CHAPTER III

METHODOLOGY

Terminology

1.1 ***work load*** The unit that measure the work load in work test with bicycle ergometer is kiloponds(kp). 1 kp is the force acting on the mass of 1 kg at normal acceleration of gravity; 100 kpm/min = 723 foot-ponds/min = 16.35 watts.) The braking power(kp) set by adjustment of belt tension, multiplied by distance pedalled(m), gives the amount of work in kilopond metres(kpm). If the distance is expressed per minute, then the rate of work in kpm per min will be obtained. In bicycle ergometer, one complete turn of the circumference of the pedals move a point on the rim 6 meters. If the pedalling frequency is 50 complete pedal turns per minute, the "track distance" covered will be 300 metres per minute. So the rate of work load is 300kpm/min or 50 watts.

1.2 ***Exercise heart rate*** is a simple and readily available index of cardiac stress, as it is directly proportional to the oxygen consumption of the heart. Provided that the work is not too heavy, respiration and circulation increase during the first few minutes and then attain a steady state. The increase in heart rate can be established by counting the heart rate once every minute. After 4-5 minutes the heart rate has generally reached the steady state. (In order to work the muscles need oxygen and nutritive substances, carbon dioxide and waste products have to be removed. This transport exert a load on respiration and circulation.) As a

rule, about 6 minutes is thus sufficient to adapt the heart rate to the task being performed. The maximal heart rate(MHR) depends on the age. We calculate the MHR by Fox's formula(1971) that is

$$\text{MHR(beats/min)} = 220 - \text{Age(year)}$$

*1.3 **Maximal oxygen consumption*** (**Vo_2max**)is defined as the rate of oxygen consumption beyond which additional energy demand must be met from anaerobic sources, resulting in an accumulation of lactic acid in working cells and in plasma(Margaria, 1976). Vo_2max is usually elicited during intense activities involving large muscle masses such as runing and cycling. Under these conditions, over 90% of the O_2 uptake in the lungs is consumed by skeletal muscle cells. In this studies, we determine Vo_2max from a nomogram by Astrand and Irma(1960), Fig.4 - Fig.7, which predict of Vo_2max from heart rate and work load on a bicycle ergometer

Subjects.

Twenty-four healthy male subjects, were divided into two groups; athletes groups and non-athletes groups, with twelve subjects in each group.

The subjects abstained from any medication for at least 3 weeks before the study and avoided changes in their usual diet and level of physical activity. The athletes must regularly exercise at least 4 days per week for at least 6 weeks and maximal Oxygen capacity must above 45 ml/kg/min.

Methods

Experimental protocol. As is shown in Fig. 8, the subjects performed two uninterruptedd incremental exercise tests until exhaustion, sitting on an electromagnetically braked bicycle ergometer (Monark Ergometer Model 818E). The first test was a pretest meant to assess the subject's maximal exercise capacity and preceded the second experiment by at least 1 week; The initial external work load of 25 W was increased by 25 W/2 min., until the subject declared he was exhaust. The highest work load performed for 2 minutes was noted. At least 1 week later the subjects came to the laboratory around 8.00 A.M. after an overnight fast. A small catheter was inserted into an antecubital vein for blood sampling. Thereafter they rested in the recumbent position for 20 minute, after which they assumed the sitting position on the bicycle ergometer, then a first blood sample was withdrawn into heparinized tubes. Finally, the exercise test was started at an initial work load of 20% of the maximal exercise capacity as determined at the pretest; the work load was increased with a further 20% each 6 minute, up to a final work load of 80%. Blood samples were again withdrawn, without interrupting exercise, at the end of the work loads of 80% of maximum. Finally, a last blood sample was withdrawn after 10 minutes rest in the sitting position.

Preparation of blood samples

After fresh heparinized blood were collected, the blood was immediately centrifuged at 1800 rpm, 4^{o} C for 10 minutes, the plasma, the buffy coat and the topmost layer of erythrocytes were removed. The

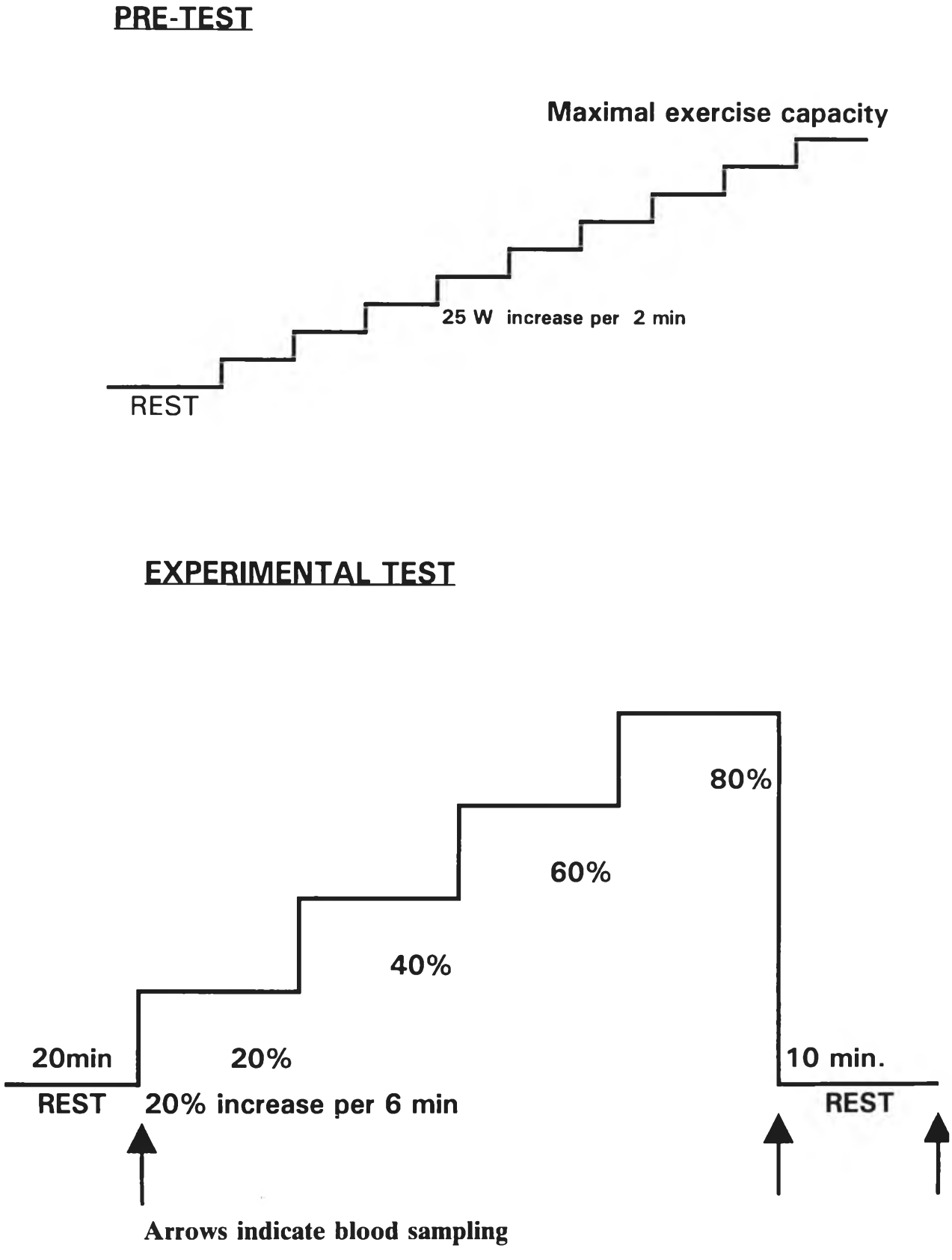

Fig. 5 Experimental protocol

remaining erythrocytes were washed with 10 volumes of ice cold, 112 mM $MgCl_2$ solution. One part of washed erythrocytes was resuspended in an equal volume of ice cold saline-histidine buffer (SHB; 155 mM NaCl, 3 mM histidine, pH 7.5), kept at -20^{o} C for further erythrocyte membrane preparation. The remaining part of erythrocyte was analysed for intracellular electrolytes and number of erythrocyte Na-K-ATPase or ^{3}H ouabain binding site (ROBS).

Plasma and intracellular electrolytes

Plasma and intracellular sodium, potassium and magnesium (PNa, PK, PMg, RNa, RK and RMg) were measured by flame photometry. To measure RNa and RK, erythrocytes were suspended in $MgCl_2$ solution to get a hematocrit of 40-50% and lysis according to the method of Mayer and Starkey, 1977.

Plasma Lactate and osmolality

Plasma Lactate was measured by enzymatic method, the plasma osmolality was measured by freezing point depression.

(^{3}H)- ouabain-binding site assay

ROBS was measured by a minor modification of a previously described techniques (Sangu-Mize, 1990). The erythrocyte as prepared above were washed in ice-cold ouabain binding buffer (OBB; 140 mM NaCl, 30 mM HEPES, 10mM dextrose, pH 7.4), to get a hematocrit of

5-10%. Cells were counted with a hemocytometer. To establish Scatchard plots, triplicate 400 µl aliquots of erythrocyte suspension were mixed with 50 µl (^{3}H)-ouabain solution (Amersham, Specific activity 47 Ci/mM, concentration 1.0 mCi/ml) to a final concentration of 8, 16, 32, 64 and 128 nM, and to a total volume of 500 µl in absence and in presence of 0.1 mM unlabelled ouabain. After incubation at 37^o C for 120 minutes, the cell pellet was washed thrice with ice-coride. Membrane bound radioactivity was extracted from the cells with 500 µl of 5% trichloroacetic acid and counted by a liquid scintillation spectrometer (Rack Beta 1219, LKB Wallacoy, Turku, Finland). Specific ^{3}H-ouabain binding was a concentration difference between bound ^{3}H-ouabain in the absence and that in the presence of 0.1 mM unlabelled ouabain. It was expressed as ^{3}H-ouabain binding sites per cell from Scatchard plots as previously described (Scatchard , 1949). Intraassay coefficient of variation from the same pool of erythrocyte obtained in the same day was less than 6.5%.

ATPase assay

Isolation of erythrocyte membranes was carried out as reported by Hanahan, Ekcholm, 1978 with a slight modification. In brief, frozen erythrocytes was thrawed and hemolysed with 0.1 mg/ml saponin in ice-cold SHB. The membrane were separated by centrifugal washing at 23,500 g, 20 minutes for 5 times and resuspended in the same buffer. A 0.1 ml aliquot of membrane suspension was incubated in 0.4 ml ATPase assay medium at 37^{o}C, 90 minutes with 0.4 ml ATPase assay medium [100mM NaCl, 50 mM Tris-HCl, 15 mM KCl, 5 mM $MgCl_2$, 5 mM

ATP, 1 mM ethylene glycol bis (beta-amino ethyl ether) N, N, N', N' - tetraacetic acid (EGTA)]. The reaction was stopped by trichloracetic acid. Membrane protein was measured by Lowry's method (Lowry et al, 1951) and it ranged from 3-5 mg/ml. The phosphorus was by Lawrence's methodd (Lawrence, 1951). The ATPase activity was expressed as nanomole inorganic phosphate released/mg membrane protein. h (nmol Pi/mg.h). The Na-K-ATPase activity was a difference between Pi released by action of erythrocyte membrane on ATP in the absence and presence of 1.0 mM ouabain. Intraassay variation from the sane membrane pool was less than 4.9% and interassay variation when the frozen erythrocytes were kept for no longer than 5 days was less than 5%.

Statistical analysis

The data at rest, during 80% of maximal exercise capacity and during recovery were analyzed by two-way analysis of variance (ANOVA), considering the levels of activity and the recovery and the subjects as sources of variation.