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AN ABSTRACT OF THE THESIS OF Peter Blair Kimmel for the
Master of Science in Biology presented August 22, 1985.

Title: The Significance of Hypovolemia in Dehydrational
Death in Anurans.

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

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The importance of hypovolemia in dehydrational death was assessed in two anuran species. Xenopus laevis, a species which experiences a significant reduction in circulating plasma volume with dehydration, was used to evaluate the role of sympathetic reflex compensation in hypovolemia. Adrenergic blockade with propranolol or phenoxybenzamine produced no significant reduction in dehydration tolerance in this species, although β -blockade with propranolol appeared to have a minor effect. The role of hypovolemic shock in the terminal circulatory collapse

that precedes death in dehydrating anurans was investigated in the toad, Bufo marinus. The activity of lysosomal proteinases (cathepsins) was used as an indicator of the shock state. An assay was developed for the determination of cathepsin activity in toad plasma and was used to demonstrate the presence of proteinases similar in pH dependence to mammalian cathepsins B1 and D. Plasma cathepsin D-type activity increased with dehydration in toads but was not significantly different from the activity in controls or in toads subjected to splanchnic artery occlusion. Toads appear to maintain plasma volume to 20% water loss at the expense of other extracellular fluids. These results do not support the hypothesis that hypovolemia is a direct cause of dehydrational death in anurans.

THE SIGNIFICANCE OF HYPOVOLEMIA IN
DEHYDRATIONAL DEATH IN ANURANS

by

PETER BLAIR KIMMEL

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
in
BIOLOGY

Portland State University

1985

TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

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GENERAL INTRODUCTION

Amphibians are highly susceptible to dehydration in terrestrial environments. Evaporative water loss from most amphibians occurs at about the same rate as from a free surface of water and they cannot produce urine hyperosmotic to their body fluids (Bentley, 1971). Adaptation to terrestrial environments in amphibians does not usually involve mechanisms to reduce water loss, but rather increased tolerance to water loss. Among anuran amphibians (frogs and toads) there is a general positive correlation between dehydration tolerance and the terrestriality of the species (Thorson and Svihla, 1943; Thorson, 1955).

The physiological basis of dehydration tolerance is presently unclear. A detailed understanding of the nature of dehydrational death is essential to determining the mechanisms of extended tolerance. As in any complex system, the component or components that fail first are the most critical to the continued functioning of the system.

The principal hypothesis as to the immediate cause of dehydrational death in anurans is that of cardiovascular failure leading to a curtailment of circulatory oxygen delivery and generalized anoxia (Shoemaker, 1964; Hillman, 1978b). It is not entirely clear, however, exactly how dehydration leads to this cardiovascular debilitation.

Dehydration has been shown to cause a significant reduction in circulating plasma volume in a variety of amphibians. However, the proportion of water lost from the plasma as compared to the whole animal varies greatly among species. The tiger salamander (Ambystoma tigrinum) loses plasma water with dehydration just as predicted from the overall water deficit (Alvarado, 1972), while the blood of Rana pipiens has a percent water loss that is almost twice that of the frog as a whole (Smith and Jackson, 1931). The importance of the maintenance of plasma volume to dehydration tolerance in amphibians has not been systematically examined, although in mammals it is thought to be a critical aspect of the relative ability to withstand desiccation (Schmidt-Nielsen, 1964).

Hypovolemia is but one consequence of dehydration in amphibians. With loss of water, there is a proportional increase in the non-permeant osmotic concentration and hematocrit of the blood. Plasma hyperosmolality has been implicated as a limiting factor in dehydrational death in anurans (Hillman, 1978b; 1980), and the increasing viscosity that accompanies rising hematocrit has been shown to further compromise cardiovascular function (Hillman, 1978b). However, nothing is known about the role of hypovolemia itself in dehydrational death in anurans.

This study was undertaken to investigate the possible importance of hypovolemia in dehydrational death and,

conversely, the significance of plasma volume maintenance to desiccation tolerance in anurans. Two approaches were taken: (1) interference with the normal compensatory mechanisms for reduced blood volume in order to determine their contribution to dehydration tolerance, and (2) assessment of the consequences of dehydration relative to hypovolemia and the possibility of circulatory shock.

If hypovolemia is an important factor in dehydrational death in anurans, then the normal physiological mechanisms that compensate for reduced blood volume should be critical for survival. Interference with these mechanisms should significantly reduce desiccation tolerance. The importance of sympathetic reflex compensation for hypovolemia in mammals is well known (Chien, 1967). The possible importance of sympathetic compensation in anurans is the subject of Chapter I.

If hypovolemia is the cause of the circulatory collapse that precedes death in dehydration (Shoemaker, 1964), then there should be some indication of circulatory shock. Shock is a generalized insufficiency of blood flow to such a degree that extensive tissue damage results from inadequate oxygen delivery (Guyton, 1981). Dehydrational death in anurans is associated with such a generalized anoxia (Hillman, 1978b), but circulatory shock has never been demonstrated in amphibians.

Any condition which reduces cardiac output may result in circulatory shock in mammals (Guyton, 1981) and over one hundred different types of shock have been described (Hardaway, 1981). Causes of shock include damage to the heart as in myocardial infarction (cardiogenic shock), massive allergic reaction (anaphylactic shock), bacterial infection (septic shock), serious physical trauma (traumatic shock), and neurological damage or anesthesia (neurogenic shock). The best known type is hypovolemic shock, in which reduced blood volume results in a decrease in venous return to the heart and a fall in cardiac output. The most important cause of hypovolemia, from a medical standpoint, is loss of blood (hemorrhagic shock), although dehydration can lead to a similar shock state (Guyton, 1981).

Shock may be divided into three distinct phases (Guyton, 1981): non-progressive, progressive, and irreversible. Non-progressive or compensated shock is characterized by an assortment of negative-feedback physiological responses that serve to maintain arterial pressure and restore blood volume. The sympathetic nervous system is of particular importance in cardiovascular adjustments at this stage (Chien, 1967; see Chapter I). With time or further loss of blood volume, shock may enter the second phase, called progressive or decompensated shock, characterized by the predominance of positive-feedback mechanisms that accelerate cardiovascular failure. The

compensatory mechanisms begin to fail in severe shock and an ever-increasing cycle of circulatory deterioration is initiated: shock causes damage to the cardiovascular system, which worsens the shock state, leading to further damage, and so on. Eventually, so much damage has been done to the cardiovascular system that death becomes inevitable.

Arterial pressure and cardiac output may be temporarily restored to near normal levels but no type of therapy can prevent death (Guyton, 1981). It is likely that deterioration of the heart is the reason for the irreversibility of this final stage of shock (Crowell and Guyton, 1962; Guyton, 1981), although collapse of the peripheral vasoconstriction is an alternative hypothesis (Bond et al., 1973).

One positive-feedback mechanism in progressive shock is believed to be a consequence of prolonged sympathetic stimulation. Tissues made ischemic by the intense vasoconstriction seen in shock may become necrotic and release toxic substances into the circulation. Of particular importance is a myocardial depressant factor (MDF) produced by the ischemic pancreas (Lefer, 1978). Vasoconstriction in the splanchnic circulation produces hypoxia in the pancreas and hypoxia promotes the breakdown of lysosomal membranes in pancreatic cells (Lefer, 1978), apparently by promoting Ca^{2+} influx into the cells (Hock et al., 1984; Lefer and Papanicolaou, 1985). Lysosomal

disruption releases a variety of proteolytic enzymes called cathepsins (Glenn and Lefer, 1971). One or more of these cathepsins cleaves a plasma protein substrate to produce MDF, a peptide having a molecular weight of 500-1000 (Lefer and Martin, 1970; Lefer and Barenholz, 1972; Leffler et al., 1973; Litvin et al., 1973). In the circulation, MDF depresses cardiac contractility by 50-80%, thus further reducing pancreatic blood flow and accelerating MDF formation (Lefer et al., 1967; Lefer and Blattberg, 1968; Lefer, 1970). MDF also specifically constricts splanchnic (superior mesenteric) arterial strips but not aortic strips (Glucksman and Lefer, 1971) and depresses its own clearance from the blood (Lefer and Blattberg, 1968).

Plasma cathepsin activity has been used previously as an indicator of the degree of circulatory shock in experimental animals (Curtis and Lefer, 1980), but never in amphibians. Chapter II of this thesis describes the development of an assay for cathepsin activity in toad plasma and Chapter III concerns the application of that assay to determine whether or not toads suffer from circulatory shock as a result of dehydration.

CHAPTER I

THE EFFECTS OF ADRENERGIC BLOCKADE ON DEHYDRATION TOLERANCE IN XENOPUS LAEVIS

INTRODUCTION

The role of the sympathetic nervous system in hypovolemia has been well documented in mammals (see Chien, 1967). A reduction in blood volume results in a fall in blood pressure which is detected by baroreceptors in the carotid arteries and aortic arch. Blood pressure is regulated by the medullary cardiovascular center via sympathetic nerves to the heart and blood vessels and catecholamines from the adrenal medulla. Reflex activation of the sympathetic nervous system with falling pressure results in (1) an increase in heart rate and force of contraction to maintain cardiac output and (2) selective vasoconstriction to redistribute the remaining blood to vital areas and to increase total peripheral resistance. Thus, sympathetic activation is an emergency response to maintain central arterial pressure and circulation to the heart and brain.

There is some evidence for sympathetic involvement in the response to dehydrational hypovolemia in anurans. Sympathetic reflexes have been implicated in the maintenance

of blood pressure (Sham et al., 1984), the redistribution of blood flow (Hillman and Sommerfeldt, 1981), and the hydroosmotic (water uptake) response (Yokota and Hillman, 1984) in a number of anuran species. It should be possible to reduce dehydration tolerance using adrenergic blocking agents if adrenergically-mediated adjustments to hypovolemia are important to survival in dehydration. In addition, since cardiac adrenoceptors in amphibians are of the β -type (Erlij et al., 1965; Stene-Larsen and Helle, 1978; Ask, 1983; Herman and Sandoval, 1983), while vascular adrenoceptors are predominantly of the α -type (Erlij et al., 1965; Herman and Sandoval, 1983), it should also be possible to examine separately the relative importance of changes in cardiac performance and peripheral vasoconstriction to desiccation tolerance.

In this experiment, Xenopus laevis treated with propranolol (a non-specific β -blocker) or phenoxybenzamine (an α_1 -blocker) and control frogs were dehydrated to determine the effects of selective adrenergic blockade on survival. Xenopus is particularly suited to this type of study because of its relatively low dehydration tolerance, insignificant bladder volume, and lack of any hydroosmotic response (Hillman, 1978a). Also, much is known about the physiological consequences of dehydration in this species (Hillman, 1978a; 1978b; 1980; Hillman and Sommerfeldt, 1981).

MATERIALS AND METHODS

Xenopus laevis were purchased from commercial suppliers. The frogs were maintained in tap water at room temperature and fed weekly with a ground liver preparation. Animals used in experimentation had a mean mass of 16.8 g (SE = 1.1 g; range 9.1-31.0 g, N=39).

The frogs were blotted and placed in pre-weighed 1-pint plastic freezer containers that had ventilation holes bored in the lids. Body mass was measured to the nearest 0.1 g by weighing the container and frog together and subtracting the mass of the container. Animals could thus be weighed without handling them. Dehydration took place at room temperature with no attempt to control humidity. The frogs were weighed periodically until an average loss of 20-25% of original body mass was achieved. They were then divided into three groups of approximately equal mean mass. Control animals were injected with 0.9% saline; experimental animals received either 1 mg/kg DL-propranolol (Sigma) or 10 mg/kg phenoxybenzamine. The volume used in all cases was 0.1 ml, and the solutions were introduced by injection into the dorsal lymph sac. The frogs were then weighed periodically until they died. The criterion of death used in this study was the critical activity point (CAP), defined as the point at which a frog is unable to right when flipped on its back (Hillman, 1978b).

Mean water loss at CAP, expressed as percent of original body mass, was compared for the three groups by analysis of variance. In addition, the control and propranolol group means were compared with a two-tailed t-test. A $P < 0.05$ was considered significant.

RESULTS

The results are presented in Table I. Frogs injected with propranolol or phenoxybenzamine reached CAP having lost less water, on the average, than untreated animals, although analysis of variance showed no significant difference between the means of the three groups ($P = 0.13$). If, however, the phenoxybenzamine data is ignored and the control and propranolol group means are compared using a two-tailed t-test, then the difference between these two means is statistically significant ($P < 0.05$).

DISCUSSION

The mean water loss at critical activity point (CAP) reported here for control Xenopus (36.1% original body mass) is greater than the value of 33.8% reported for Xenopus by Hillman (1978b; 1980). Frogs used in the present study were considerably smaller than those used by Hillman, however, and tolerance to water loss appears to be greater for frogs

TABLE I
WATER LOSS AT C.A.P., EXPRESSED AS PERCENT OF
ORIGINAL BODY MASS, FOR CONTROL AND
PHARMACOLOGICALLY-TREATED
XENOPUS LAEVIS

	Mean (2 SE)	Range	N
Control	36.1 (1.0)	33.5 - 39.2	13
Propranolol	34.7 (0.7)	32.1 - 36.7	13
Phenoxybenzamine	35.5 (1.2)	32.0 - 39.2	13

of lower body mass (Thorson, 1955). The frogs in the present study lost water at a rate of 10-30% original body mass per day, as opposed to the 6-9% Hillman reported.

The results of the pharmacological treatments are inconclusive. Statistically, the reduction in dehydration tolerance brought about by adrenergic blockade is not significant (by ANOVA). However, there is an indication that β -adrenergic blockade with propranolol may interfere slightly with adjustments to hypovolemia (see t-test results). It must be stressed that it is not statistically appropriate to use a t-test to test the difference between two means within a group of three. Still, it is possible that a much larger data base would provide evidence that the effects of propranolol are real.

Drug concentrations used in this study were within the range of those used in previous experiments with amphibians. