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JOSEPH ROQUE EMMANUEL VALADARES

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SEX DIFFERENCES IN THE ELECTROLYTE CONTENT

OF THE CANINE HEART

APPROVED BY

Paul W. Smith

H. A. Bloemaker

H. W. Lyman

M. R. Shetlar

A. T. Beyer

DISSERTATION COMMITTEE

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SEX DIFFERENCES IN THE ELECTROLYTE CONTENT OF THE CANINE HEART

CHAPTER I

INTRODUCTION

It has been known for many years that toxic doses of the cardiac glycosides produce changes in the rhythm of the heart which pass progressively through varying degrees of nodal block, to ventricular tachycardia, ventricular extrasystoles, auriculo-ventricular dissociation and ultimately, ventricular fibrillation.

In 1957, Grinnell and Smith (1) demonstrated a sex difference in the response of dogs to a toxic dose of digoxin. They found that the male is the more sensitive of the two sexes as evidenced by an earlier onset of a complete idio-ventricular tachycardia. They also found that the spayed female is equally as sensitive as the male and that her resistance is restored by treatment with an estrogenic compound. These authors were unable to offer an explanation for this phenomenon.

Grinnell (2) found that the potassium content of the blood increased and the sodium content decreased after a period of digoxin-induced arrhythmia, and that these changes were more pronounced in the

male than in the female. No specific inferences could be drawn from these results, however, since it had been shown before (3,4,5,6) that cardiac glycosides induce ion shifts in other tissues as well as the heart.

There seemed to be two possible explanations for the above phenomena; first, that digoxin actually does induce more pronounced cardiac intracellular-extracellular ion exchanges in males than in females; or second, that the initial levels of the major ions might be basically different in the hearts of the two sexes. It is obvious that these facts can only be elucidated by direct analysis of heart muscle. Consequently, the study reported in this dissertation was instituted and designed specifically to test the second of these two possibilities.

Several of the chemical methods which had been selected for use in this study because of their satisfactory performance when applied to the determination of cations in blood and other body fluids, proved to be erratic and inaccurate when applied to cardiac tissue. The modifications which were necessary to make them suitable for this purpose, and the degree of accuracy which was obtained, constitute a part of this dissertation.

CHAPTER II

HISTORY

Medical scientists are aware of the differential response to drugs by the two sexes of many species. While for the most part, these sex differences have been observed in laboratory animals rather than in man, they are of obvious importance to those engaged in the detailed toxicity tests which determine whether or not an agent is suitable for clinical trial.

The sex differences which have been recorded in the literature vary greatly in degree. The 10 per cent greater susceptibility of the young, adult, female mouse to neoarsphenamine is near the lower extreme (7). Differences of this order of magnitude are likely to be detected only by careful quantitative work. At the other extreme is the effect of chloroform. Eschenbrenner (8) subjected mice to varying doses of chloroform administered by stomach tube, and observed no sex difference in susceptibility to necrosis of the liver. A striking sex difference was seen, however, in the occurence of renal necrosis, which was present in all of the males but absent in all females, at comparable dosage levels. Deringer, Dunn and Heston (9) showed that exposure of strain C3H mice to air containing 5 mg of chloroform per liter, for one, two or three hours, resulted in lesions of the kidney of all of the males but in none of the females. Hence, it appears that the sex

of the animal determines whether it shall receive a lethal injury from damage to its kidneys, or survive with no detectable renal lesions.

A second, well-known difference exists between the sexes in rats given certain barbiturates, principally those possessing one short and one long branched side chain, a cyclohexenyl group, or a methylated nitrogen. Nicholas and Barron (10) were the first to observe that there was a marked difference in the amount of sodium amytal which can be safely employed in male and female rats for the production of anesthesia. The dosages which these workers established were 10 mg per 100 gm for females, and 20 mg per 100 gm for males. This sex difference in the reaction to amytal was observed only when the animals weighed above 60 gm. Below this weight there was no significant difference between the response of males and females. These and subsequent observations indicate clearly that sexual maturity is related to the appearance of a differential response to drugs. Barron (11) investigated this differential response to sodium amytal more deeply, and discovered that this difference in response was due to an androgenic influence, inasmuch as it could be largely removed by castration of the mature males. Rats display a similar sex difference with regard to an isomer of amytal, Pentobarbital, which is pharmacologically twice as potent and twice as toxic. Females sleep longer than do males, and less easily acquire tolerance to repeated doses following one another at short intervals (12, 13, 14).

The organo-phosphorus compounds are another group of pharmacological agents to which either the male or the female rat may be the more susceptible. In certain instances there may be no sex difference

at all. Hagan and Woodard (15) reported that there was a difference in the mortality rate of male and female rats given hexaethyl tetra-phosphate. The comparative susceptibility to the same toxic dose was: 14 deaths among 20 female test animals and 2 deaths among 20 males. Dubois, Doull, Salerno and Coon (16) obtained similar results with respect to Parathion, whether injected intraperitoneally or administered orally. These investigators, in consideration of the possibility that sex hormones influence the toxicity of this insecticide, conducted the following experiments: Each of twelve male rats was given 1.2 mg/kg of diethyl stilbestrol, subcutaneously in 3 doses of 0.4 mg/kg, on alternate days. To test the influence of a male sex hormone on the susceptibility of female rats to Parathion, each of a group of 12 females was given 2.5 mg/kg of testosterone propionate, administered subcutaneously in divided doses of 0.8 mg/kg, on alternate days. They found that administration of the male sex hormone to female rats and an estrogenic hormone to male rats tended to equalize the susceptibility of the two sexes to Parathion. The greater susceptibility of the female rat to Parathion has been confirmed by Aldridge and Barnes (17). Frawley, Hagan and Fitzhugh (18) in a study of the toxicity of anti-cholinesterase drugs in rats, found that the oral L D 50's for certain of these compounds varied in the two sexes, as follows: D F P (Di-isopropyl fluro-phosphate), 13.5 mg/kg for the male and 7.7 mg/kg for the female; Parathion, 30.0 mg/kg for the male and 3.0 mg/kg for the female; E P N (ethyl p-nitro phenyl thionobenzene phosphonate), 91.0 mg/kg for the male and 14.5 mg/kg for the female; O M P A (octa methyl pyrophosphoramide), 13.5 mg/kg for the male and 35.5 mg/kg for the

