CHAPTER III

MATERIALS AND METHODS

Animals preparation

Six healthy nonpregnant swamp buffaloes (Bubalus bubalis) of 5 to 8 years of age and ranging in weight from 300 to 400 kg were used in the experiment. The animals were fed in naturally conditions. On the day of experiment the animals were fed normally and water was given ad.lib. in the morning. During the experiment, water and feed were withheld.

The experiments were carried out on unanesthetized animals in the standing position on the concrete floor in the room which could be adjusted temperature and air ventilation. Prior to the experiment, polyethylene catheter (PE 200) was inserted into jugular vein by using a Medicut intravenous canula. The catheter was left in place throughout the experiment to facilitate both infusion and blood sampling. The animal was fitted up with a temporary stainless steel canula (O.D. 5.0 mm) in the rumen, for injection of polyethylene glycol (PEG) and rumen sampling. Urethral catheter which made of rubber was inserted for collection of urine. Each animal was prepared before the experiment began in order to eliminate the additional stress of handling.

Experimental procedure

The experiment was divided into two periods; control and acute heat stress. The elapsed time between two periods were at least 2 weeks and it took about 6 hours for each experimental period.

Control period

The animal was housed in the room held at ambient temperature of 28.83 ± 1.57 to 32.7 ± 1.35°c. Measurements were between 10.00 and 17.00 hours, on the beginning of experiment tritiated water (TOH) at the dose of 3,000 μCi per animal and 20 ml of 0.5 % T-1824 solution were infused intravenously via catheter. Heparinized blood samples were periodically collected after infusion of TOH and T-1824 for measurement of total body water, water turnover rate, plasma volume, plasma electrolytes (Na, K, Cl), creatinine concentration, plasma aldosterone levels and haematocrit values. Collection of urine samples were made every hour for 6 hours after TOH infusion for analysis of urinary electrolytes and creatinine values in order to obtain the values of fractional excretion of electrolytes. Polyethylene glycol (PEG M.W. 4,000 Nakarai Chemicals Ltd. Japan) at the amount of 40 g dissolved in 500 ml of distilled water was administered into rumen after TOH and T-1824 infusion. Rumen samples were hourly collected after PEG administration for determination of ruminal fluid volume ; its outflow rate and ruminal fluid concentration of electrolytes. Measurement of respiratory rate, heart rate and rectal temperature were obtained every hour until the end of the experiment.

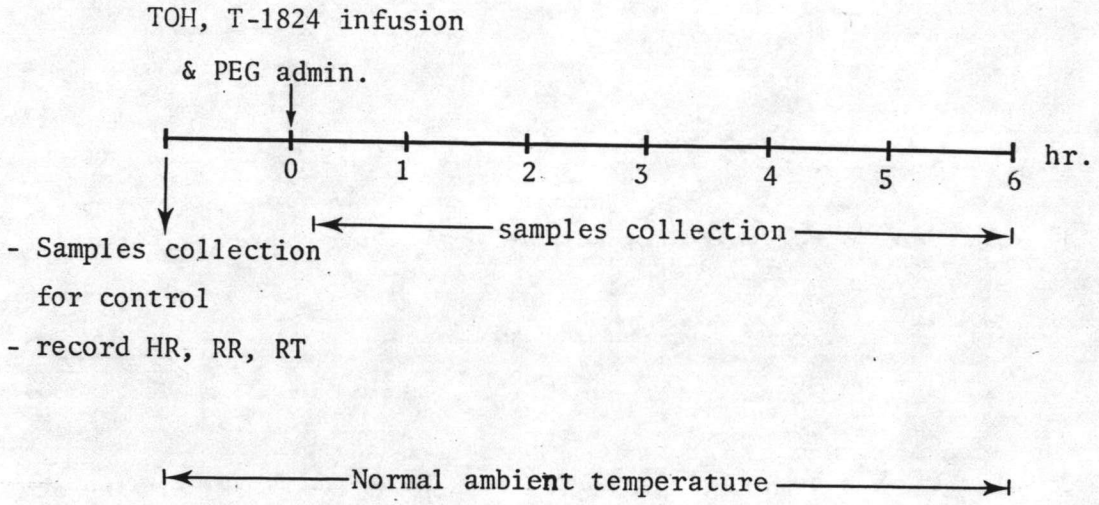
Acute heat stress period

One hour before the experiment began, blood sample, rumen sample, urine sample were collected and respiratory rate, heart rate, rectal temperature were recorded for control values. T-1824 was infused intravenously before animal exposed to heat for measuring plasma volume and blood volume. After that, the animal was kept in the room which had already been adjusted the temperature to 42°c, relative humidity at 45 % and the temperature was maintained at 42°c until the end of the experiment. After the animal was kept in the room for 1 hour, the sequence of experiment was carried out the same as control period except that infusion of T-1824 was done on the fourth hour after heat exposure.

