rats (World Health Organization, 1969) and men (Saxena *et al.*, 1969) led us to study the circadian behaviour of two testis enzymes, hexokinase and pyruvate kinase. These enzymes, in addition to the primary rôle played in the sequence of glycolytic reactions, possess specific isoenzymes with electrophoretic and kinetic characteristics (Sosa, Altamirano, Mercado, Hernández, Reyes & Rosado, in preparation) that can be studied as part of the circadian behaviour of testis metabolism.

Male albino Wistar rats, weighing 150 to 170 g, were maintained for 20 days in a 12 hr light/12 hr dark cycle; after this time, groups of three to five rats were killed at six different times of day, the testes were rapidly removed and some of the tissue was fixed in Zenker's solution for histological study. Tunica albuginea-free tissue was homogenized in an equal volume of cold 0.01 M-phosphate buffer, pH 7.0, with a Potter-Elvehjem homogenizer equipped with a Teflon pestle. The buffer contained 5×10^{-3} M-EDTA and 5×10^{-3} M-2-mercaptoethanol. After homogenization for 1 min, adequate samples were taken for protein, RNA and DNA determinations, the remaining homogenate was centrifuged at 100,000 g for 60 min and the resultant supernatant solution used without further treatment for protein and enzyme assays. For the determination of the activities associated with subcellular particulate and soluble fractions of normal rat testis homogenate separated by differential centrifugation, the method of Katzen, Soderman & Wiley (1970) was followed for tissue obtained from two groups of rats killed at 06.00 and 18.00 hours.

Hexokinase activity was assayed at two different concentrations of glucose, 25×10^{-3} M and 1×10^{-4} M, respectively, according to the spectrophotometric assay of DiPietro & Weinhouse (1960); when fructose was used as substrate, an excess of phosphohexose isomerase was included in the assay incubation mixture (Grossbard & Schimke, 1966).

Pyruvate kinase was assayed following the decrease in absorbance at 340 nm in the coupled reaction with lactate dehydrogenase at room temperature, according to a small modification of the method of Bucher & Pfeleiderer (1955).

Histological studies of the tissue, stained with Harris haematoxylin and eosin technique, were used to assess normal spermatogenic function in all the samples.

Hexokinase activity in testis seems to be high when compared with some

TEXT-FIG. 1. (a) Circadian variation of hexokinase and pyruvate kinase activities of rat testis. The upper line represents the variation in the activity of pyruvate kinase in relation to the time of day (indicated in the abscissa of Text-fig. 1b). The activity at 14.00 hours is significantly different ($P < 0.05$) from the activity at 06.00 hours. The two lower curves represent the hexokinase activity using 25×10^{-3} M-glucose (middle line) and 25×10^{-3} M-fructose (lower line). All the points in the graphs are significantly different from the previous and subsequent ones, except the activity found at 02.00 hours which does not differ from the activity at 06.00 hours.

(b) Circadian variation of protein, DNA and RNA in rat testis. The traces represent the values of RNA (upper line), protein (middle line) and DNA (lower line) found at the times indicated. Only the amount of RNA showed a significant variation, with a decrease at 14.00 hours ($P < 0.01$) and an increase at 22.00 hours ($P < 0.05$). Every point on the graphs represents the mean of three to five determinations $\pm$ one standard deviation. The stippled part represents the interval during which the rats were kept in darkness.

other rat tissues, at least ten times as high as in rat liver (Farina, Adelman, Lo, Morris & Weinhouse, 1968). As can be seen in Text-fig. 1 (a), the activity of this enzyme in the high speed supernatant from centrifuged rat testis homogenate

showed a remarkable variation in relation to the time of day at which it was assayed, varying from $4.4 \mu\text{mol/mg protein/hr}$ at 06.00 hours to almost $7.0 \mu\text{mol/mg protein/hr}$ at 10.00 hours. Afterwards, the activity decreased, with a new and smaller peak occurring at 18.00 hours.

In contrast, pyruvate kinase displayed no significant variation in relation with time, with the possible exception of the small decrease observed at 14.00 hours.

It can also be seen in Text-fig. 1(a) that hexokinase activity, though lower in the presence of fructose, follows the same time-course pattern as with glucose, indirectly showing that the same enzyme phosphorylates both substrates.

Rat testis hexokinase is almost all soluble (Text-fig. 2), almost 80% of the activity being found in the soluble fraction of the homogenate. Among the

TEXT-FIG. 2. Hexokinase activity in soluble and particulate fractions of rat testis homogenate. Apart from microscopic observations (which showed heavy contamination of the 1100 g precipitate by whole spermatozoa), no other control of fraction purity was made. The columns indicate the mean of the four determinations. No significant difference in the hexokinase activity assayed at 06.00 or 18.00 hours was observed in any of the isolated fractions.

other fractions, only nuclei possess a significant amount of activity (about 18%) but this is probably due to contamination of the fraction with whole spermatozoa.

The circadian rhythm of rat testicular hexokinase, and perhaps also of rat testicular pyruvate kinase, is probably related to gonadotrophic control acting directly or indirectly through the stimulation of testosterone synthesis. It has been found previously (Sosa *et al.*, in preparation) that developmental changes of hexokinase and particularly of sperm hexokinase can be related to the increase in 17β -hydroxysteroid dehydrogenase activity and the decrease in pregn-4-en-5 α -reductase that permits an efficient conversion of androstenedione to testosterone.

It has been proposed that the activity of hexokinase *in vivo* may be controlled by the relative distribution between soluble and particulate forms and this equilibrium is considered to be governed by the intracellular ATP, glucose-6-

phosphate and phosphate (Wilson, 1968; Katzen *et al.*, 1970). However, the possibility that the cyclical changes observed in rat testis hexokinase activity could be caused by some other cyclic process in the tissue can be excluded since rat testis hexokinase is nearly all soluble (Text-fig. 2) and because no significant change was observed in its subcellular distribution at 06.00 and 18.00 hours, while during the same period a significant change was present in soluble activity (Text-fig. 2).

Copulatory activity in the rat is critically determined by the photoperiod. Dewsbury (1970) has shown that copulation takes place as soon as the light is turned off and that males reach ejaculation and resume copulation after ejaculation with shorter latencies towards the end of the dark phase of their cycle.

It is known that copulation produces a release of LH and that peripheral levels of this hormone are higher during copulation periods (Taleisnik, Caligaris & Astrada, 1966). The decrease in hexokinase activity which occurs during the dark period may, therefore, be due to the passage of spermatozoa from intimate contact with the Sertoli cells to the seminiferous tubules, induced by the high blood levels of LH (Donoso & Santolaya, 1969) more than to a direct inhibitory action of this hormone upon hexokinase activity.

This hypothesis is supported by the finding that simultaneously with the decrease in hexokinase activity, a significant increase in RNA synthesis ($P < 0.01$) is observed during the dark phase of the photoperiod (Text-fig. 1b) that may be related as a precursor-product effect to the increase in enzymatic activity observed during the light period. Experiments are at present being carried out in our laboratory to test if the observed changes in enzyme activity and biochemical composition reflect active *de novo* synthesis of proteins and RNA.

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