female. The male rat appears to be the more resistant to all of these insecticides except O M P A, to which he shows greater susceptibility. This again could be reversed by prior castration.

In 1927, Winton (19) showed that female rats succumb to doses of red squill only one-half as great as those needed to kill males. Crabtree, Ward and Welch (20) confirmed this finding and demonstrated that the presence or absence of the ovary has no effect on the susceptibility of the female to this toxic principle. They further showed the presence of the testes or of the testicular hormone, testosterone, to be the determining factor in the increased resistance of the male rat to powdered red squill.

Deuel and Gulick (21) reported that a more severe ketosis develops during fasting in the human female than in the male. Lorenz, Chaikoff and Entenman (22) showed that the concentration of liver lipids was the same in the mature and immature male fowl and in the immature female bird. The onset of maturity led to a pronounced increase in neutral fat in the liver of the female. This appears to be a direct result of increased ovarian activity. A similar difference was observed by MacKay and Carne (23) in rats. They found that more fat was deposited in the livers of female rats than in those of male rats during the first 24 hours after partial hepatectomy. Farber, Koch-Weser and Popper (24) demonstrated a clear cut sex difference in the response of the liver to the administration of ethionine in rats. Total liver lipids following the administration of ethionine were considerably higher in the female than in the male. The value for the males was only slightly higher than for the controls. The lack of response of the male

seems to be mediated by an androgen because, as these authors showed, testosterone propionate protected castrate males and intact and spayed females against the fatty metamorphosis of the liver produced by ethionine.

The lethal effect of large doses of epinephrine on male and female albino rats was tested by Astarabadi and Essex (25). They found the female to be remarkably resistant as compared to the male. Castration was without significant effect on the resistance of the male but resulted in the female becoming as susceptible as the male four weeks after oophorectomy.

The most adequately investigated examples of selective toxicity in the sexes have usually been shown to be under hormonal control. Either the male or the female hormone may be dominant, or they may both influence toxicity. Many such instances of hormone dependence have been reviewed by Hurst (26) who points out that "Generally speaking, a sex difference is not evident in the very young animal and appears only with sexual development." Although, like others, the sex difference in the toxicity of ouabain develops only with maturity, it is exceptional in remaining unaffected by gonadectomy in rats of either sex. This was shown by Holck and Kimura (27). More recently, Grinnell and Smith (1) showed that male and castrate female dogs are more susceptible to a toxic dose of digoxin than normal females in anestrus. They also showed that administration of estrogens to spayed females induces in these animals a state of resistance equal to that of the normal female. The present investigation was designed in an attempt to reveal the mechanisms which underlie this sex-difference.

CHAPTER III

METHODS

Biological

Twenty-four healthy, adult, male and female dogs of mixed breeds, weighing from six to twelve kilograms, were used for these experiments. The animals were held in quarantine for one week, and retained in the animal quarters for one to three additional weeks before use. They were fed a diet consisting of "Friskie's Dog Meal", supplemented with meat scraps twice a week.

Twelve females were spayed by a technique which included hysterectomy. A minimum of five weeks was permitted for post-operative recovery and for the establishment of a stable physiological state. Six of these animals were treated with 17 β-estradiol, 0.5 mg/kg/day, for seven days, which proved to be adequate to produce a state of estrus as indicated by the vaginal smear technique.

The acute experiments were carried out in the same manner for all the animals: six normal females, six normal males, six castrate females and six estrogen-treated castrate females. Food was withheld for twenty-four hours prior to the crucial experiment. Water was allowed ad libitum during the fasting period.

Since it was intended that the data obtained in this investi-

gation should be used later as basal values in a projected study requiring that an anesthetized animal be observed for three hours before cardiac analysis, these experiments were performed in a comparable manner.

The anesthetic consisted of a mixture of alpha chloralose, 100.0 mg/kg and pentobarbital sodium, 5.0 mg/kg administered intravenously. The chloralose was dissolved in polyethylene glycol (Carbowax 200) in a concentration of 150.0 mg/cc.

Immediately before use, the required amounts of chloralose and pentobarbital sodium solutions were mixed in a syringe and a few cubic centimeters of saline were added to reduce the viscosity. This mixture proved to be highly satisfactory. The action of chloralose is comparatively long lasting, and cardiovascular and respiratory reflexes are depressed only slightly. Pentobarbital sodium contributes a rapid anesthetizing action, and at this dosage level, lacks the vagolytic action and reflex depressant effects which otherwise would make it undersirable if used as the sole anesthetic agent.

The anesthetized animal was then placed in the supine position on an operating table. A vaginal epithelial smear was taken at this time if the animal was a female, and the slide examined to determine the stage of estrus. The animal was catheterized and the bladder thoroughly irrigated with de-ionized water. The completion of this step marked the start of the three-hour time period of the crucial experiment. The left femoral vein was cannulated with a polyethylene tube for withdrawal of blood samples and the artery on the same side cannulated and attached to a Statham strain gage transducer for recording of blood pressure.

Conventional limb electrodes were connected to the animal and the electrocardiogram was monitored on a four-beam oscillograph. Lead aV_F was routinely employed. Front-occipital leads were used consistently for monitoring the electroencephalogram as a means of gauging the depth of anesthesia. The blood pressure, electrocardiogram and electroencephalogram were recorded at intervals by means of a Grass Model 5, four-channel polygraph. Blood and quantitative urine collections were made hourly. The blood was collected in heparinized tubes and centrifuged at 1800 revolutions per minute for fifteen minutes in a Precision Vari-Speed Centricone. Hematocrit values were calculated and the plasma preserved for subsequent determination of sodium, potassium, calcium and magnesium. The urine samples were also retained for analysis of sodium and potassium only. The animal was watched carefully throughout the three-hour period for cyanosis or other signs of respiratory depression. If such occurred, the animal was artificially respired.

At the end of the three-hour experimental period the animal was killed by means of air-embolism. The heart was removed as quickly as possible, trimmed of its fat and auricles, rinsed with de-ionized water to wash off superficial blood and pressed between sheets of absorbent paper. The ventricles were then passed through a meat grinder and the fine mince of ventricular muscle was thoroughly and evenly mixed by means of a spatula. The adrenal glands were also excised, carefully dissected free of all adhering fascia, and weighed. Internal organs were examined for gross pathology. The presence of both ovaries in the normal female and the complete absence of ovarian tissue in the spayed female was verified in each instance.

Chemical

Triplicate samples of ventricular muscle of approximately 1.5 gm each were taken for the determination of sodium, potassium, calcium, magnesium and total phosphorus. These samples, in glazed translucent silica crucibles of 20 cc capacity, were placed in a drying oven at 95° C for twenty-four hours. The samples were again weighed and the dry weight percentages calculated and recorded. For the analysis of the acid-soluble phosphorus compounds, duplicate samples of the heart muscle weighing approximately one gram each, were ground in cold mortars with chemically inert sand (20 mesh), and extracted with 9.0 cc of cold 10 per cent trichloroacetic acid by a technique similar to that of Schneider (28). The final volume in each instance, therefore, equalled 9.0 cc plus the calculated water content of the muscle sample. The extracts were filtered through Whatman No. 2 filter paper, and 3.0 cc aliquots of each extract were pipetted into silica crucibles.

Ashing Technique

In general, three methods are available for processing tissues for determination of inorganic ions: extraction with water or dilute acid, wet-ashing in concentrated acid with the aid of an oxidizing agent and incineration. The first of these is widely used for the extraction of sodium and potassium, but is not satisfactory for calcium, magnesium or phosphorus. Wet ashing has the disadvantage that the end product is a highly acid solution unsuitable for the determination of calcium and magnesium by the methods used in this study. Incineration has long been a standard method of preparing biological material for analysis of

certain inorganic ions, but certain precautions are necessary to avoid losses of sodium, potassium and magnesium.

As recently as 1955, for instance, Glick, Swigant, Nayyar and Stecklein (29) stated that losses of potassium as great as 20 per cent are inherent in both digestion and incineration procedures. It has been found in this laboratory that careful pre-treatment of the biological material with sulfuric acid, as described below, avoids these losses. The complete ashing procedure is as follows:

To each of the three crucibles containing the dry muscle 1.0 cc of approximately 50 per cent v/v H_2SO_4 is added. To each of the two crucibles containing the acid-soluble phosphate extract 0.5 cc of concentrated H_2SO_4 is added. The five crucibles are then heated on a sand-bath; this heating process is a critical step in the ashing procedure and must be done slowly and carefully. The temperature of the sand is maintained at 200° C at the mid-point of a two inch total depth. The crucibles are embedded to a depth of approximately $\frac{1}{2}$ inch at the start, and gradually pushed deeper as the digestion proceeds. The material is thus thoroughly reacted with sulfuric acid, the excess driven off, and a dull, dry, charred mass results. This process converts the cations to the stable sulfate form and prevents the losses which are otherwise inherent in the incineration procedure. This step in the processing of the samples requires approximately one hour.

The crucibles are then placed in a furnace at 500° C for twenty-four hours, after which time ashing is essentially complete. Because some samples are not completely free of all organic material at this stage, it has been a routine practice to add two drops of concen-

trated H_2SO_4 and two drops of concentrated HNO_3 to each of the crucibles and to return them to the furnace for about two hours. It is important to note that the 500° C temperature is crucial, inasmuch as higher temperatures will result in some loss, particularly of potassium. The fused masses are cooled, 5.0 cc of 5N HCl are added, the crucibles are placed on a sand-bath at 200° C and excess HCl is evaporated off. This process takes approximately forty minutes. The muscle residues are taken up in 8.0 cc of de-ionized water, while the trichloroacetic acid extractable phosphate fractions are restored to their original volume of 3.0 cc.

Blank runs, in which crucibles were carried through the entire ashing procedure, yielded negative results for all the ions under investigation.

Sodium and Potassium

These cations are determined by flame spectrophotometry. It is a well known fact that in flame photometry various compounds interfere with the excitation band of the ion under inspection. For example, sodium increases the intensity of excitation of potassium. Any one of four methods can be used to partly circumvent these undesirable effects: further diluting the sample, increasing the concentration of the cation affected, narrowing the slit width in the measuring instrument or using a mixed standard solution, the composition of which approximates the composition of the solution under analysis. The mixed standard solution used in these analyses contains the major ions of interest in approximately the same concentration as is found in normal ventricular muscle: sodium, 40 milliequivalents per liter; potassium, 70 meq per liter;

calcium, 60 mg per liter; magnesium, 200 mg per liter; phosphorus, 2.0 gm per liter. A 1:50 dilution of the ashed-tissue solution was satisfactory for the determination of both ions. At this dilution, sodium and potassium are present in an average sample in concentrations of 3 and 10 parts per million respectively. Since the detection limits of these ions on the Beckman DU spectrophotometer are 0.0002 ppm and 0.0001 ppm respectively, the intensity of the flame produced at the 1:50 dilution permits the use of a narrow slit and a high degree of resolution results.

Since it is practically impossible to duplicate, even approximately, the constitution of urine, because of variability of its organic contents, the samples collected in these experiments were run against the above mixed standard. For the estimation of these ions in blood plasma the commercial Beckman Flame standard (sodium, 145 meq/1 and potassium, 4.5 meq/1), was used as the reference. All final dilutions for flame analysis were made up to contain 0.02 percent of Acationox, since the presence of this detergent improves the stability of the spray pattern.

A standard curve for each of these ions was constructed using a series of dilutions of the mixed flame standard. Concentrations of the ions in the unknown solutions were determined by reference to these curves.

The accuracy with which these ions can be determined in cardiac tissue is demonstrated in Tables I and II.

Calcium

The technique of Ferro and Ham (30) is a simple, rapid and

TABLE I

DETERMINATION OF SODIUM[a]

A. Normal Sodium Content

Sample Number	Wet Wt. gm	Final Dilution After Digestion cc	Sodium mg/gm	Average Sodium mg/gm	Average Per Cent Deviation
0	1.4953	8.0	0.8029		
1	1.5010	8.0	0.7998		
2	1.5149	8.0	0.7667		
3	1.5532	8.0	0.7922	0.7904±0.0087[b]	1.49

B. Recovery of Sodium Added before Ashing Procedure

Sample Number	Wet Wt. gm	Final Dilution After Digestion cc	Muscle Contribution mg[c]	Sodium Added mg	Calculated Total	Found	Recovery Per Cent	Average Recovery Per Cent
4	1.5143	10.0	1.1969	0.460	1.6569	1.6932	102.19	
5	1.5346	10.0	1.2130	0.460	1.6730	1.6675	99.67	
6	1.4966	12.0	1.1829	0.920	2.1029	2.1390	101.71	
7	1.5152	12.0	1.1976	0.920	2.1176	2.1390	101.01	101.15

[a]Samples all taken from one minced heart

[b]S. E.

[c]Calculated from the average sodium content of 0.7904 mg/gm

TABLE II

DETERMINATION OF POTASSIUM[a]

A. Normal Potassium Content

Sample Number	Wet Wt. gm	Final Dilution After Digestion cc	Potassium mg/gm	Average Potassium mg/gm	Average Per Cent Deviation
0	1.4953	8.0	3.1245		
1	1.5010	8.0	3.0475		
2	1.5149	8.0	3.0791		
3	1.5532	8.0	3.0963	3.0868±0.0194[b]	0.76

B. Recovery of Potassium Added before Ashing Procedure

Sample Number	Wet Wt. gm	Final Dilution After Digestion cc	Muscle Contribution mg[c]	Potassium Added mg	Calculated Total	Found	Recovery Per Cent	Average Recovery Per Cent
4	1.5143	10.0	4.6743	1.3685	6.0428	6.0605	100.29	
5	1.5346	10.0	4.7370	1.3685	6.1055	6.2071	101.66	
6	1.4966	12.0	4.6197	2.7370	7.3567	7.2315	98.30	
7	1.5152	12.0	4.6771	2.7370	7.4141	7.4075	99.91	100.04

[a]All Samples taken from one minced heart

[b]S. E.

[c]Calculated from the average potassium content of 3.0868 mg/gm

accurate one for the determination of calcium in blood plasma. The method as outlined by the above authors was not dependable for analysis of this cation in cardiac tissue digests. A slight modification of the technique made it quite satisfactory for tissue analysis. Two cc of the chloranilic acid reagent are added to 4.0 cc of the heart muscle solution and this mixture is placed in a refrigerator at 5° C for a minimum period of seventy-two hours. This period of time has been found to be adequate for complete precipitation of calcium in the solution as the chloranilate complex. The solution is centrifuged at 1800 rpm for fifteen minutes and the supernatent discarded. The precipitate is then washed with an excess of 50% isopropyl alcohol to remove all traces of free chloranilic acid, re-centrifuged and the supernatent discarded. The contents of the tube are then dried, a drop of de-ionized water is added to re-suspend the precipitated complex, and exactly 3.0 cc of a 5 per cent tetra sodium ethylenediamine tetracetate solution are added. The stoppered tube is inverted three or four times to effect complete chelation of all calcium, thereby setting free the chloranilic acid as the sodium salt, which imparts its color to the solution. The test samples are compared to a standard solution treated in exactly the same manner. Optical density was determined on a Beckman DU spectrophotometor at a wave length of 530 mμ. Table III depicts the recovery experiments for this cation.

Magnesium

The method of Neill and Neely (31), which is a modification of the original titan yellow method of Běcka (32), works well for the estimation of this ion in blood plasma.