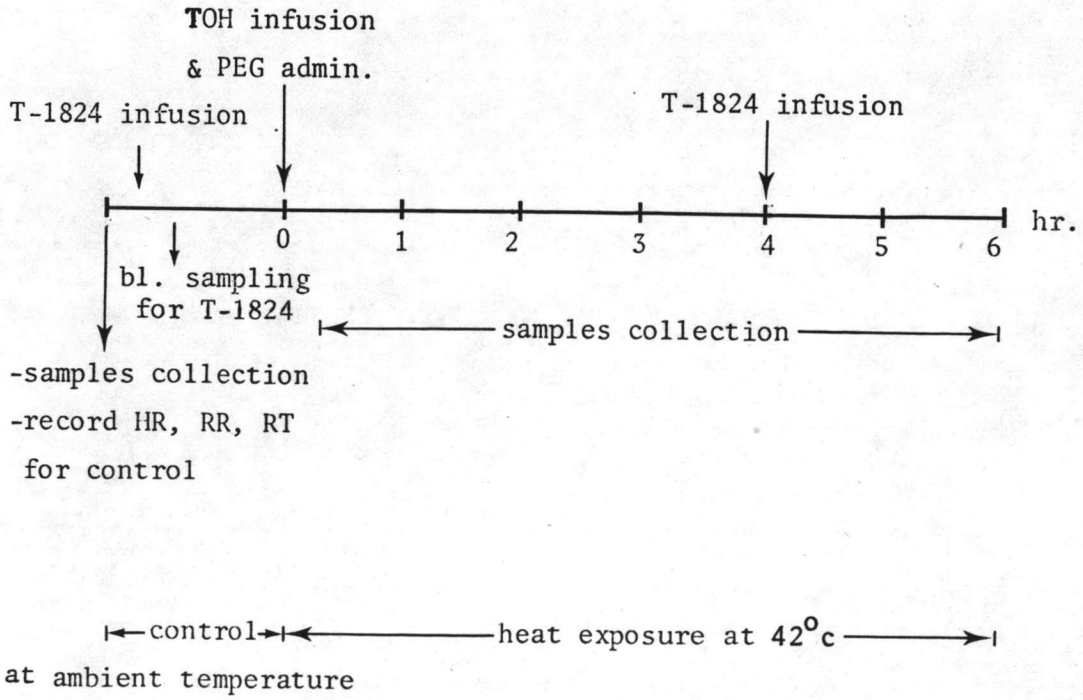
All samples were kept at -20°c for further analysis.

Protocol of experiment

Control period



Heat stress period



Method of measurement

Heart rate was recorded by electrocardiogram lead II, rate of respiration by counting the flank movements and rectal temperature by using a clinical thermometer. Plasma, urinary and ruminal fluid electrolytes were measured by flame photometer for Na and K (Klina flame operating; Beckman instrument) and by chloridometer for Cl (Buchler digital chloridometry; Beckman instrument). Packed cell volume (Hct.) was determined by using Clay-Adam micro-haematocrit centrifuge and microhaematocrit reader.

Determination of plasma and blood volume

Plasma volume was determined by Evan's blue dye dilution technique and blood volume was calculated indirectly from the estimated plasma volume and haematocrit value. In order to determine plasma volume, the dye concentration of a control sample of blood which was collected before the dye injection and the blood samples of 20, 30, 40 and 60 mins blood after dye injection were read by spectrophotometry. Dilution of dye at zero time was determined by extrapolation. Blood volume was determined from plasma volume and haematocrit value (Kolmer et al., 1951) :

$$\text{Blood volume} = \frac{\text{Plasma volume} \times 100}{100-\text{Hct}}$$

Determination of ruminal fluid volume and its outflow rate

Ruminal fluid volume and its outflow rate from rumen to reticulum were measured by using polyethylene glycol by the method

