CHAPTER III

MATERIALS AND METHODS

Animal Preparation

Adult male mongrel dogs, weighing 9-18 kilograms, were used in all experiments. The animals were deprived of food but not of water for 12 hours prior to study. On the day of the experiment, the animal was anesthetized with sodium pentobarbital (Nembutal, Ceva Sanofi sante animale s.a., Paris, France) 30 mg/kg.bw. intravenously injection initially, and supplemented subsequent doses of 1-2 mg/kg.bw. when necessory to maintain light anesthesia throughout the experiment.

Surgical and Experimental Proceduce

The animal was tracheostomized and endotracheal tube was inserted to secure free airways. Two femoral veins were cannulated with polyethylene tubes (PE 180). One for infusion of the clearance solution, the other for infusion of sodium metavanadate solution or injection of compounds for loading in a series of experiment. The glomerular filtration rate was calculated from the clearance of inulin (In) (Sigma chemical company, St.Louis, MO, U.S.A.) and the effective renal plasma flow was calculated from the clearance of p-aminohippuric acid (PAH) (Sigma chemical company, St.Louis, MO, U.S.A.). For the renal clearance studies, the priming solution containing PAH 1.2 gm% and inulin 5 gm% in isotonic normal saline was administered 0.5 ml/kg.bw. then the sustaining solution composed of 120 mg% and 500 mg% of PAH and inulin respectively, was infused with a constant peristaltic infusion pump

(Eyla model 3) at a rate of 1.4 ml/min. To maintain potency of the other femoral lines an infusion of isotonic normal saline was given through each line as the rate of sodium metavanadate infusion. Sodium metavanadate ($NaVO_3$) (M.W.=121.9, Sigma chemical company, St.Louis, MO, U.S.A.) diluted in isotonic normal saline, 0.2 μmole/kg/min, was intravenously infused for 120 minutes during experimental periods at the time indicated below, using a constant peristaltic infusion pump. One of femoral artery was cannulated with polyethylene tube (PE 200) for blood collection and connected to the pressure transducer (PE 23 AA, Statham Instruments) and recorder (Polygraph Model 79, Grass Instruments Co.), which allowed continuous monitoring of arterial blood pressure and heart rate. Left ureter reached by paracostal incision with a retroperitoneal approach and cannulated with polyvinyl catheter (PV190) for urine collection. The intraoperative fluid losses were replaced by isotonic normal saline intravenously equivalent to 0.5 ml/kg.bw. when the experiment began. The left kidney was exposed and a hooked shape 23-gauge needle attached to polyethylene catheter (PE50) was placed at the base of left renal artery, antrograde to renal arterial flow, for infusion of isotonic normal saline in group I and dissolved infusion of various drugs (prazosin, atenolol, acetylcholine, and verapamil) diluted with isotonic normal saline in a series experiment with a constant syringe pump (Harvard apparatus intravenous syringe pump). As shown in Figure A.

An equilibration period of at least an hour after the clearance infusion and urine flow rate stabilization was allowed, thus, the experiment protocols began. Urine sample was collected during two control, four experimental, and two recovery clearance periods of 30 minutes 's time duration. Blood samples from femoral artery was obtained at the midpoint of each clearance period. Plasma and urine were frozen until analyzed as described below.