TABLE III

DETERMINATION OF CALCIUM[a]

A. Normal Calcium Content

Sample Number	Wet Wt. gm	Final Dilution After Digestion cc	Calcium mcg/gm	Average Calcium mcg/gm	Average Per Cent Deviation
0	1.4953	8.0	45.98		
1	1.5010	8.0	44.13		
2	1.5149	8.0	45.39		
3	1.5532	8.0	45.92	45.35±0.5791[b]	1.35

B. Recovery of Calcium Added before Ashing Procedure

Sample Number	Wet Wt. gm	Final Dilution After Digestion cc	Muscle Contribution mcg[c]	Calcium Added mcg	Calculated Total	Found	Recovery Per Cent	Average Recovery Per Cent
4	1.5143	10.0	68.67	30.0	98.67	98.68	100.01	
5	1.5346	10.0	69.59	30.0	99.59	101.90	102.31	
6	1.4966	12.0	67.87	60.0	127.87	126.06	98.58	
7	1.5152	12.0	68.71	60.0	128.71	129.90	100.92	100.46

[a]Samples all taken from one minced heart

[b]S. E.

[c]Calculated from the average calcium content of 45.92 mcg/gm

Initial attempts to use this method in tissue analysis proved unsuccessful. The ammonium phosphate method of Denis (33) was tried next, and this also proved unsatisfactory. A sample of the mixed standard previously referred to and an aliquot of a heart muscle electrolyte solution were submitted to another laboratory in the Medical Center for analysis by the method of Zak (34) which was in routine use for magnesium determination. This method proved not to be sufficiently sensitive. It was therefore decided to attempt to perfect the titan yellow method, which is highly sensitive for magnesium, for the quantitative determination of this cation in tissues. An investigation revealed that the phosphate anion was the interfering factor in the analysis of magnesium by the titan yellow technique. Various ion-exchange resins were then utilized in an attempt to remove this interference. Amberlite IRA-410, a chloride-charged anion-exchange resin finally proved to be the most satisfactory.

The chromatographic columns used were designed especially for this study. They measure 14.0 cm in height and 1.2 cm in internal diameter and are equipped with standard burette-type stop-cock with teflon plugs. The resin bed is supported by a fritted disc, and the inherent dead-space between disc and stop-cock is kept minimal. A 1 cc aliquot of the ashed-tissue solution is pipetted into the column on top of a resin bed 8 cm long and permitted to flow through at the rate of two drops per minute. After the meniscus of the solution has completely disappeared, approximately 5 cc of de-ionized water are added and allowed to percolate through the column at the same drop rate. After these 5 cc have gone through, additional de-ionized water is added to

the column and the drop rate doubled until 30 cc of eluate are collected. The use of this volume of water insures the quantitative recovery of magnesium.

The beakers containing the eluates are then placed on a sand-bath and allowed to evaporate to dryness. The residues are reconstituted in 2.0 cc of de-ionized water and a 1 cc aliquot of this is taken for final development of the color in glass stoppered tubes. The procedure outlined below is essentially the same as that followed for blood. To the 1.0 cc aliquot, which represents half of the original amount taken for the chromatographic process, an additional 5.0 cc of de-ionized water are added, followed by 1.0 cc of a 0.1 per cent polyvinyl alcohol solution. Next, 1.0 cc of a 0.05 per cent titan yellow solution is added, followed by 2.0 cc of 4 N NaOH. This normality is critical. At every step twirling of the tube is essential so as to insure thorough mixing. The tubes are stoppered, mixed thoroughly, and read against a blank prepared similarly, in a Beckman DU spectrophotometer at 540 mμ. For the analysis of magnesium in blood, 1.0 cc of $CaCl_2$ solution containing 0.05 mg of calcium, is used in the blank in place of plasma to compensate for the intensifying effect of calcium on the color of the magnesium-titan yellow complex. In the tissue blank, no calcium is necessary because the Mg:Ca ratio is high and the calcium interference effect is essentially absent. Table IV shows the effect of removal of the phosphate ion and the accuracy which is obtainable by this method in the determination of tissue magnesium.

TABLE IV

DETERMINATION OF MAGNESIUM[a]

A. Normal Magnesium Content

Sample Number	Wet Wt. gm	Final Dilution After Digestion cc	Before IRA-410 Treatment mcg/gm	After IRA-410 Treatment mcg/gm	Average Magnesium mcg/gm	Average Per Cent Deviation
0	1.4953	8.0	187.25	238.07		
1	1.5010	8.0	178.54	239.84		
2	1.5149	8.0	180.34	239.75		
3	1.5532	8.0	187.74	238.21	238.96 ± 1.1933[b]	0.34

B. Recovery of Magnesium Added before Ashing Procedure

Sample Number	Wet Wt. gm	Final Dilution After Digestion cc	Muscle Contribution mcg[c]	Magnesium Added mcg	Calculated Total	Found	Recovery Per Cent	Average Recovery Per Cent
4	1.5143	10.0	361.86	100.00	461.86	467.49	101.22	
5	1.5346	10.0	366.71	100.00	466.71	467.45	100.16	
6	1.4960	12.0	357.63	200.00	557.63	555.56	99.63	
7	1.5152	12.0	362.07	200.00	562.07	560.94	99.80	100.20

[a]All samples taken from one minced heart

[b]S. E.

[c]Calculated from the average magnesium content of 238.96 mcg/gm

Phosphorus

Total and acid-soluble phosphates of cardiac muscle are determined by the method of Fiske and Subba Row (35). The results of these determinations are expressed in terms of elemental phosphorus. A typical recovery experiment is illustrated in Table V.

TABLE V

DETERMINATION OF PHOSPHORUS[a]

A. Normal Phosphorus Content

Sample Number	Wet Wt. gm	Final Dilution After Digestion cc	Phosphorus mg/gm	Average Phosphorus mg/gm	Average Per Cent Deviation
0	1.4953	8.0	1.2340		
1	1.5010	8.0	1.2687		
2	1.5149	8.0	1.2430		
3	1.5532	8.0	1.2940	1.2599 ± 0.0257[b]	1.69

B. Recovery of Phosphorus Added before Ashing Procedure

Sample Number	Wet Wt. gm	Final Dilution After Digestion cc	Muscle Contribution mg[c]	Phosphorus Added mg	Calculated Total	Found	Recovery Per Cent	Average Recovery Per Cent
4	1.5143	10.0	1.9079	1.0841	2.9920	2.8595	95.57	
5	1.5346	10.0	1.9334	1.0841	3.0175	2.9733	98.53	
6	1.4966	12.0	1.8796	2.1682	4.0478	4.0724	100.60	
7	1.5152	12.0	1.9090	2.1682	4.0772	4.0315	98.87	98.39

[a]All samples taken from one minced heart

[b]S. E.

[c]Calculated from the average phosphorus content of 1.2599 mg/gm

CHAPTER IV

RESULTS

Tables VI through IX present the cardiac electrolyte values for the twenty-four animals that made up the four experimental groups. Sodium and potassium are expressed in terms of milliequivalents per kilogram of wet tissue; calcium, magnesium and phosphorus, in milligrams per kilogram of wet tissue. The specific ratios, calcium/potassium (Ca/K), calcium/magnesium (Ca/Mg) and acid-soluble phosphorus/total phosphorus (AP/TP) are also included in these tables. Table X presents the group average for each of the electrolytes determined along with the standard error for each.

It is obvious from an inspection of Table X that levels of certain ions vary comparatively little from group to group, while others show striking differences. In order to ascertain the real effect of hormone balance on the electrolyte content of the heart, four specific comparisons were made: normal females were compared with males and with castrate females. These two comparisons include the animals whose hormone pattern can be assumed to differ most widely. Normal females were compared with estrogen-treated, spayed females because the later treatment represents an attempt to duplicate the normal hormonal pattern of the female. Males were compared with castrate females because their hormone pattern is known to be qualitatively similar.

TABLE VI

IONIC CONTENT OF VENTRICULAR MUSCLE
IN NORMAL FEMALE DOGS

Experiment Number	Na meq/kg	K meq/kg	Ca mg/kg	Mg mg/kg	Total P mg/kg	Acid Soluble P mg/kg	Ratios		
							Ca:K	Ca:Mg	AP:TP
44	26.83	80.50	36.45	230.38	1876.7	1023.0	0.01158	0.158	0.545
28	28.81	89.54	39.62	241.31	1784.3	1031.7	0.01132	0.164	0.578
31	31.69	77.26	33.88	267.65	1660.8	875.2	0.01122	0.127	0.527
35	33.80	76.72	32.97	270.53	1784.8	877.5	0.01099	0.122	0.492
38	32.40	79.98	31.87	244.25	1810.1	1028.8	0.01019	0.130	0.568
40	26.67	80.54	35.63	248.48	1767.7	978.7	0.01131	0.143	0.554
Average	30.03	80.75	35.07	250.43	1780.7	969.2	0.0110	0.140	0.554

TABLE VII

IONIC CONTENT OF VENTRICULAR MUSCLE
IN NORMAL MALE DOGS

Experiment Number	Na meq/kg	K meq/kg	Ca mg/kg	Mg mg/kg	Total P mg/kg	Acid Soluble P mg/kg	Ratios		
							Ca:K	Ca:Mg	AP:TP
22	32.27	86.21	49.50	250.91	1548.2	453.1	0.01468	0.197	0.293
24	30.56	85.42	43.13	237.40	1508.0	534.2	0.01291	0.182	0.354
29	19.88	80.55	48.24	239.74	1530.9	464.8	0.01532	0.201	0.304
34	32.23	76.82	48.55	237.17	1528.9	466.0	0.01616	0.205	0.305
39	28.03	79.16	48.50	235.28	1560.0	467.2	0.01567	0.206	0.299
43	28.44	78.06	47.62	236.37	1579.0	587.3	0.01560	0.201	0.372
Average	30.23	81.03	47.59	239.47	1542.6	495.4	0.01502	0.199	0.321

TABLE VIII

IONIC CONTENT OF VENTRICULAR MUSCLE
IN CASTRATE FEMALE DOGS

Experiment Number	Na meq/kg	K meq/kg	Ca mg/kg	Mg mg/kg	Total P mg/kg	Acid Soluble P mg/kg	Ratios		
							Ca:K	Ca:Mg	AP:TP
23	33.72	84.43	46.86	265.17	1570.3	729.3	0.01419	0.177	0.464
32	31.34	75.63	49.66	276.66	1181.5	527.0	0.01679	0.179	0.446
33	37.91	79.44	47.82	260.26	1180.3	501.4	0.01540	0.184	0.425
37	30.02	80.35	46.55	236.98	1229.6	485.5	0.01482	0.196	0.395
51	29.70	86.29	46.66	237.68	1580.0	715.0	0.01383	0.196	0.453
52	31.20	79.31	47.22	249.87	1531.8	673.2	0.01523	0.189	0.439
Average	32.32	80.91	47.46	254.44	1378.9	605.2	0.01500	0.187	0.439

TABLE IX

IONIC CONTENT OF VENTRICULAR MUSCLE IN ESTROGEN-TREATED CASTRATE FEMALE DOGS

Experiment Number	Na meq/kg	K meq/kg	Ca mg/kg	Mg mg/kg	Total P mg/kg	Acid Soluble P mg/kg	Ratios		
							Ca:K	Ca:Mg	AP:TP
21	32.96	84.68	45.96	256.51	1472.4	545.65	0.01388	0.179	0.371
26	44.04	70.69	40.34	263.82	1340.4	460.20	0.01459	0.153	0.343
27	36.37	79.96	33.62	246.29	885.0	370.50	0.01075	0.137	0.419
30	36.00	73.83	38.57	255.44	1402.1	543.80	0.01336	0.151	0.388
45	32.58	76.47	37.61	226.25	1344.2	466.20	0.01258	0.166	0.347
57	32.76	80.27	37.66	245.48	1387.9	539.60	0.01200	0.153	0.389
Average	35.78	77.65	38.96	248.97	1305.3	487.65	0.01286	0.156	0.373

TABLE X

SUMMARY OF TABLES VI, VII, VIII, IX
AVERAGE VALUES OF ELECTROLYTES
IN CANINE VENTRICULAR MUSCLE

	Na meq/kg	K meq/kg	Ca mg/kg	Mg mg/kg	Total P mg/kg	Acid Soluble P mg/kg	Ratios Ca:K	Ratios Ca:Mg	Ratios AP:TP
Normal Females.	30.03	80.75	35.07	250.43	1780.70	969.20	0.01	0.14	0.54
S. E.±	1.25	1.94	1.14	6.42	29.10	30.10	0.00022	0.0094	0.013
Normal Males.	30.23	81.03	47.59	239.47	1542.60	495.40	0.02	0.20	0.32
S. E.±	0.78	1.66	0.93	2.53	11.75	22.01	0.00066	0.0	0.014
Castrate Females.	32.32	80.91	47.46	254.44	1378.90	605.20	0.02	0.19	0.44
S. E.±	1.23	1.56	0.51	6.43	81.94	46.02	0.00066	0.0027	0.0
Estrogen-Treated Castrate Females.	35.79	77.65	38.96	248.97	1305.30	487.65	0.01	0.16	0.37
S. E.±	1.77	2.50	1.66	5.29	86.42	28.40	0.00052	0.0081	0.025

The similarities and differences which were revealed by these comparisons of the data summarized in Table X were subjected to statistical analysis, the results of which are tabulated in Table XI. Three degrees of statistical significance have been applied in the evaluation of these results: questionable significance ($^{a}p < 0.1 > 0.05$), borderline significance ($^{b}p < 0.05 > 0.02$) and high significance ($^{c}p < 0.01$).

Sodium values for males and normal females are essentially identical. Castrate females and estrogen-treated castrates show slightly higher values. The average sodium level for estrogen-treated castrates is higher than that for normal females, but this difference is of borderline significance only. Potassium levels show no significant differences among the four comparisons. Calcium values are lowest in normal female animals, intermediate in estrogen-treated castrates, and highest in males and castrate females. Differences in calcium levels are highly significant in every comparison except in that between castrate females and normal males. Cardiac magnesium levels vary little from group to group. The greatest difference exists between spayed females and males, and this is of doubtful statistical significance. Total phosphorus content of ventricular muscle is highest in the normal female, lowest in the estrogen-treated castrate and intermediate in the other two groups. The same relationship holds true for the acid-soluble phosphorus levels. Total phosphorus levels are significantly higher in normal females than in males, and a difference of questionable significance exists between castrate females and males. The differences in acid-soluble phosphorus values are highly significant in three of the

TABLE XI

t - VALUES FOR DIFFERENCES IN CARDIAC ELECTROLYTE CONTENT

Groups	Na	K	Ca	Mg	Total P	Acid-Soluble P
Normal Female vs. Normal Male	0.14	0.52	8.53[c]	1.59	7.60[c]	10.46[c]
Castrate Female vs. Normal Male	1.44	0.05	0.12	2.16	1.98[a]	2.15[a]
Normal Female vs. Estrogen-treated Castrate Female	2.66[b]	1.10	3.35[c]	0.18	1.65	11.64[c]
Normal Female vs. Castrate Female	1.31	0.65	9.91[c]	0.14	1.46	6.63[c]

t-values calculated as $$t = \frac{(\bar{x} - \bar{y})}{\sqrt{\frac{(\Sigma x^2 + \Sigma y^2) - N[(\bar{x})^2 + (\bar{y})^2]}{N_1 + N_2 - 2}} \cdot \sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}$$

$N_1 = N_2 = 6$

[a] $P < 0.1 > 0.05$

[b] $P < 0.05 > 0.02$

[c] $P < 0.01$

comparisons but of only questionable significance when castrate females are compared with normal males.

Cardiac Ca/K ratios in males and castrate females are double those for normal females and estrogen-treated spayed females. The Ca/Mg ratios vary little from group to group. The AP/TP ratio is highest in normal females, lowest in males and intermediate in the other two groups. Because of the difficulties inherent in the interpretation of a statistical treatment of ratios, the significance of the observed differences is not clear.

The dry weight percentages of heart muscle; adrenal weights; hematocrit values; blood levels of sodium, potassium, calcium and magnesium; and the hourly urinary excretion of sodium and potassium showed no significant variation from group to group and these data have not been recorded in this dissertation.

CHAPTER V

DISCUSSION

The objective of this investigation was to study the underlying causes that contribute towards the differential response by male and female dogs to a toxic dose of a digitalis glycoside. It is obvious that an understanding of the mechanisms which are responsible for this difference in response to drugs as important as the cardiac glycosides, would be of great physiological and pharmacological significance.

These results have been analyzed in a search for any differences in cardiac ionic content that could conceivably be responsible for this phenomenon. The constituents studied were sodium, potassium, calcium, magnesium and phosphorus, inasmuch as these inorganic ions have been shown to have an impact on physiological phenomena such as the action potential, ion influx and efflux, contractility, rhythmicity and excitability. Phosphate distribution was also studied because of its obvious importance in connection with all of the normal physiological properties of heart muscle.

Cattell and Goodell (3) showed that the digitalis glycosides cause a reduction in muscle potassium and advanced the theory that an increase in the calcium/potassium ratio might be responsible for the characteristic action of these drugs. The calcium/potassium ratios for normal males and castrate females are definitely higher than for the

other two groups and if the above mentioned hypothesis is valid, one could deduce, that because of this increased ratio, normal males and spayed females displayed an arrhythmia earlier, in the experiments of Grinnell and Smith (1).

Calcium, however, is the cation which shows variations of most marked significance within the four categories of experimental animals. Table X demonstrates that cardiac calcium values are highest for normal males and castrate females and lowest for normal females. It is highly significant that there is abundant evidence in the literature to the effect that the calcium ion concentration alone, without regard to the calcium/potassium ratio, may be the most important determinant of the responsiveness of the myocardium to digitalis.

It is well known that cardiac muscle loses its excitability in the absence of calcium. Under these circumstances Clark (36) has shown that digitalis is without effect. Hoff, Smith and Winkler (37) and Clarke (38) showed that an increase in calcium leads to the production of ventricular extrasystoles and ventricular fibrillation. Calcium ions and digitalis have a synergistic action on the heart as shown experimentally by Nyiri and Du Bois (39) by Gold and Edwards (40) and by Salter, Sciarini and Rubin (41). Bower and Mengle (42) and Rogen (43) reported cases in which death from ventricular fibrillation have occurred after intravenous administration of calcium gluconate to fully-digitalized patients. Blumenfeld and Loewi (44) state that digitalis changes the state of the heart in such a manner that it becomes hypersensitive to calcium and therefore reacts to the normal calcium concentration of the medium like a heart without digitalis reacts to high calcium concen-

trations.

The synergism between calcium and digitalis has also been demonstrated by an entirely different technique. Sapeika (45), Smith and Grinnell (46) and Page and Real (47), showed that ethylenediamine tetraacetic acid due to its ability to chelate and therefore inactivate calcium ions, is capable of reversing the arrhythmia produced by a toxic dose of digitalis. It is extremely interesting that the highest values for calcium occur in the two categories of animals which are the most susceptible to the production of an arrhythmia by a toxic dose of digitalis.

It has been pointed out that the castrate female becomes resistant to digitalis after administration of estrogens. The results in this study show that estrogen treatment lowers the myocardial calcium values of the castrate animal to a level which approximates that of the normal female. The fact that estrogens influence the mobilization, transport and deposition of calcium is well documented. For instance, Kyes and Potter (48) discovered that at certain times, the bone marrow cavities of female pigeons became so filled with spicules of bone that it was no longer possible to obtain specimens of marrow from them. This phenomenon was never encountered in male pigeons. Later, Pfeiffer and Gardner (49) showed that this hyperossification could be duplicated in male pigeons by the administration of estradiol benzoate. These results and those reported here would seem to imply that estrogens mobilize calcium from soft tissues and facilitate the deposition of this cation into bone.

While these results show that there are definite and significant

differences in total and acid-soluble phosphate content and distribution in the animals included in this investigation, it is not possible at this time to correlate these differences with sensitivity to digitalis. For instance, estrogen treatment of the castrate female, which lowers her calcium and increases her resistance to digitalis, does not re-establish the normal phosphate pattern.

Certain other differences in ionic content have emerged from this study: 1) A higher sodium content of hearts from estrogen-treated castrates when compared with normal females and 2) A lower magnesium content of hearts of normal males when compared to normal females. Both of these differences are of borderline or questionable signigicance respectively and their importance with respect to the differential sensitivity of these types of animals to digitalis cannot be evaluated at this time.

CHAPTER VI

SUMMARY AND CONCLUSION

Summary

1. Sodium, potassium, calcium, magnesium, total phosphorus and acid-soluble phosphorus have been determined, by improved chemical methods, in ventricular muscle from female, male, castrate female and estrogen-treated, castrate, female dogs.

2. The average cardiac content of sodium, potassium and magnesium was found not to vary significantly in these four types of animals.

3. Total and acid-soluble phosphorus were found to be highest in the hearts of normal females.

4. The acid-soluble phosphorus/total phosphorus (AP/TP) ratio was highest in the normal female and lowest in the male.

5. Estrogen treatment of the castrate female did not restore the phosphorus distribution to that of the normal female.

6. The lowest calcium values were found in the hearts of normal females.

7. Calcium values were found to be highest, and almost identical in the hearts of male and castrate female animals.

8. Estrogen treatment lowered the calcium content of the heart

of the castrate female to a value which approached but did not equal that of the normal female. The difference between the calcium content of the hearts of these two groups was found to be statistically significant.

Conclusion

Two major differences in electrolyte content of the heart have been found to exist in the four types of animals studied. Of these, the higher calcium values in the male and castrate female would appear most likely to explain their greater susceptibility to the arrhythmic action of a toxic dose of digitalis.

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