of Hyden (1961). Rumen samples at the time of 1,2,3,4,5 and 6 hour after PEG administration were centrifuged in order to remove any small quantity of feed particles, and PEG concentration was determined in the supernatant according to the method of Smith (1959). For volume of rumen liquor of 5 ml, 2 ml 0.3 N $Ba(OH)_2$ was added, followed by 2 ml of 5 % (W/V) $ZnSO_4.7H_2O$, and 0.5 ml 10% (W/V) $BaCl_2.2H_2O$ was added , the mixture was made up to 10 ml by D.W. and well shaken. After 5 min the mixture was centrifuged and filtered. Three ml of filtrate was pipetted into a test tube and the volume was made up to 5 ml by D.W. Five ml of a solution containing trichloroacetic acid (30% W/V) and $BaCl_2.2H_2O$ (5.9% W/V) was added, the tube was shaken and allowed to stand for 5 min. At the same time standard tubes were prepared by measuring exactly 5 ml of solution containing 0,0.125, 0.25, 0.5 and 0.75 mg/ml polyethylene glycol respectively followed by 5 ml of the TCA/$BaCl_2$ solution , the tube were shaken and allowed to stand for 5 min. Turbidity of the solutions were read by spectrophotometer at the wavelength of 540 nm. Polyethylene glycol concentration at the time of dosing (zero time) was calculated by least square regression method. From this value and the amount of PEG added to the rumen, ruminal fluid volume was calculated. The ruminal fluid outflow rate was calculated by the slope of this line.

Calculations :

$$\text{Ruminal fluid volume} = \frac{\text{the amount of PEG added}}{\text{PEG conc}^{n}\text{ at zero time}}$$

The outflow rate of ruminal fluid can be calculated from the theoretically assumption that

$$X_t = X_o e^{-ut/v}$$

where X_o is the initial concentration of PEG. X_t is the concentration after time t, u is the rate of flow of fluid into and out of the rumen and v is the volume of ruminal fluid.

$$\text{i.e. } u = \frac{0.693v}{T_{\frac{1}{2}}}$$

where $T_{\frac{1}{2}}$ is the time the concentration was half reduced.

Method of determination total body water and water turnover rate

Water turnover rate and total body water were measured by using tritiated water (TOH) as the method described by IAEA/FAO (1979). The animal was infused intravenously with 3 ml of 1,000 µCi of tritiated water. Collected blood samples at the time of 20,40, 60 mins, 2, 3, 4, 5, 6, 20, 30, 44 and 54 hours respectively after TOH infusion and assayed tritiated water by the method of internal standardization by Vaughan and Boling (1961). Since precipitation of plasma protein occurred on adding 1 ml of plasma directly into 17 ml of dioxane scintillator system, counting was carried out on the supernatant fluid only. Simultaneous correction both for loss of radioactivity in the discarded precipitate and for quenching was achieved as follows: An amount of liquid scintillant sufficient for all samples, blanks and internal standards was devided into two portions. Tritiated water was added to the one portion, in the ratio of 10 µl of high specific

activity tritiated water to 20 ml of liquid scintillant, giving a counting rate of 10,000 cpm per 20 ml. This is the "internally standardized" portion. The other portion had nothing added to it. Seventeen millilitre quantities of scintillant were pipetted into the centrifuge tubes. Two pipettings were made of each sample, one into internally standardized scintillant, the other into nonstandardized scintillant. In addition, duplicate tubes for a reagent blank and duplicate tubes for the internal standard were prepared at this time. Tube were capped, shaken and centrifuged for 15 min at 1,000 g. The supernatant was poured directly into counting vials, then counted.

Calculations : the correct counting rate by internal standardization is divided as follow :

$$X_c = \bar{X}. \frac{S}{(X + S) - (\bar{X})}$$

where $\bar{X}$ = sample net counting rate in nonstandardized scintillant; $(X + S)$ = net counting rate of sample in internally standardized scintillant; S = net counting rate of internal standard (17 ml of standardized plus 1 ml of nonstandardized scintillant); X_c = sample counting rate corrected for quenching and loss in precipitate.

$$\text{total body water (V)} = \frac{\text{dose of TOH administered}}{\text{activity of TOH at zero time}}$$

where activity of TOH at zero time was determined by extrapolation.

$$\text{water turnover rate} = V \times k$$

where $k = \frac{0.693}{T_{1/2}}$

$T_{1/2}$ = the time activity was half reduced.

Determination of aldosterone level

Aldosterone levels were obtained from radioimmunoassay method by using Coat-A-Count No extraction aldosterone kit of Diagnostic Products Corporation U.S.A. All samples were counted for 1 minute in a gamma counter.

Calculation :

For calculation of fractional excretion of electrolytes, determination of endogenous creatinine in plasma and urine was carried out according to the method of Kennedy as described by Smith (1962). The following calculation was performed.

$$\text{Fractional electrolyte excretion (\%)} = \frac{U_e/P_e}{U_{cr}/P_{cr}} \times 100$$

which U_e = urinary concentration of electrolytes (mEq/L)

P_e = plasma concentration of electrolytes (mEq/L)

P_{cr} = plasma concentration of creatinine (µg/ml

U_{cr} = Urinary concentration of creatinine (µg/ml)

Statistics

The conventional paired t-test was used to estimate the statistical significance of differences between value obtained from the same animals under control and heat-stress conditions.