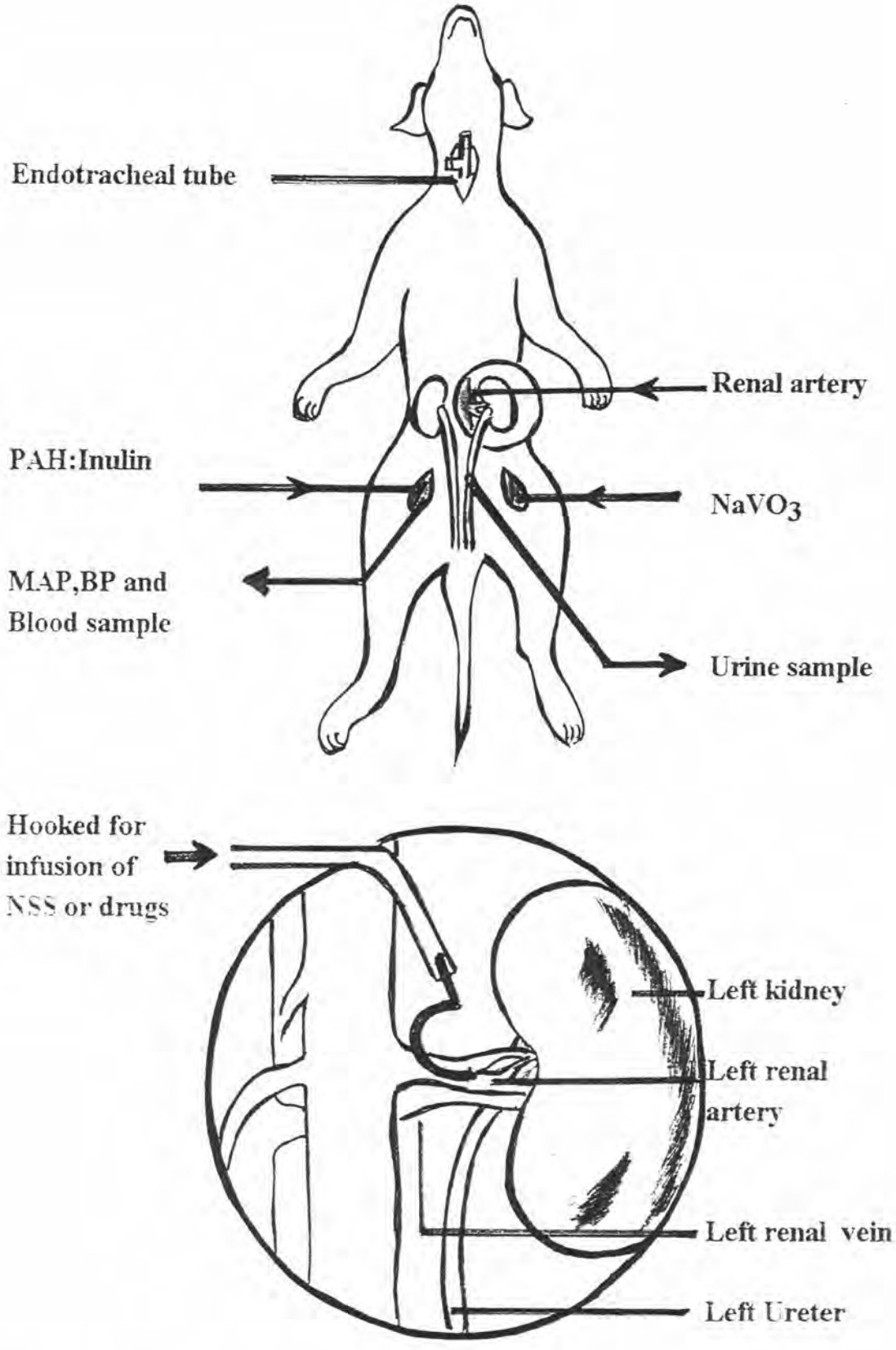

Figure A. Scheme of experiment

Experimental protocols

The experimentals were devided into two series.

Series I : This series was studied to elucidate the actions and machanisms responsible for cardiovascular, renal hemodynamics, and renal functions during intravenous sodium metavanadate infusion in anesthetized dogs. Thirty male mongrel dogs were divided into six groups. As shown in Figure B.

Group I : **Animals Pretreated With Isotonic Normal Saline Solution Before Sodium Metavanadate Infusion.**

Five dogs were used as control animals. After an equilibration period of an hour, animals were infused into the renal artery with isotonic normal saline solution at a rate of 1 ml/min throughout the experiment. Two periods of 30-min each in the control were collected. In experimental period, sodium metavanadate ($NaVO_3$) diluted in NSS was infused immediately by intravenous infusion with the dose of 0.2 μmole/kg/min for 120 minutes. Four periods of 30-min each in the experimental clearance collections were obtained. After removal of $NaVO_3$ infusion, two periods of 30-min each in the recovery clearance collections were observed.

Group II : **Animals Pretreated With Prazosin (Pra) Before Sodium Metavanadate Infusion.**

Five dogs were used in this group. In the 30-min control period, animal was infused into the renal artery with isotonic normal saline solution at a rate of 1 ml/min. One 30-min in the control period was collected. Thirty minutes before infusion of

sodium metavanadate solution, the animal was pretreated with prazosin (M.W.=419.9, Sigma chemical company, St.Louis, MO , U.S.A.) which was performed by intrarenal-arterially infusion in the dose of 2 μg/kg/min diluted in NSS at a rate of 1 ml/min in replacing of NSS throughout the experiment period. One 30-min in the pretreated with the drug period was collected. In experimental period, sodium metavanadate ($NaVO_3$) diluted in NSS was performed immediately by intravenous infusion with the dose of 0.2 μmole/kg/min for 120 minutes. Four periods of 30-min each in the experimental collections were obtained. Then, after removal of prazosin and $NaVO_3$ infusion, two periods of 30-min each in the recovery clearance collections were observed.

Group III : Animals Pretreated With Atenolol (AT) Before Sodium Metavanadate Infusion.

Five dogs were used in this group. A protocol identical to group II was followed, except that animal was pretreated with atenolol (M.W. = 266.3, Sigma chemical company, St.Louis, MO , U.S.A.) which was infused intrarenal-arterially in the dose of 2 μg/kg/min diluted in NSS at a rate of 1 ml/min in replacing of NSS throughout the experiment period.

Group IV : Animals Pretreated With Enalapril Maleate (MK 422) Before Sodium Metavanadate Infusion.

Five dogs were used in this group. A protocol identical to group II was followed, except that animal was pretreated with enalapril maleate (M.W. = 492.5, Sigma chemical company, St.Louis, MO, U.S.A.) which was injected intravenously in a single dose of 10 mg/kg at 30 minutes before given sodium metavanadate.

Group V : **Animals Pretreated With Acetylcholine (ACh) Before Sodium Metavanadate Infusion.**

Five dogs were used in this group. A protocol identical to group II was followed, except that animals was pretreated with acetylcholine (M.W. = 181.67, Sigma chemical company, St.Louis, MO, U.S.A.) which was infused intrarenal-arterially in the dose of 100 μg/min in replacing of NSS throughout the experiment period.

Group VI : **Animals Pretreated With Verapamil (Ver) Before Sodium Metavanadate Infusion.**

Five dogs were used in this group. A protocol identical to group II was followed, except that animals were pretreated with verapamil (M.W.= 491.1, Sigma chemical company, St.Louis, MO, U.S.A.) which was infused intrarenal-arterially in the dose of 50 μg/min diluted in NSS at a rate of 1 ml/min in replacing of NSS throughout the experiment period.

Equilibium Period	Control Period	Experimental Period	Recovery Period
⟵ Inulin 5 gm% and PAH 1.2 gm% ⟶			
NSS (IV)	NSS (IV)	$NaVO_3$ 0.2 μmole/kg/min (IV)	NSS (IV)

-60 -30 0 30 60 90 120 150 180 210 240

Time Elapse (minutes)

Group I NSS (IR)	NSS (IR)	NSS(IR)
Group II NSS (IR)	Prazosin 2 μg/kg/min (IR)	NSS (IR)
Group III NSS (IR)	Atenolol 2 μg/kg/min (IR)	NSS (IR)
Group IV NSS (IR)	↑MK422 10 mg/kg single dose (v) injection	NSS (IR)
Group V NSS (IR)	Acetylcholine 100 μg/kg/min(IR)	NSS (IR)
Group VI NSS (IR)	Verapamil 50 μg/kg/min (IR)	NSS (IR)

Figure B. **Diagrammatic illustration of experimental protocols in Series I.**

Series II : This series was studied to define the ***in vivo*** effect of sodium metavanadate intravenously infusion on renal function especially proximal and distal tubular functions in five anesthetized dogs during control period, $NaVO_3$-infused period and recovery period. The animals were examined the transtubule movement maximum of glucose reabsorbtion, PAH secretion and bicarbonate electrochemical gradient time limit transport by loading these agents. As shown in Fingure C.

