SYMPATHO-ADRENAL ACTIVITY UNDER CONDITIONS OF MILD HYPOXIA

by

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REVIEW OF LITERATURE

A. General Introduction:

The activity of the sympatho-adrenal system at times of emergency has been well established. W. B. Cannon and his collaborators (1,2) have shown that the adrenal medullary secretion is increased in fear, rage and pain. These findings have been confirmed by a number of other investigators. It has been shown that the adrenal medulla is stimulated by a number of conditions which are severely stressful to the organism. These stimuli include asphyxia (3-9), severe hemorrhage and hypotension (10,11), hypothermia (12), and insulin hypoglycemia (13-15). These conditions are rather severe and would be infrequently encountered under normal circumstances. Because of inadequacies of method there have been too few studies of unanesthetized, untraumatized animals subjected to mild stress, e.g. hypoxia, mild muscular exercise, and less drastic changes in blood volume and blood sugar.

Walter Cannon concluded that the sympatho-adrenal system was not active in the normal animal under normal conditions because of his inability to demonstrate epinephrine secretion from the adrenal glands (16). Furthermore, animals which had been adrenal demedullated and sympathectomized did relatively well provided they lived in a sheltered environment (17). More recently the "resting" secretion of the adrenal medulla has been estimated by several investigators (15,18). These studies were done on anesthetized animals so the results are open to question. Anesthetic agents have been shown to have a variable effect on secretion of epinephrine and norepinephrine (19). Wada, Seo, and Abe were able to demonstrate resting secretion in specimens of adrenal venous blood from

unanesthetized dogs in which the veins were exteriorized (12). The physiologic significance of the minute quantities secreted under these conditions has been questioned by von Euler (20). However, recently it has been demonstrated that a positive arterio-venous difference for epinephrine exists in the peripheral plasma taken from resting subjects (21,22). This further suggests that there is some physiologic significance for the resting adrenal secretion. Thus, there has been some question whether the adrenal medulla and the sympathetic nervous system is functional in the normal animal under the ordinary stresses of life.

Evidence of altered sympatho-adrenal function has been sought in the adrenal medulla and adrenergic nerves, the adrenal vein effluent, the peripheral blood, and the urine. Of these only the peripheral blood and urine are relatively accessible in the intact animal. Elmadjian et al (23) reported that 0.5-1% of the infused epinephrine and 3.0-5.0% of infused norepinephrine appeared unaltered in the urine. The proportion excreted remained constant unless the infusion rate became excessive. These values are in agreement with the earlier studies of von Euler et al (24,25) and more recently those of R. T. Jones (26). Thus, it has been demonstrated that a constant proportion of infused epinephrine and norepinephrine, and presumably endogenous catecholamines also, is excreted unchanged in the urine.

It has been stated thatin urine epinephrine and norepinephrine occur in the ratio of approximately 1:5. von Euler (20) has stated that the major source of norepinephrine is the adrenergic nerves and that the adrenal gland is responsible for the epinephrine in urine. In support of this he cites experiments in which they studied the urinary excretion of epinephrine and norepinephrine in 14 patients before and after bilateral adrenalectomy (27). There was no significant change in

norepinephrine excretion but the epinephrine excretion fell to about one-fifth of the original amount. The actual magnitude of change was from 5.5 to 1.0 µgm./24 hrs.

B. Regarding Sympatho-adrenal Activity in Mild Stress and in Some Abnormal States:

Recently studies have been done to evaluate sympatho-adrenal activity in mild stress, e.g. moderate exercise, slight cold, and emotional excitement.

von Euler et al (28) and Sundin (29) were able to demonstrate increased amounts of norepinephrine and epinephrine in the urine when subjects were tilted to 75° on a tilt-table. Increased excretion in the urine has been observed in severe muscular exercise. von Euler and Hellner (30) observed increases up to ten times the resting level with severe exercise but were unable to show any changes with moderate work. Karki (31) obtained similar results in a larger series of subjects. More recently Gray and Beetham (32) have demonstrated elevations of epinephrine and norepinephrine in peripheral venous plasma after strenuous muscular exercise. In view of the known shifts of blood from the splanchnic regions to the brain, heart, lungs and muscle during exercise, it would seem logical to implicate epinephrine and norepinephrine as the mediators of this change. It is known that exogenous epinephrine and norepihephrine can produce these changes.

Elmadjian et al (23) noted increased excretion of epinephrine and norepinephrine in emotional states associated with anxiety. These findings confirm those of von Euler and Lundberg (33) who studied urinary excretion in air force personnel.

Halme et al (34) in studying 102 patients undergoing surgery noted elevations in urinary norepinephrine which persisted 4-7 days after surgery. The increased epinephrine levels returned to normal within 2-3 days. These results demonstrate that sympatho-adrenal discharge can be rather prolonged.

In some abnormal states there has been a questionable increase in sympatho-adrenal activity. It has long been felt that many of the manifestations of hyperthyroidism were due to sympathetic overactivity or to sensitization of sympathetically innervated structures. Diller and Kilpatrick (35) studied 17 patients with hyperthyroidism and found a significant increase in urinary epinephrine. This increase correlated well with the I^{131} uptake by the thyroid. There is sufficient reason to believe that the renal clearance of epinephrine remains unchanged (26); therefore, the amounts of epinephrine formed must be increased or the inactivating mechanisms must be less efficient.

There is some indirect evidence to indicate that there may be sympathetic overactivity in congestive heart failure. Raab (36) feels that the catecholamines are responsible for some of the physiologic derangements in congestive heart failure. It is well established that heart failure is aggravated by exercise, emotional stress, cold and pain. These same stresses, if severe, act in the normal subject to produce sympatho-adrenal discharge. Eckstein et al (37) demonstrated that epinephrine had deleterious effect on myocardial metabolism by provoking an inefficient utilization of oxygen. However, more recently Feinberg and Katz (38) have obtained results which led them to the opposite conclusion.

These studies led to the formulation of the problem to study sympatho-adrenal activity in normal animals in response to mild stress

and in animals with decreased cardiac reserve subjected to the identical stressful condition. It would seem that the same stress on the circulatory system might evoke a greater response in animals with a decreased cardiac reserve than in normal animals.

C. Methods of Producing Experimental Heart Failure:

Heart failure can be achieved either by decreasing the output of the heart or increasing the work load on the heart chronically. Both of these approaches have been used in attempts to reproduce congestive heart failure in experimental animals.

Valvular defects, either those of stenosis or insufficiency, have been used to increase the cardiac work load (39-41). Also stenosis of the great vessels of the heart, i.e. the pulmonary artery (42-44), the aorta (45), and the inferior vena cava (46), have been utilized. The work load on the heart can also be increased by producing a severe anemia (47) or an arterio-venous fistula (48).

Attempts have been made to reduce cardiac output through the production of myocardial damage. Injection of sclerosing agents (49), implantation of radio-active materials (50), and ligation of coronary vessels (51) all have been utilized for this purpose. The final approach to be mentioned is the production of myocardial infarction by embolizing the coronary circulation. Roos and Smith (52) first attempted this using starch granules ranging from 12-72 micra in diameter. They were unable to get very satisfactory preparations. C. M. Agress et al (53) embolized the coronary circulation with plastic microspheres and produced myocardial infarcts which were similar to those seen in humans. The spheres which they injected ranged from 190-450 micra in diameter. Bing et al (54) used this method to study the immediate hemodynamic and

metabolic changes associated with coronary embolization. They also performed pathologic studies on 23 animals at varying time intervals (20 min.-6 wks.) after embolization and obtained evidence of severe myocardial damage in all instances. Munro et al (55) produced a definite decrease in exercise tolerance and obtained evidence of myocardial ischemia on the electrocardiogram in 7 of 11 dogs surviving the embolization. In a larger series Marcus et al (56) embolized 70 dogs of which 43 died within 48 hours. They claimed the development of congestive heart failure in an unspecified number of the surviving dogs. Autopsy findings revealed the consistent production of myocardial infarcts in chronic preparations.

All methods have been unsatisfactory. The chief drawback has been the inconsistency in producing clinical congestive heart failure.

D. Assay Methods for Epinephrine and Norepinephrine:

One persistent problem in the study of the sympatho-adrenal system has been primarily technical, viz. the accurate estimation of epinephrine and norepinephrine. Both of these sustances belong to a larger class of closely related compounds called catecholamines, several of which occur in biological systems. In the earlier studies it was not realized that two adrenergic mediators existed and the substance released by the adrenergic nerve endings and the adrenal medulla was described as epinephrine. Holtz et al (57) presented evidence in 1947 that the adrenal medulla contained norepinephrine in addition to epinephrine. In 1948 von Euler (58) was able to conclusively demonstrate the presence of norepinephrine in adrenergic nerves. The realization that two such closely related compounds had to be differentiated increased appreciably the complexities of both biological and chemical methods for the assay of these compounds.

The assay procedures available for the estimation of catecholamines can be divided into two general categories, biological and chemical. (Hereafter in this thesis the term catecholamine shall be used to denote the mixture of both epinephrine and norepinephrine). One of the earliest biological methods used was based on the supersensitivity to epinephrine of sympathetically denervated structures in vivo. This supersensitivity of denervated structures is manifest by a decreased threshold, an increased sensitivity of the response, or a prolongation of response (59). Cannon and his co-workers used this method extensively in their studies of the denervated nictitating membrane, dilator muscle of the pupil, and the heart (2,59). This method was only roughly quantitative, and at the time the studies were done the existence of norepinephrine was unknown.