After an equilibium period and neither $NaVO_3$ nor loading, one 30-min each in the control clearance collections were obtained. After 120 min of $NaVO_3$ intravenously infusion with the dose of 0.2 μmole/kg/min and before loading, one 30-min each in the experimental clearance periods was carried out. After removal of $NaVO_3$ infusion and before loading, one 30-min each in the recovery clearance periods were observed.

Glucose loading was carried out by raising blood glucose concentration, with 25gms% glucose in water (D(+)Glucose(monohydrat), E. Merck AG., Darmstadt, Germany). Bicarbonate loading was carried out by raising blood bicarbonate concentration, with 7.5 gms % sodium bicarbonate ($NaHCO_3$, May & Baker Ltd. dagenham, England). PAH (Sigma chemical company, St.Louis, MO, U.S.A.), 12 mg/ml in normal saline, was infused at a rate of 1.4 ml/min calculated to maintain the plasma concentration of PAH around 15 mg/dl. Thereafter, the plasma concentration of PAH was increased by using an infusate containing PAH at a concentration of 25 mg/ml. Start loading with the priming dose of the combined these agents solution was administered 0.3 ml/kg.bw. then the sustaining solution was infused intravenously with a constant syringe pump (Harward apparatus intravenous syringe pump) in a stepwise fashion of three periods of 10-min each at increasing rate of 0.03 ml/kg/min to 0.06 ml/kg/min and to 0.12 ml/kg/min respectively in the control period, experimental period after 120 min of $NaVO_3$-infusion, and in the recovery

period. At each period, three 10-min clearance were obtained.

For all PAH calculation, it was assumed that 40% of the plasma PAH was protein bound, and appropriate corrections of plasma PAH concentrations were made according to Smith (1962).

Equilibium Period	Control Period			Experimental Period				Recovery Period		
<== Inulin 5 gm% and PAH 1.2 gm% ==>										
NSS (IV)	NSS (IV)			$NaVO_3$ 0.2 µmole/kg/min (IV)				NSS (IV)		
	A	B	R		A	B	R	R	A	B
60	30	30	30	120	30	30	30	30	30	30

Time Duration (minutes)

Abbreviations : A, Before loading period; B, Stepwise fashion loading period; R, Rest period

Figure C. Digrammatic illustration of experimental protocols in Series II.

Determination of blood and urine samples

The glomerular filtration rate (GFR) was calculated from the clearance of inulin and the effective renal plasma flow (ERPF) was calculated from the clearance of PAH by using the Fick's principle. Inulin concentration in plasma and urine were determined by the anthrone method which modified the method of Young and Raisz (1952). Determination of PAH concentration in plasma and urine were carried out with applied method of Bratton and Marshall (1939) as described by Smith et al. (1962).

The compositions in the plasma and urine were measured as following : sodium and potassium concentrations by flame photometer (Clinical Flame Photometer 410C; Corning Ltd., Halstead Essex, England), chloride concentration by chloridometer (Chloride Analyzer 925; Corning Ltd., Halstead Essex, England), and osmolality by the freezing point osmometer (Advanced Digimatic Osmometer model 3D 2).

The pH measurement in plasma and urine were determined by the electrometric techniques with glass electrode pH meter (Hanna Instrument 8520). Bicarbonate concentration in blood and urine were assessed equally the total carbondioxide content when P_{CO_2} changed in 20-60 mmHg. The total carbondioxide content were determined by the gasometric or monometric techniques with microgasometer (Kopp-Natelson microgasometer, Models #660; Scientific industriesinc., Bohemia., N.Y., U.S.A.) as described by Natelson and Manning (1955).

Glucose concentration in blood and urine were determined by Ortho-Toluidine without acetic acid method as discribed by Snegoski and Freier (1973).

Packed cell volume was determined by the preparation of blood in an international microcapillary tube and then centrifuged by microcapillaries centrifuge (Adams micro hematocrit centrifuge, Model 850 Ta), and determined by international microcapillary reader (Howkley micro hematocrit reader).

The urinary weak acid titratable acidity ($H_2PO_4^-$) and ammonium (NH_4^+) were titrated with strong base 0.05N sodium hydroxide and using 1% phenolphthalein as an indicator which modified the method as discribed by Connors (1975).

Titration of weak acid with strong base procedure :

1. Transferred 2.5 ml. of urine sample to the flask and added 1 gm. of potassium oxalate powder (M.W.=184.24 g/mol.; E.Merck, Darmstadt, Germany) to precipitate the calcium which would otherwise interfere with the end point since calcium phosphate precipitated on neutralization of the urine.

2. Added 1 drop of 1% phenolphthalein indicator solution (M.W.=318.33 g/mol.; E.Merck, Darmstadt, Germany) in alcohol and shook well for 2-3 minutes.

3. The direct titration of titrable acid (TA) was carried out by adding the standard 0.05 N sodium hydroxide solution (M.W.=40 g/mol.; E.Merck, Darmstadt, Germany), from a buret, to the solution of sample acid containing a suitable indicator. The end point was markedby the first permanent pink color (pH 8.2). The volume of titrant requiredto reach the end point was read from the calibrated scale of theburet and calculated the normality of titrable acid and the urinary titratable acid excretion.

4. A back titration (residual titration) was useful if the sample should be a volatile base. Then a known volume of standard acid, in excess of that required for the stoichiometric reaction. 37% formaline solution 2 ml., was added in the above titrated mixture. The excess of acid ammonium (NH_4^+) was back-titrated with standard

0.05 N NaOH to the first permanent pink color. The volume of titrant required to reach the end point was read from the calibrated scale of the buret and caculated the normality of ammonium and the urinary ammonium excretion as following :

$$\text{Normality of TA or } NH_4^+ = \frac{\text{0.05N NaOH} \times \text{Volume of NaOH was used}}{\text{Volume of urine (2.5 ml.)}}$$

$$\text{Urinary TA or } NH_4^+ \text{ excretion} = \frac{\text{Normality of TA or } NH_4^+ \times \text{urine flow rate}}{\text{Time collection of urine} \times \text{kg.bw.}}$$

$$= \mu Eq/min/kg.bw$$

Calculations :

$$\text{Mean arterial blood pressure (MAP)} = P_d + 1/3\,(P_s - P_d)$$

$$\text{Glomerular filtration rate (GFR)} = \frac{U_{In}V}{P_{In}}$$

$$\text{Effective renal plasma flow (ERPF)} = \frac{U_{PAH}V}{P_{PAH}}$$

$$\text{Effective renal blood flow (ERBF)} = \frac{ERPF \times 100}{(100\text{-}PCV)}$$

$$\text{Filtration fraction (FF)} = \frac{GFR \times 100}{ERPF}$$

$$\text{Renal vascular resistance (RVR)} = \frac{MAP}{ERPF}$$

Filtered load = $GFR \times P_e$

Urinary electrolyte excretion = $U_e \times V$

Tubular reabsorbtion (T_e) = $(GFR \times P_e)-(U_e \times V)$

Tubular secretion (T_{PAH}) = $(U_{PAH} \times V)-(GFR \times P_{PAH})$

Fractional excretion of electrolyte (FE_e) = $\frac{U_e \times V / P_e \times 100}{GFR}$

Plasma anion gap = $P_{Na}-(P_{Cl}+P_{HCO_3})$

Urine anion gap = $(U_{Na}+U_K)-U_{Cl}$

Partial pressure of CO_2 (P_{CO_2}) = $\frac{[CO_2]T}{0.03\ (1+10^{pH-6.10})}$

Net acid excretion (NAE) = $(U_{TA}V+U_{NH_4})-U_{HCO_3}V$

Fractional water excretion = $\frac{V \times 100}{GFR}$

Osmolar clearance (C_{osm}) = $\frac{U_{osm}V}{P_{osm}}$

Free water clearance (C_{H_2O}) = $V-C_{osm}$

Statistical Analysis

All the data are presented as the means±SEM. Statistical singnificance of differances between period in the same group was determined by the Student's paired t-test. The Student's unpaired t-test was used to estimate the statistical significance of the difference between group means. Linearity was assessed by linear regression analysis. P values less than 0.05 was considered significant.