After it was discovered that there were two main adrenergic mediators, i.e. epinephrine and norepinephrine, the biological assay techniques were refined to estimate differentially the catecholamines (60-62). These tests are based on the fact that epinephrine and norepinephrine have quantitatively different actions on many structures so that two suitable preparations can be selected to determine epinephrine and norepinephrine differentially. Preparations which have been used are the cat's blood pressure, hen's rectal caecum, rat's colon, rat's uterus and cat's nictitating membrane (20). The cat's blood pressure responds to as little as 0.05-0.10μgm. of norepinephrine and the hen's rectal caecum responds to as little as 1.0 mμgm. of epinephrine in a 5.0 ml. bath. These values given are those obtainable under the most ideal conditions. The response varies among different animals or preparations and with respect to time. The most distressing shortcoming of these methods is the relative lack of specificity. It is true that epinephrine

and norepinephrine have a markedly different response in many of these preparations but it is also true that many other substances can influence the response. The presence of potassium salts, calcium salts, histamine and acetylcholine present in extracts can markedly influence the response (63).

The next major method of assay is the chemical method. This consists of colorimetric and fluorimetric techniques. The colorimetric methods presently available are too insensitive to detect the amounts of catecholamines present in plasma and urine. The only major use they have acquired is in the estimation of catecholamines in adrenal gland extracts. More recently the fluorimetric methods have been developed to the point where they are useful in the differential estimation of the catecholamines in blood, urine, and tissue extracts.

There are two fluorimetric methods which have been used extensively in recent years to estimate epinephrine and norepinephrine. The first of these depends upon the condensation of the catecholamines with ethylenediamine (64). It is postulated that epinephrine and norepinephrine are oxidised to adrenchrome and noradrenchrome, respectively. These oxidation products then condense with ethylenediamine to produce a highly fluorescent compound. When these compounds are excited by ultraviolet light it is found that products of epinephrine and norepinephrine emit light of different wave lengths. Weil-Malherbe and Bone (65) utilised this difference and developed a method for the differential estimation of epinephrine and norepinephrine. The sensitivity of this method is claimed to be 1.0 mμgm., which would correspond to a concentration of approximately 0.01 μgm.% when 10-15 ml. of sample is used (66).

The other fluorimetric technique is dependent upon the fact that epinephrine and norepinephrine produce a strong, if transient,

fluorescence in alkaline solutions. Ehrlén (67) first postulated that the fluorescent substance formed from epinephrine was an indole. Lund (68) and Harley-Mason (69) isolated and identified the compounds derived from epinephrine and norepinephrine as 3,5,6-trihydroxy-1-methylindole (adrenolutin) and 3,5,6-trihydroxyindole (noradrenolutin), respectively. Lund (70,71) was the first to utilize the fluorescence for the estimation of epinephrine and norepinephrine in plasma. The reaction is dependent upon the oxidation of epinephrine and norepinephrine to adrenochrome and noradrenochrome, respectively. In alkaline solution in the presence of ascorbic acid these oxidation products rearrange to adrenolutine and noradrenolutine, respectively. Lund found that the oxidation of epinephrine and norepinephrine was dependent upon the pH. The oxidation of epinephrine was complete at pH 3.0 and 6.5, but the oxidation of norepinephrine at pH 3.0 was only 5% of that at pH 6.5. On the basis of this observation he was able to estimate differentially epinephrine and norepinephrine in mixtures. Lund originally used manganese dioxide for oxidation. Since then there have been various modifications using other more convenient oxidizing agents such as potassium ferricyanide (72) and iodine (73,74).

More recently it has been noted that the activation spectra of adrenolutine and noradrenolutine were different (22,75). Based on this observation a further modification of Lund's method has been developed. The catecholamines are allowed to completely oxidize at pH 6.0-6.5 and are converted to adrenolutine and noradrenolutine. Then the sample is excited at two different wave lengths of ultraviolet light and the emission is read in the visible region (22,76). This allows differential estimation of epinephrine and norepinephrine in the same mixture. The sensitivity is said to be sufficient to detect concentrations of 0.01-0.05 µgm.% using 10-15 ml. of sample (22,76).

Of the two fluorimetric methods available Lund's method, which is dependent upon the formation of trihydroxyindoles, is more specific than the ethylenediamine condensation method. Lund's method is stated to be specific for B-catecholethanolamines (22,66,70). The ethylenediamine condensation method has been shown to produce appreciable fluorescence with a number of mono- and dihydroxyphenyl compounds in addition to epinephrine and norepinephrine (80). The method has not been applicable to urine because it produces fluorescence with 3-hydroxytyramine and 3,4-dihydroxyphenylacetic acid which are present in appreciable quantities in normal urine (81). The values for epinephrine and norepinephrine concentrations in plasma by the ethylenediamine method are generally higher than those obtained by the trihydroxyindole method (22,66,80) or by biological assay.

E. Methods for Extraction of Catecholamines from Blood and Urine:

A problem in the analysis of hormones present in minute quantities is that of separation for assay. The most successfully used method for the separation of catecholamines from blood and urine is adsorption on aluminum hydroxide or aluminum oxide. Shaw (77) originally used aluminum hydroxide but subsequently aluminum oxide has been used more extensively because of greater convenience. In 1949 Lund (70,71) described a procedure for the extraction of epinephrine and norepinephrine from plasma. He found that the adsorption of catecholamines onto the alumina was nearly quantitative when the pH of the sample was adjusted to approximately 8.0 and elution occurred when the pH was reduced to 3.0. The major difficulty with this method is that the catecholamines are relatively unstable at neutral and alkaline pH so that the procedure must be carried out rapidly. This procedure has been modified by von Euler

and Orwén (78) and Pitkänen (79) so that it is applicable to urine and tissue extracts. It has been claimed that alumina adsorption, under the conditions specified, is highly specific for separation of dihydroxyphenyl compounds, but this has been questioned more recently (80).

No attempt will be made here to discuss the applications of paper and ion exchange chromatography for the separation of catecholamines. These methods have been particularly useful in separating mixtures of epinephrine and norepinephrine. In general these methods are more time consuming and less adaptable to the routine analysis of plasma and urine.

OBJECTIVES

1. To study the response of the sympatho-adrenal system in animals subjected to mild hypoxia using the plasma and urinary catecholamines as indices of activity.
2. To determine if the response of the sympatho-adrenal system in animals with decreased cardiac reserve is quantitatively or qualitatively different from that of the normal animal.

METHODOLOGY

This section is divided into the description of the assay procedure for epinephrine and norepinephrine and the description of the experimental procedures performed on the animals.

A. Assay Procedure:

A Farrand spectrofluorometer was used in the fluorescence determinations of the samples. This instrument consists of a xenon light source, an exciting monochromator, sample chamber, analyzing monochromator and a photomultiplier tube with recording circuit. Blocking filters were inserted between the light source and exciting monochromator and between the sample chamber and the analyzing monochromator to reduce light scatter. A Corning CS 7-54 (transmits ultraviolet light and excludes visible light above 410 mμ.) was inserted between the light source and the exciting monochromator and a Corning CS 3-72 (excludes ultraviolet and transmits visible light above 440 mμ.) was used between the sample chamber and the analyzing monochromator. A 1-P21 photomultiplier tube was used because of greatest sensitivity to visible light.

The wavelength of ultraviolet light activating the sample can be varied by a motor which turns a cam on the activating monochromator and the fluorescent emission is read at a predetermined wavelength of visible light by setting the analyzing monochromator. This gives the activation spectrum of the sample. To obtain the emission spectrum, the activating monochromator is preset at a particular wavelength of ultraviolet light and the motor then turns a cam on the analyzing monochromator to record the character of the emitted light.

The extraction of catecholamines from blood and urine samples was accomplished by using a modification of Lund's technique. The pH of a 10 ml. sample was adjusted to 7.5-7.8 and it was mixed with 0.5 gm. of "Woelm" non-alkaline alumina for 3 minutes. The pH of the resulting mixture was 8.0-8.5. Since the catecholamines are unstable at this pH, this stage must be accomplished within 10 minutes. The elution of the catecholamines from the alumina was accomplished with 6 ml. of 0.2M acetic acid after washing the alumina 2-3 times with 10 ml. portions of distilled water. This part of the procedure is essentially that described by R. T. Jones (26).

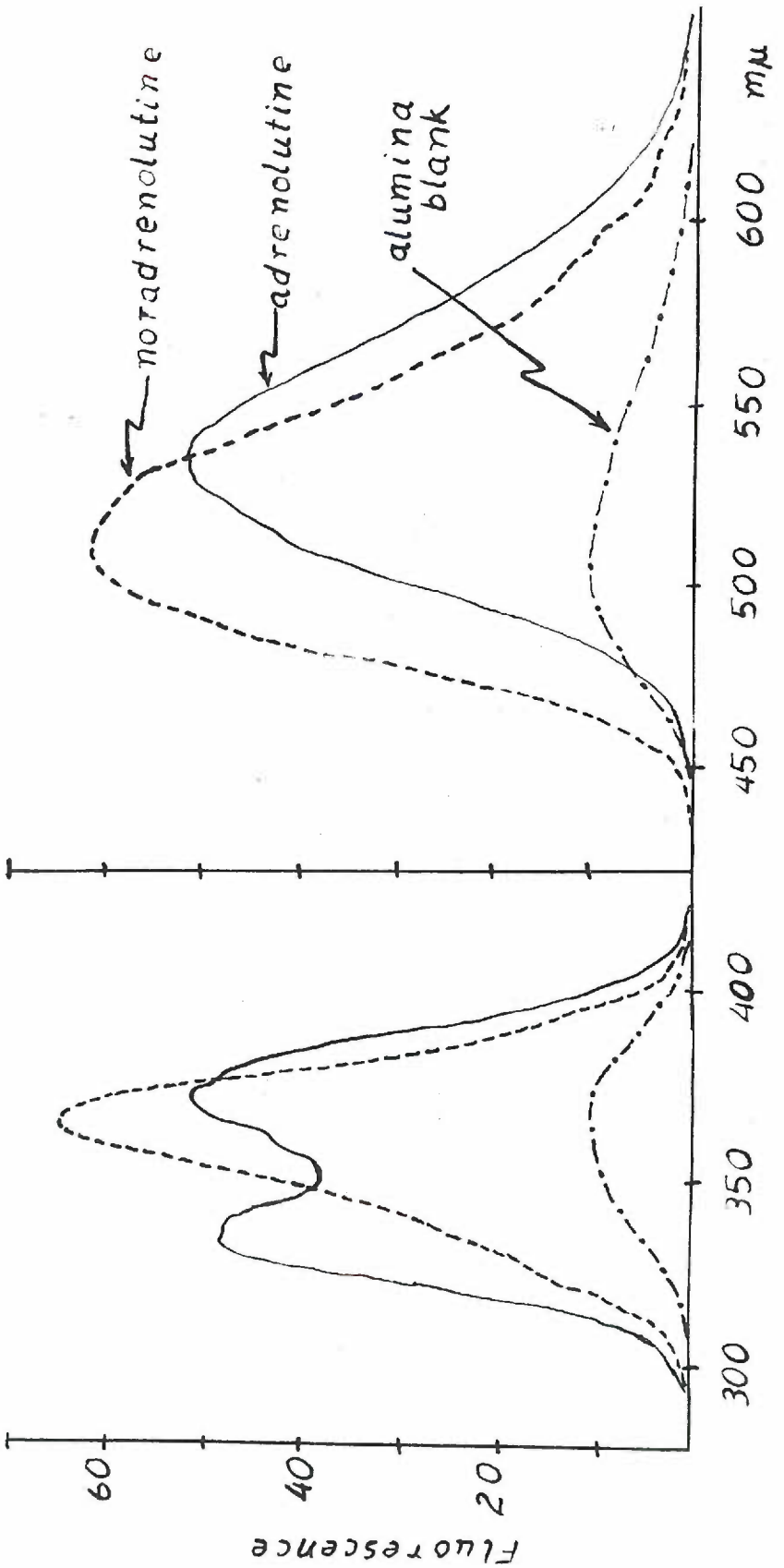
The pH of the eluate obtained from the alumina was then adjusted to 6.0. Then the sample was oxidized for 5 minutes using 0.1 ml. 250 mg.% potassium ferricyanide. It was found that the optimum oxidation time was 5 minutes instead of the 2-3 minutes recommended by von Euler and Floding (72). After the oxidation was completed, 1 ml. of a mixture of ascorbic acid and sodium hydroxide was added to form the trihydroxy-indoles. The blank was formed by adding the sodium hydroxide without the ascorbic acid and thus destroying the fluorescence due to epinephrine and norepinephrine.

The fluorescence spectra of adrenolutine and noradrenolutine were determined with the spectrofluorometer (fig. 1). It was found that the peak activation of epinephrine occurred at 335 mμ. and 375 mμ. and that the activation peak for norepinephrine was at 370 mμ. The peak emission for epinephrine was at 535 mμ. and that for norepinephrine was at 515 mμ. In the analysis of samples the emission was read at 525 mμ. while the samples were activated at 335 mμ. and 375 mμ. The activation at 375 mμ. gives maximum values for epinephrine and norepinephrine but at 335 mμ.

Figure 1

Left: Activation spectra of adrenolutine and noradrenolutine taken with the analyzing monochromater set at 525 mμ. Samples contained 2.5 μgm.% epinephrine and norepinephrine.

Right: Emission spectra of adrenolutine and noradrenolutine taken with the activating monochromator set at 375 mμ. Activation and emission spectra of a blank passed through alumina are included for comparison. All spectra presented are uncorrected instrumental values.


noradrenolutine
adrenolutine
alumina blank
Fluorescence
60
40
20
300
350
400
450
500
550
600
mμ

the reading for epinephrine is approximately 2½ times greater than that of norepinephrine.

Standard curves for aqueous solutions of epinephrine and norepinephrine are plotted in fig. 2 for values varying from 0.1-5.0 µgm.%. The values are tabulated in table 1 to demonstrate the precision.

The ability to differentiate epinephrine and norepinephrine in mixtures was checked. Epinephrine and norepinephrine were added in mixtures varying the ratio from 1:10 to 10:1. The amounts added and the amounts found by assay are presented in table 2.

Other related compounds were tested for fluorescence according to the method used. The relative fluorescence of these compounds is shown in table 3. 3-hydroxytyramine and 3,4-dihydroxyphenylacetic acid are known to occur in urine in appreciable quantities (81); however, these substances gave relatively little fluorescence when compared with epinephrine and norepinephrine. Dopa (3,4-dihydroxyphenylalanine) was the only other compound which gave a large amount of fluorescence but the presence of this compound in urine and plasma has not been verified (82).

The recoveries of epinephrine added to dog plasma are presented in table 4. The results for norepinephrine were comparable. The recoveries of norepinephrine added to urine are presented on table 5.

The exact procedure for analysis of plasma and urine samples is given in the appendix.

B. Experimental Procedures:

Trained mongrel female dogs, weighing 11-25 kgm. were used in these studies. These dogs were trained to lie supine on an animal board while urine and blood collections were being made.

Figure 2

Standard curves for pure aqueous solutions of epinephrine and norepinephrine. Fluorescence readings were taken at 525 mμ. when activated by ultraviolet light at 335 mμ. and 375 mμ.

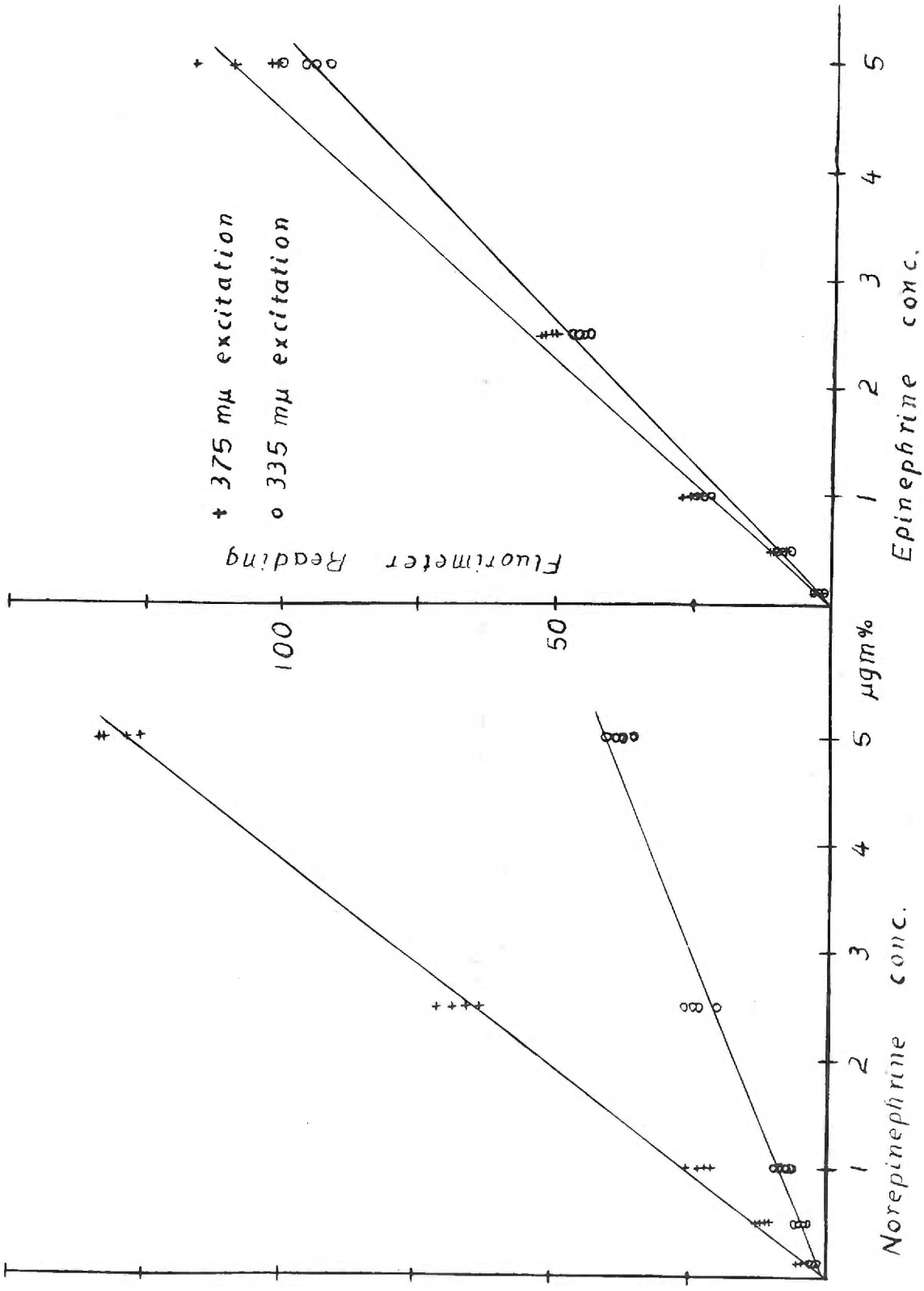

+ 375 mμ excitation
o 335 mμ excitation
Fluorimeter Reading
100
50
1
2
3
4
5
μgm%
Norepinephrine conc.
Epinephrine conc.

Table 1

Fluorescence Reading of Standard Solutions

Conc. µgm.%	Number Samples	335 mµ. $\bar{x}$	S.D.	375 mµ. $\bar{x}$	S.D.
Norepinephrine					
5.0	4	38.1	1.8	130.5	3.6
2.5	4	23.6	2.3	67.2	3.5
1.0	4	7.9	1.0	22.9	1.9
0.5	4	4.0	0.2	11.7	0.6
0.1	4	1.9	0.6	3.1	0.9
Epinephrine					
5.0	4	94.9	4.3	106.1	7.2
2.5	4	46.0	1.3	51.4	1.1
1.0	4	19.5	0.6	19.9	1.6
0.5	4	9.4	1.6	9.5	1.7
0.1	4	2.1	0.6	2.4	0.5

Table 2

Assay of Epinephrine-Norepinephrine Mixtures

Amount Added (µgm./9 ml.)			Amount Found (µgm./9 ml.)		
Epineph.	Norepi.	Total	Epineph.	Norepi.	Total
0.025	0.250	0.275	0.022	0.250	0.272
0.125	0.250	0.375	0.138	0.244	0.382
0.250	0.250	0.500	0.320	0.205	0.525
0.250	0.050	0.300	0.248	0.048	0.302
0.250	0.025	0.275	0.246	0.081	0.327

Table 3

Fluorescence of Related Compounds

Compound	Activation Peak mμ.	Emission Peak mμ.	Reading/μgm./9ml. 335 mμ.	375 mμ.
l-norepinephrine	370	515	152.5	522.0
l-epinephrine	335, 375	535	379.5	424.5
3,4-dihydroxy-phenylalanine	350	510	258.0	107.0
3-hydroxytyramine*	350	500	1.28	1.05
3,4-dihydroxy-phenylacetic acid*	365	510	0.80	0.24
3-methoxyepinephrine*	365	510	0.60	0.84
5-hydroxytryptamine*	350	520	3.60	1.64

*Because of the small amount of fluorescence emitted, 10-25 μgm. were added to 9 ml. and the fluorescence measured at 525 mμ. after reacting in the same manner as epinephrine and norepinephrine.

Table 4

Recoveries of Epinephrine Added to Dog Plasma

Conc. μgm.%	Number of Recoveries	Mean % Recovered	1 Standard Deviation
2.5	4	80.2	3.0
1.0	4	76.3	6.5
0.5	4	71.6	4.2

Table 5

Recoveries of Norepinephrine Added to Urine

Conc. μgm.%	Number of Recoveries	Mean % Recovered	1 Standard Deviation
5.0	4	64.2	6.6
1.0	4	67.8	7.4

Urine was collected with an urethral catheter. The bladder was rinsed with 10 ml. of distilled water followed by air at each collection. Blood samples were obtained from the femoral artery with a 20 ml. syringe which had previously been wet with heparin. The area over the femoral artery was infiltrated with 1% Procaine before each collection. The blood samples were centrifuged at 2500 r.p.m. for 15 minutes within 20 minutes of the collection. The plasma and urine were stored in the refrigerator until the analysis. Hematocrits were determined by the Wintrobe technique (83).

The plan was to study the dogs during three phases, i.e. normal, after the production of myocardial damage by coronary embolisation, and after embolization plus severe anemia to produce additional stress in the animal. In each experiment the dogs were studied in a control period lasting 19-61½ minutes, during hypoxia lasting 94-195 minutes, and during a recovery lasting 21-41 minutes. The effects of hemorrhage and carbamylcholine stimulation of the adrenal medulla, both of which are known to provoke a sympatho-adrenal discharge (84,85), were studied in the animals to test the validity of the overall procedure.

Coronary embolization was accomplished by the technique described by C. M. Agress et al (53). The animals were anesthetized with 30 mg. Nembutal/kgm. given intravenously, the left carotid artery was isolated, and injections were made after inserting a catheter down the carotid artery into the ascending aorta. The catheter which is described by Agress et al consists of two lumens with an inflatable balloon tip on the outer bore. Thus, injections can be made into the coronary circulation while the general circulation is occluded. Ungraded plastic microspheres ranging from 150-600 micra in diameter were suspended in

15% acacia. The concentration of spheres used was 1 mg./ml. The dosage injected varied from 1-3.8 mg./kgm. It was found that the injection was facilitated when positive pressure respiration was applied to reduce the inflow of blood into the heart and to reduce aortic pressure. This decreased the likelihood of leakage of spheres around the balloon cuff. In later studies the electrocardiogram was used to monitor changes which occurred with the injections. The dogs were allowed to recover at least 1 week before being studied.

To subject the animals to hypoxia a chamber designed and built by Dr. R. E. Mammen was used. This consisted of an air-tight plywood box 2' x 2' x 4' in dimensions with a removal door. There were provisions for absorption of CO_2 and a fan for circulation of air. In preliminary studies an O_2 electrode was used to monitor the O_2 concentration. It was found that the animals became cyanotic, hyperpneic, and agitated when the pO_2 fell below the range of 50-55 mm. Hg (7.5% O_2). Therefore, 8-10% O_2 was selected as the mild stress. A mixture of 8% O_2 in nitrogen was used to flush the chamber for 10-15 minutes; then the concentration was maintained at 8-10% by allowing this mixture to flow in at the rate of approximately 2 L./minute.

The effect of hemorrhage was studied in unanesthetized animals by taking plasma samples before and immediately after hemorrhage. The amounts of blood withdrawn ranged from 14.6-41.8 ml./kgm. The procedure required 20-40 minutes to complete. The blood was withdrawn through a Cournand needle in the femoral artery. The blood withdrawn was replaced with an equal volume of normal saline. The effect of rapid exsanguinating hemorrhage was studied in 3 anesthetized dogs.

The effect of adrenal medullary stimulation by injection of carbamylcholine was studied in 4 unanesthetized dogs. The dogs were atropinized with 0.3 mg. atropine sulfate/kgm. body weight 10-20 minutes before the procedure. Then 0.2-0.5 mg. "Carcholin"/kgm. was injected into the femoral vein over 15-30 seconds. Blood samples were collected from the femoral artery before the injection and from 30 seconds-10 minutes after completion of the injection.

EXPERIMENTAL RESULTS

A. General Description of Experimental Animals:

All of the experimental animals were subjected to a routine series of situations which were designed to bring forth the same responses which would be expected of a mammalian organism in a mild stress. Each animal was subjected to mild hypoxia on three separate occasions: 1) before any interference with cardiovascular function; 2) after the production of multiple small myocardial infarcts; and 3) after the production of anemia in the animals with the cardiac lesions. The following paragraphs deal with some of the behavioral characteristics of these animals which serve as evidence that these procedures did, in fact, produce alterations in various functions consistent with the assumption that a mildly stressful state was produced. It was stated earlier that reduction of oxygen concentration to 7.5% in the inspired air produced evidences of functional alteration which were so severe that it was felt that this level of hypoxia could not be tolerated. Consequently, levels of 8-10% oxygen (57-71 mm. Hg) were utilized for the production of a mild hypoxia. At this level of hypoxia none of the normal animals showed and signs of obvious respiratory distress, i.e. no cyanosis, hyperpnea, dyspnea, or undue agitation. In all instances the dogs appeared to be resting comfortably. The hypoxia appeared to be tolerated just as well by the same dogs after the production of myocardial damage. When chronic anemia was added to the myocardial damage, the dogs showed a mild degree of hyperpnea during the hypoxia period of the experiment. In spite of the lack of externally observable signs of distress, it seems probable that these animals were forced to make compensatory alterations at the chosen

level of hypoxia, and thus may be regarded as reacting to mild stress (88).

The hematocrit was checked in 16 experiments during which the animals were subjected to hypoxia. It was determined in each experiment on blood samples taken in a control period, 2-7 minutes after removal from the hypoxic conditions, and in a recovery period 29-47 minutes after removal from the hypoxic conditions. All hematocrit values from each period were pooled to determine the mean difference in hematocrit during hypoxia as compared to the average of the control and recovery periods. The mean increase was 2.7% with a standard deviation of 2.5. This difference was found to be highly significant using the t test ($P<0.0025$). The results are presented in table 6.

A total of 13 dogs were embolized with the plastic microspheres. Of these 6 died in acute cardiac arrest immediately (5 min.-12 hrs.) after the injection. One dog died in acute pulmonary edema and congestive heart failure in 48 hours. Another dog had to be sacrificed because of severe cerebral damage. Five dogs survived for periods ranging from 5-17 weeks after the injection before they were sacrificed. In 4 embolized dogs that were studied, there were signs suggestive of myocardial ischemia on standard and unipolar limb leads of the electrocardiogram. These consisted of T wave inversion and ST segment depression on serial tracings. However, none of the dogs showed any definite decrease in exercise tolerance. No signs of congestive heart failure appeared, i.e. no pulmonary congestion, dyspnea, peripheral edema, ascites, venous distention, or weight gain.

After these 4 dogs were made severely anemic in addition to the embolization, they all showed decreased exercise tolerance in climbing 8 flights of stairs. They would appear dyspneic after climbing 2-4 flights and refuse to climb any further without resting; whereas, the normal dogs would climb the 8 flights without stopping and with no signs of distress. The resting

Table 6

Effect of Hypoxia on Hematocrit

Dog	Type*	B_1 Control	B_2 Hypoxia	B_3 Recovery	$B_2 - \frac{B_1 + B_3}{2}$
C	C	41.0	42.0	41.0	+1.0
C	E	37.0	37.0	35.5	+0.8
C	E & A	26.5	26.5	25.0	+0.8
B_1	C	44.0	50.5	48.0	+4.5
B_1	E	23.0	29.5	25.0	+5.5
B_1	E & A	19.0	25.0	19.0	+6.0
B_2	C	45.0	49.0	46.0	+4.5
B_2	E	30.5	32.5	29.0	+2.8
B_2	E & A	20.3	20.0	19.0	+0.4
F	C	40.0	49.0	46.5	+5.8
F	E	47.5	49.0	47.5	+1.5
F	E & A	26.5	28.0	26.5	+2.0
Y_1	C	47.0	44.5	-	-3.5
Y_2	C	49.0	53.0	50.5	+3.3
H	C	44.5	48.0	-	+3.5
G	C	42.5	47.0	42.0	+4.8
					$\bar{x} = 2.7$
					S.D. = 2.5

$\bar{x}$ significantly greater than zero at $P < 0.0025$

* C = control, E = post-embolization, E & A = post-embolization plus anemia

pulse rates in the control animals ranged from 70-100 beats/minute. Following the development of anemia, resting pulse rates rose to a persistent value of 100-160 beats/minute. No signs of congestive heart failure developed. The hematocrit in these animals ranged from 19.0-28.0% as compared to 40.0-53.0% in the controls.

At autopsy gross examination of the hearts revealed multiple myocardial infarcts in all the animals. Histopathological examination of the hearts of 3 animals revealed the small infarcts to be areas in which there was fibrosis and small amounts of lymphocytic infiltrate.

B. Ability of the Method to Demonstrate Physiological Change in Catecholamines:

Despite the high degree of sensitivity characteristic of this chemical assay procedure, it seemed important to establish the fact that it is adequate to detect changes in catecholamine concentration under the conditions of its use in this laboratory and in the species used for these experiments. Accordingly, two series of experimental animals were subjected to procedures which could be expected reliably to result in activation of adrenal medullary secretion. One series was subjected to varying degrees of acute hemorrhage. Another series was given large doses of carbamylcholine, which could be expected to activate postganglionic sympathetic discharge by direct action on these cells and on the cells of the adrenal medulla.

The effect of hemorrhage on plasma catecholamine concentration was studied in 5 unanesthetized dogs (12 experiments). The blood was withdrawn over a period of 20-40 minutes. The withdrawal of 14.6-19.1 ml. of blood/kgm. body weight (6 experiments) produced no significant elevation of the plasma catecholamines (table 7). These animals showed no signs of obvious distress during or after the hemorrhage. When 30.8-41.8 ml. of blood/kgm. body weight was removed, there was significant rise in plasma catecholamine concentration (table 7). These animals all appeared to be in shock as manifested by

Table 7

Effect of Hemorrhage on Plasma Catecholamines

Amount of hemorrhage	Plasma Catecholamine Conc. (µgm.%) Before	After
Moderate		
18.9 ml./kgm	0.00	0.00
17.3	0.14	0.07
15.0	0.00	0.00
14.6	0.21	0.12
19.1	0.17	0.14
18.6	0.00	0.00
	$\bar{x}$ = 0.09	$\bar{x}$ = 0.06
Severe		
30.8	0.10	0.19
41.8	0.05	0.12
40.6	0.00	0.29
37.3	0.00	0.50
37.3	0.07	0.17
37.5	0.05	0.19
	$\bar{x}_1$= 0.05	$\bar{x}_2$= 0.24
	S.D. = 0.04	S.D. = 0.14

$\bar{x}_2$ significantly greater than $\bar{x}_1$ at $P < 0.005$

$\bar{x}$ = mean
S.D. = 1 standard deviation

a rapid thready pulse, collapse of veins, distant heart tones, dilated pupils, and deep, labored respirations. They all showed prompt recovery when the amount of blood withdrawn was replaced with normal saline.

In three anesthetized dogs the effect of rapid exsanguinating hemorrhage was studied. In these experiments the blood was removed through a large cannula in the femoral artery so the rate of hemorrhage was much more rapid. The results obtained are presented in table 8. The increases in plasma concentration were more marked than in the previous experiments. In figure 3 the fluorescent spectra of plasma samples taken before and after rapid hemorrhage in one experiment (Dog C) are shown.

Carbamylcholine stimulation of the adrenals in atropinized dogs produced demonstrable changes in the peripheral plasma concentration of catecholamines. A total of seven experiments were done on four unanesthetized dogs. Marked increases in the plasma catecholamine concentration were noted immediately after the injection and the concentrations returned to the control levels in about two minutes. The increase appeared to be more marked with larger doses of the drug. The hematocrit also showed a consistent increase in all the animals. The individual experiments are presented in table 9. Figure 4 demonstrates the fluorescent spectra of plasma samples obtained at various times in one dog (Dog B_2).

C. Catecholamine Determinations:

A total of nine dogs were used to test the response of normal animals to hypoxia. The rate of urinary excretion of catecholamines during the control period prior to hypoxia averaged 8.7±7.1 mμgm./min. (mean±1 standard deviation). During hypoxia it was 7.5±5.1 mμgm./min. and during the recovery phase 8.6±8.0 mμgm./min. No significant differences (P=0.05) were detected in these means (refer to table 10). The proportion of epinephrine present in the urine was highly variable ranging from 0-100% (refer to table 1 in appendix).

Table 8

Effect of Rapid Exsanguinating Hemorrhage

Dog	Amount Bled ml.	Amount Bled ml./kgm.	Time Required (min.)	Plasma Catecholamine Concentration (ugm.%) Before Total	After Total*	After Epineph.	After Norepineph.
C	500	39.2	5	0.05	0.52	0.40	0.12
	750	59.0	10	0.05	0.82	0.45	0.37
B_1	250	17.2	1	0.00	0.00	-	-
	650	44.8	3	0.00	0.48	0.48	0.00
B_2	250	16.2	2	0.05	0.05	-	-
	500	32.4	5	0.05	0.24	-	-

*Differential estimates were not made when total concentration was less than 0.30 ugm.%.

Figure 3

Left: Activation spectra of plasma samples taken before and at various times after hemorrhage in one dog. Note the similarity with spectra of standard solutions on fig. 1. Wavelength in mμ is represented on the abscissa and fluorescent intensity in arbitrary potentiometer units on the ordinate.

Right: Fluorescent emission spectra of same plasma samples.

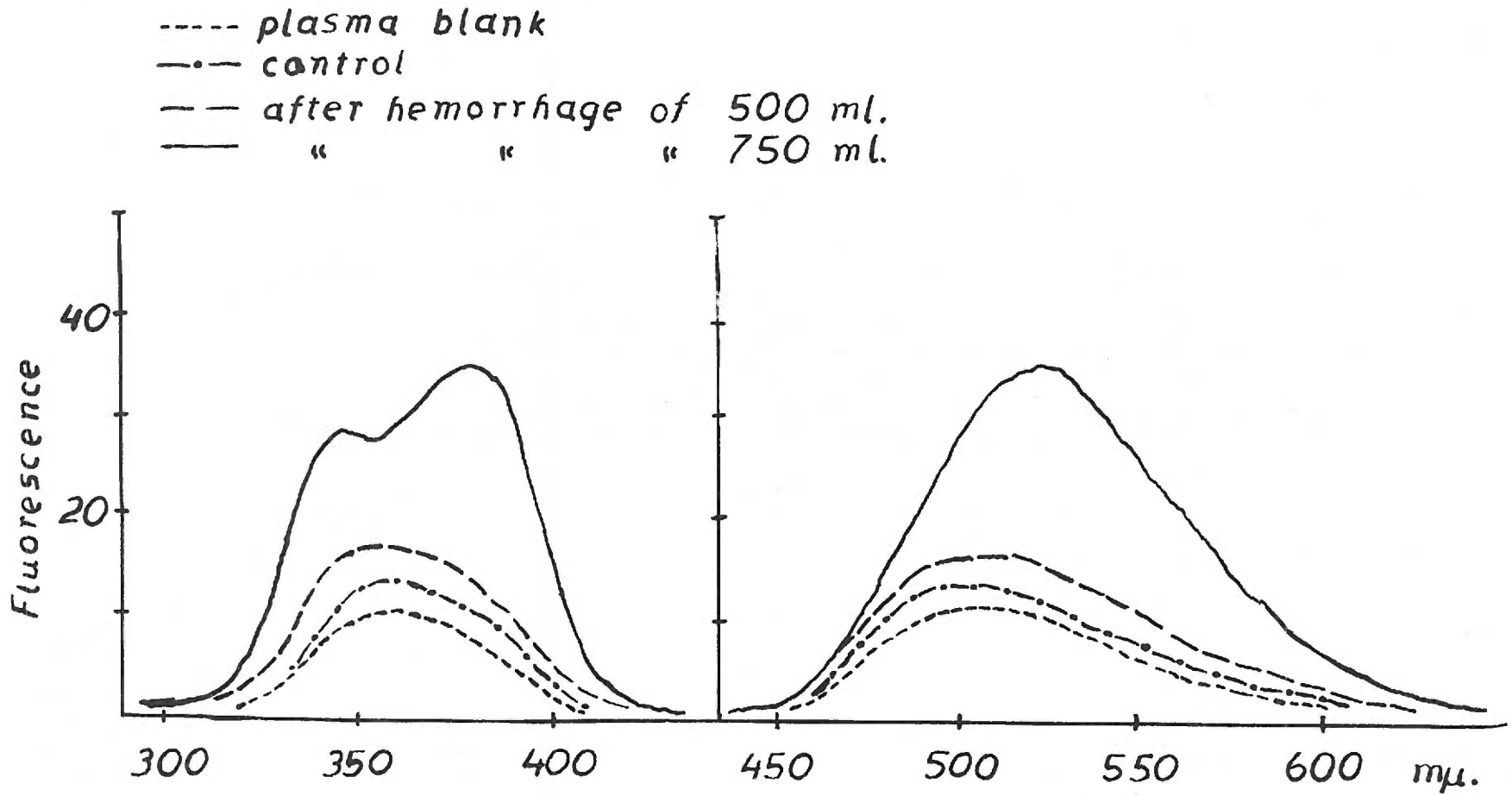

----- plasma blank
—·— control
— — after hemorrhage of 500 ml.
—— « « « 750 ml.
Fluorescence
40
20
300
350
400
450
500
550
600
mμ.

Table 9

Effect of Carbamylcholine Injection

Dog	Carbamylcholine mg./kgm.	Time Samples Taken	Plasma Catecholamine Concentration (µgm.%) Total	Epineph.	Norepineph.	Hematocrit %
B_1	0.20	Control	0.00			23.5
		30 sec.	0.05			25.5
		120 sec.	0.10			25.0
		10 min.	0.05			20.5
C	0.20	Control	0.05			40.0
		30 sec.	0.37	0.29	0.08	-
		60 sec.	0.05			47.0
B_2	0.25	Control	0.05			20.5
		30 sec.	0.21			22.5
		60 sec.	0.00			23.5
		5 min.	0.00			23.0
B_1	0.33	Control	0.05			-
		90 sec.	4.79	3.81	0.98	-
C	0.50	Control	0.05			37.5
		30 sec.	1.04	0.39	0.65	41.0
		90 sec.	0.32	0.24	0.08	43.0
		5 min.	0.00			43.0
B_2	0.50	Control	0.14			26.5
		30 sec.	1.09	0.80	0.29	28.0
		90 sec.	0.27			30.0
		2½ min.	0.05			31.0
F	0.50	Control	0.00			39.5
		30 sec.	2.17	1.20	0.97	44.5
		90 sec.	0.41	0.15	0.26	45.5
		3 min.	0.00			46.5

Figure 4

Left: Activation spectra of plasma samples taken before and at various times after injection of 0.50 mg./kgm. carbamylcholine.

Right: Fluorescent emission spectra of same plasma samples.

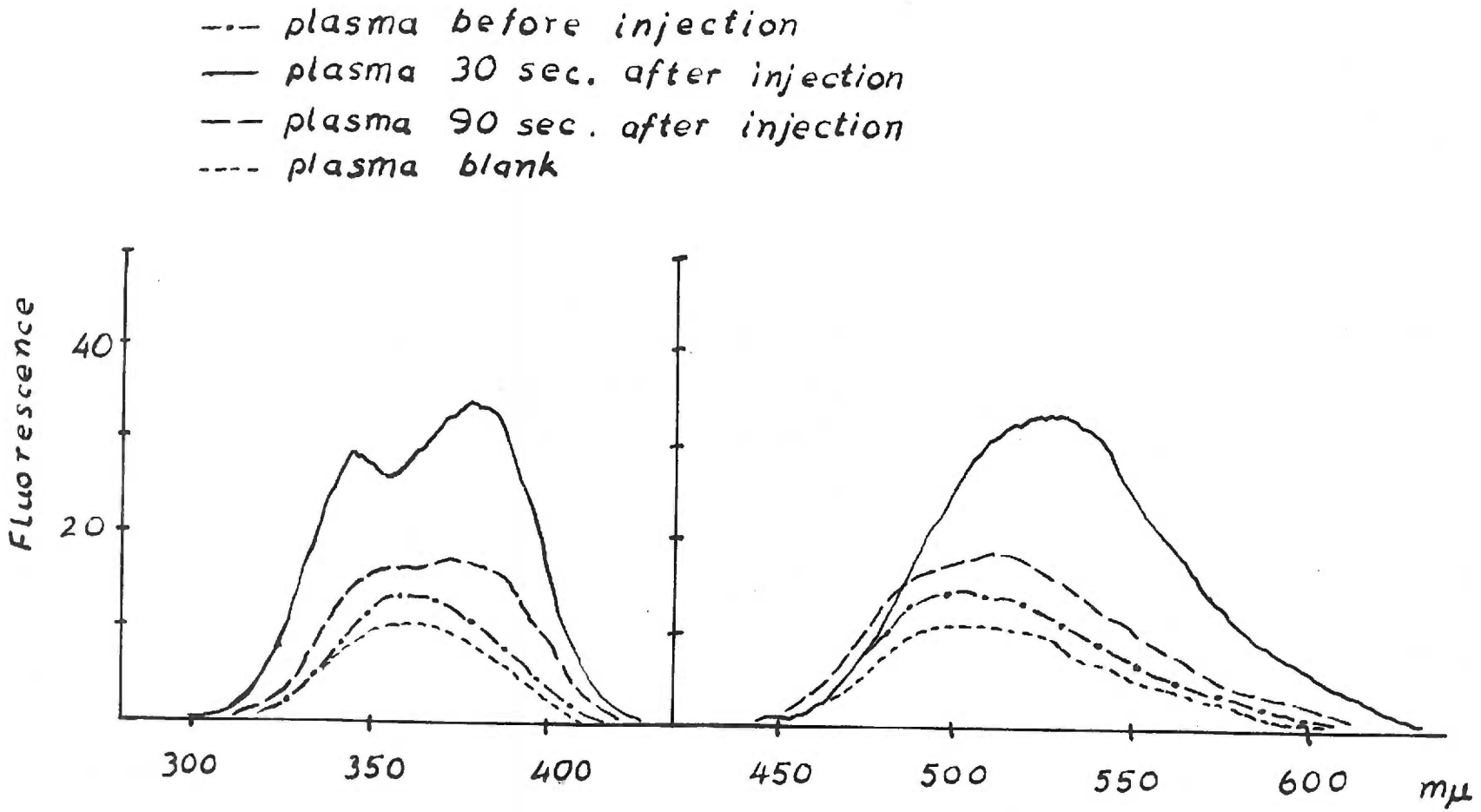

plasma before injection
plasma 30 sec. after injection
plasma 90 sec. after injection
plasma blank
Fluorescence
40
20
300
350
400
450
500
550
600
mμ

Table 10

Effect of Hypoxia on Urinary Excretion Rate of Catecholamines

Phase of Study	No. of Expts.	Urinary Excretion of Catecholamines (mμgm./min.) Control	Hypoxia	Recovery
Control	9	8.7±7.1	7.5±5.1	8.8±8.0
Post-embolization	4	10.2±6.8	7.4±6.8	4.7±1.7
Post-embolization plus anemia	4	4.5±1.1	3.9±2.0	2.5±1.2

Values were not expressed differentially because of extreme variability in the ratio of epinephrine : norepinephrine.

Table 11

Plasma Catecholamine Concentration in Hypoxia

Dogs	Number	Plasma Concentration (μgm.%) Control	Hypoxia	Recovery
Control	9	0.21±0.15	0.16±0.14	0.11±0.14
Experimental*	8	0.13±0.12	0.12±0.13	0.11±0.07

*This group includes dogs subjected to coronary embolization and to coronary embolization plus anemia. Results were pooled because no significant differences were found between the groups. Results given are the means±1 standard deviation.

After embolization there was no change in the response to hypoxia (table 10). In four experiments the excretion rate of total urinary catecholamines was 10.2±6.7 mμgm./min. during the control period, 7.4±6.8 mμgm./min. during hypoxia, and 4.7±1.7 mμgm./min. in the recovery period. There was no significant difference among the means at P=0.05.

After the production of severe anemia in addition to the embolization in four dogs, there was still no difference in the urinary excretion rate of catecholamines (table 10). The excretion during hypoxia was not significantly different from control and recovery periods (P=0.02).

Because of the inability to detect any differences among groups, the data were analyzed for possible differences in individual dogs during the three different phases, i.e. normal, post-embolization, and post-embolization plus anemia. There were four dogs in which the studies were complete. The differences in urinary excretion rate of catecholamines were not significantly different from zero (P=0.02) in all three periods of the experiments, i.e. the control, hypoxic, and recovery. The data are presented in table 12.

The plasma catecholamines showed no consistent change in the normal animal when subjected to hypoxia. The values obtained during the control period, immediately after hypoxia, and during the recovery period are given in table 11. It has been mentioned earlier that 2-7 minutes were required to obtain the blood samples after the animals were removed from the hypoxic conditions. When the animals were studied after embolization and after embolization plus anemia, they showed no significant change either in the resting levels of plasma catecholamines or in the levels immediately after hypoxia.

Table 12

Paired Comparison of Data:

I. Difference in urinary excretion rate of catecholamines before and after coronary embolization:

Dog	(Embolized -Normal) = Difference (mμgm./min.) Control	Hypoxia	Recovery
C	+12.9	+2.4	-1.9
B_1	+ 8.3	+4.0	+4.6
B_2	+ 0.8	-9.2	-3.1
F	+ 0.5	-3.8	-1.0
$\bar{x}$ =	5.6	-1.6	-0.4
S.D. =	6.1	6.0	3.4
not significant at P =	0.05	0.05	0.05

II. Difference between normal values and value obtained after coronary embolization plus anemia.

Dog	(Experimental -Normal) = Difference (mμgm./min.) Control	Hypoxia	Recovery
C	-1.7	+3.5	-2.3
B_1	-0.6	-9.9	-1.6
B_2	-0.2	-10.8	-4.4
F	+1.8	-3.2	-1.9
$\bar{x}$ =	-0.2	-5.1	-2.5
S.D. =	1.3	6.7	1.3
not significant at P =	0.05	0.05	0.02

DISCUSSION

A. Assay Procedure:

A modification of Alf Lund's trihydroxyindole method has been found to be applicable to the estimation of endogenous epinephrine and norepinephrine in plasma and urine. The sensitivity and precision of the method are sufficient to estimate accurately the concentration of 0.10 ugm% using 10 ml. of sample. This was somewhat less than the sensitivities reported by Price and Price (22) and Cohen and Goldenberg (76). They were able to estimate concentrations as low as 0.01-0.05 ugm% using 10-15 ml. of plasma with the same method.

The specificity of the method for epinephrine and norepinephrine has been found to be high. Dopa (3,4-dihyroxyphenylalanine) gave appreciable fluorescence; however, it has not been demonstrated in urine or plasma using chromatographic techniques (82, 86). Isopropylarterenol is known to produce fluorescence with the trihydroxyindole method (22, 70, 76), but it has been detected only in trace quantities in the adrenals themselves (87). No evidence exists for its presence in blood or urine. 3-hydroxytyramine and 3,4-dihydroxyphenylacetic acid, which are both known to occur in urine (81, 86) gave almost no fluorescence with the method.

In the experiments reported here, it has been found that the ratio of epinephrine : norepinephrine in the urine is highly variable. These observations are not consistent with the results of similar studies which have been reported in the literature (20, 23, 31, 86). In human urine, it is said that the ratio approximates 1:5 under normal conditions. Comparable values for dog urine have not been reported. Since the values for human urine have been

derived through the use of methods which differ in several respects from the one used in these studies, several 24 hour human urine specimens have been assayed. Using the same method used for the dog studies, the results confirmed the ratio value already published for man. Consequently, we are forced to postulate a species difference with regard to the relative amounts of these two substances excreted in the urine. Whether this means that the secretory products are differently related in man and dog, or that some conflicting urinary constituent is present in one species only remains to be determined.

B. Response to Hypoxia:

In view of the inability to note any changes in circulating or urinary catecholamines when the animals were subjected to hypoxia, it might be questioned whether 8-10% oxygen is truly stressful to the dog. This concentration of oxygen in the inspired air would reduce the partial pressure of alveolar oxygen to the same level as would be found at altitudes of 18,000-22,000 feet. In man there is no question that acute exposure to this degree of hypoxia produces an increased work load on the circulatory system (88). Even if it were assumed that the hypoxia did not produce an increased work load in the normal dog, it certainly would have done so in the dogs that had the myocardial damage and anemia. The latter animals all demonstrated an obvious decrease in exercise tolerance in a stair climbing test. In the dogs that had been embolized only, although there was gross and microscopic evidence of myocardial damage, it might be questioned whether the damage was physiologically significant because of the lack of any objective signs of cardiac decompensation.

Since there was a consistent increase in hematocrit when the dogs were subjected to hypoxia, it seems likely that there was increased sympathetic nervous system activity. Sympathetic activity is associated with vasoconstriction in the splanchnic circulation. The spleen in particular has a high

concentration of red cells and, thus, would contribute to the increase in hematocrit under conditions of sympathetic activation (89). These considerations add confidence to our conclusion that sympathetic excitation did occur in these animals.

The results obtained provide indirect evidence of a dissociation of activity of the sympathetic nerves and the adrenal medulla. Celander (90) studied the relative potency of direct stimulation of sympathetic vasomotor fibers and the splanchnic nerves to the adrenal medulla. He found that vasoconstriction in a localized area was much more intense with direct nerve stimulation than with stimulation of the adrenals. Furthermore, the frequencies of stimulation of the nerves to the adrenals required to produce any response in the general circulation were much higher than those required in the vasomotor nerves. Although one investigator is of a different opinion (91), it is generally felt that under physiological conditions sympathetic discharge does not necessarily release significant quantities of epinephrine and norepinephrine from adrenergic terminals into the general circulation (20, 90), even though this may result from intense electrical stimulation of many nerve trunks (20). Thus, if there were increased activity only in the sympathetic nerves when the dogs were subjected to hypoxia, the amounts of catecholamines released from postganglionic neuroeffector junctions might have been too small or too rapidly destroyed to be detectable in the general circulation or in the urine.

The ability of the method to detect physiological changes in adrenal medullary secretion has been proven. The results obtained with hemorrhage are quantitatively consistent with those of Manger et al (84). It was found that removal of 34.2-46.5% of the blood volume (assuming a normal blood volume of 90 ml./kgm.) produced a significant rise in the circulating catecholamines; whereas removal of 16.2-21.2% of the blood volume produced no significant change.

The studies of Lund (11) and Saito (10), who both noted increases in catecholamines in the adrenal vein effluent after hemorrhage, indicate that the increased amounts are released from the adrenal medulla. Stimulation of the adrenal medulla with carbamylcholine produced marked increases in the plasma catecholamine concentration. This response to the same drug has previously been observed by Bertler et al (85) and by Butterworth and Mann (92) who succeeded in localizing the source of the catecholamines to the adrenal medulla. Thus, all of these investigations have offered convincing evidence that the major portion of the response to hemorrhage and to pharmacologic stimulation can be ascribed to secretion from the adrenal medulla. There does not appear to be any reason why the increase in plasma catecholamines in the present experiments should not also be interpreted as a glandular response.

The results obtained in our studies seem to be at variance with those of von Euler et al (28) and Sundin (29) who both found that the urinary excretion of epinephrine and norepinephrine was significantly increased in man by tilting the subjects to 75° on a tilt-table. This procedure must certainly be considered as a mild stress except in those individuals who develop marked orthostatic hypotension. The findings of Elmadjian et al (23) indicate that moderate stress producing anxiety, usually in the absence of any increase in physical activity, provokes increased urinary excretion of catecholamines. Thus, the results of the studies in man stand in direct contradiction to the results of the present studies in the dog.

The difference between human and dog in reacting to mild stress may be related to the differences in the magnitude of change in the environment to which they may normally be exposed and which they are able to withstand. In man, acute exposure to altitudes of 24,000-25,000 feet, equivalent to 7.5% oxygen at one atmosphere, is said to produce unconsciousness (93); however, in our dogs, although it did produce obvious respiratory distress, it was not

associated with loss of consciousness. At altitudes of 18,000-22,000 feet man's performance is impaired (93), but the dogs appeared to be resting comfortably at these altitudes. Dill (94) mentions the remarkable ability of a dog to run at 15 miles/hour at altitudes of 12,000-16,400 feet in the Andes without any apparent fatigue. Thus, in man who lives in a relatively sheltered environment mild degrees of stress may provoke increased adrenal medullary secretion. Conversely, in the dog, which generally is exposed to greater changes in the environment and is able to withstand greater degrees of change, there may be no significant change in adrenal medullary secretion with mild stress.

C. Conclusions:

1. When normal dogs are subjected to mild hypoxia, there is no increase in circulating catecholamines.
2. When the cardiac reserve is decreased in dogs through the production of myocardial damage, there is no detectable change in resting levels of plasma or urinary catecholamines.
3. When animals with a decreased cardiac reserve are subjected to hypoxia, they show no increase in plasma or urinary catecholamines.
4. It is suggested that there may have been increased activity of the sympathetic nerves in these experiments, which was not detectable by observation of catecholamine concentrations in the plasma and urine.
5. It is also suggested that the adrenal medulla of the dogs does not participate in the response to the procedures described.

SUMMARY

This project was undertaken in order to study the effect of mild hypoxia on the sympatho-adrenal activity in normal animals and in animals with cardiac decompensation. Plasma concentration and urinary excretion of epinephrine and norepinephrine were used as indices of sympatho-adrenal activity.

A fluorimetric method was modified to estimate epinephrine and norepinephrine. This method allowed the differential estimation of epinephrine and norepinephrine based on differences in the activation spectra of the fluorescent compounds formed.

Dogs were studied before and after the production of myocardial damage by coronary embolization. Clinical cardiac decompensation was not attained in any of the animals studied but all showed evidence of myocardial damage at autopsy. None of the animals, either before or after the experimental procedures, showed any significant response to the stress of hypoxia. After the addition of chronic anemia to the myocardial damage there was still no significant change in plasma or urinary catecholamines even though the animals all showed a decreased exercise tolerance.

The ability of the method to detect physiological change in catecholamine concentration was checked by carbamylcholine injection and acute hemorrhage both of which procedures provoked a sympatho-adrenal discharge manifest by a rise in plasma epinephrine and norepinephrine concentration.

The results of the study suggest that the method used will detect major changes in catecholamine concentration which occur when adrenal medullary secretion occurs, but will not detect minor alterations associated with release of these substances from postganglionic neuroeffector junctions. The results

also suggest that the increased sympathetic activity in these dogs was not sufficient to provoke adrenal medullary secretion.

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APPENDIX

Outline of procedure for estimating catecholamines in plasma and urine:

10 ml. of sample is adjusted to pH 7.5-7.8 with 0.1-1.0N. sodium hydroxide using a Beckman pH meter, model G with microelectrodes. Mix resulting solution with 0.5 gm. "Woelm" non-alkaline aluminum oxide (activity grade 1) in a 15 ml. centrifuge tube for 3 minutes. Then centrifuge mixture and discard supernatent. Wash the aluminum 2-3 times with 10 ml. portions of distilled water. Elute the catecholamines from the alumina with two 3 ml. portions of 0.2M acetic acid. Remove the eluates by centrifugation and aspiration with a pipette and bulb. Save the combined eluates. The eluate is stable for several days at refrigerator temperature and several weeks in the freezer.

Add 2.0 ml. of 2M. sodium acetate to the eluate and adjust the pH to 6.0 with 1.0M. sodium hydroxide. Make up volume to 10 ml. in a 15 ml. graduated centrifuge tube. Place 5 ml. of this mixture into another graduated centrifuge tube. Add 0.1 ml. of 250 mg.% potassium ferricyanide to one tube, mix, and allow to oxidize for 5 minutes. After 5 minutes add 1 ml. of a mixture 0.5 ml. 1% ascorbic acid and 2.0 ml. 20% sodium hydroxide. Dilute volume to 9 ml., invert the tube several times, and centrifuge 1 minute to remove any alumina particles. Pour sample into cuvette and read in fluorimeter in 5 minutes. Set analyzing monochromator at 525 mμ and read with the exciting monochromator at 335 mμ and 375 mμ. To the second tube add 0.8 ml. 20% sodium hydroxide and allow to fade for 10 minutes. Then add 0.1 ml. 250 mg.% potassium ferricyanide and 0.2 ml. 1% ascorbic acid, dilute volume to 9.0 ml., centrifuge and read in fluorimeter in same manner.

Fluorescence intensity is proportional to potentiometer deflection. The fluorescence of the first tube is equal to the fluorescence of the catecholamines plus non-specific fluorescence. The fluorescence of the second tube is that due to non-specific fluorescence because fluorescence of the catecholamines is destroyed by the addition of alkali without ascorbic acid. Thus, subtracting the reading obtained from the second tube from that of the first gives the fluorescence reading for epinephrine and norepinephrine.

The reading is compared to the fluorescence reading obtained from standard solutions of epinephrine and norepinephrine to calculate the amount of each catecholamine present in the sample. The standards are kept as stock solutions of 25 mg.% and diluted to 1 µgm.% for use.

Calculations:

$R_{335} = yN_{335} + xE_{335}$

$R_{375} = yN_{375} + xE_{375}$

R_{335} = reading at 335 mµ.

R_{375} = reading at 375 mµ.

y = amount norepinephrine

x = amount epinephrine

N_{335}, N_{375}, E_{335}, E_{375} = fluorescence reading/µgm. norepinephrine (N) or epinephrine (E) standard solution at the wavelength indicated by the subscript.

Dog	Wt. (kgm.)	Time (min.)		Urine Flow (ml./min.)	Urinary Excretion of Catecholamines (mμgm./min.)	Per Cent Epinephrine in Urine	Plasma Conc. Catecholamines (μgm.%)	Hematocrit Per Cent
I. Normal Animals								
K	12.5	49	C*	0.17	6.9	67	0.34	-
		195	H	0.20	2.0	71	0.25 (5)**	-
		30	R	0.23	2.2	100	-	-
Y_1	20.9	30	C	0.32	17.7	95	0.00	47.0
		102	H	0.21	6.4	100	0.00 (5)	44.5
		21	R	0.26	16.4	100	0.20	-
Y_2	18.9	58	C	0.13	3.3	0	0.20	49.0
		135	H	0.12	2.1	0	0.00 (4)	53.0
		41	R	0.12	3.6	0	0.00	50.5
H	16.2	$58\frac{1}{2}$	C	2.55	23.2	56	0.43	44.5
		$100\frac{1}{2}$	H	1.42	15.2	51	0.36 (5)	48.5
		25	R	2.64	26.7	43	-	-
G	19.5	32	C	3.38	8.5	100	0.07	42.5
		111	H	3.16	5.8	100	0.24 (5)	47.0
		28	R	2.30	10.1	48	0.10	42.0
C	12.0	19	C	0.62	1.9	100	0.26	41.0
		14	C	1.40	9.5	100	-	-
		156	H	0.87	3.3	84	0.26 (6)	42.0
		18	R	2.33	7.0	100	0.14	41.0
		20	R	0.15	4.9	0	-	-

* C = control, H = hypoxia, R = recovery

** denotes time in minutes required to obtain blood sample after removal from hypoxic conditions.

Dog	Wt. (kgm.)	Time (min.)		Urine Flow (ml./min.)	Urinary Excretion of Catecholamines (mμgm./min.)	Per Cent Epinephrine in Urine	Plasma Conc. Catecholamines (μgm./l)	Hematocrit Per Cent
B_1	15.8	20	C	0.59	3.0	100	0.36	44.0
		12	C	1.62	5.2	100	-	-
		108	H	2.70	13.5	100	0.29 (4)	50.5
		22	R	0.32	2.5	100	0.36	48.0
B_2	16.6	15½	C	0.74	1.6	100	0.10	45.0
		15	C	1.60	10.1	100	-	-
		99½	H	1.80	12.8	56	0.24 (7)	49.0
		18	R	0.88	5.6	81	0.07	46.0
		13½	R	1.70	8.4	59	-	-
F	12.5	30	C	0.16	2.9	53	0.10	40.0
		28	C	0.46	2.2	100	-	-
		100	H	1.31	6.6	46	0.00 (3)	49.0
		30½	R	1.05	4.9	28	0.00	46.5
II. After Coronary Embolization								
C	12.8	12	C	4.35	19.6	100	0.17	37.0
		9½	C	4.55	17.7	44	-	-
		107½	H	2.50	5.7	0	0.10 (4)	37.0
		14	R	1.25	4.5	31	0.10	35.5
		13	R	0.85	3.5	14	-	-
B_1	15.8	8	C	1.91	6.3	21	0.32	23.0
		11	C	3.36	18.5	24	-	-
		94	H	2.74	17.5	100	0.24 (3)	29.5
		11½	R	0.96	10.4	100	0.13	25.0
		14	R	0.32	3.9	100	-	-

Dog	Wt. (kgm.)	Time (min.)		Urine Flow (ml./min.)	Urinary Excretion of Catecholamines (mμgm./min.)	Per Cent Epinephrine in Urine	Plasma Conc. Catecholamines (μgm.%)	Hematocrit Per Cent
B_2	16.2	27½	C	0.55	6.7	79	0.24	30.5
		156½	H	0.33	3.6	45	0.26 (2½)	32.5
		21½	R	0.37	3.8	100	0.24	29.0
F	12.4	34	C	0.23	3.1	0	0.14	47.5
		27½	C	0.38	3.2	32	-	-
		139½	H	0.90	2.8	26	0.05 (2)	49.5
		34½	R	0.32	3.9	94	0.12	47.5
III. After Coronary Embolization and Anemia								
C	11.4	32	C	0.39	4.0	66	0.12	26.5
		125	H	0.74	6.8	86	0.29 (3½)	26.5
		33½	R	0.23	3.6	90	0.10	25.0
B_1	14.3	31	C	0.48	3.5	100	0.00	19.0
		90	H	0.73	3.6	100	0.00 (2)	25.0
		29	R	0.28	0.9	100	0.05	19.0
B_2	16.3	33½	C	0.25	6.1	38	0.05	20.3
		101	H	0.23	2.0	16	0.00 (4)	20.0
		35	R	0.25	2.5	13	0.12	19.0
F	12.5	32	C	0.27	4.4	23	0.00	26.5
		108	H	0.69	3.3	69	0.00 (3)	28.0
		32	R	0.16	3.0	62	0.00	25.5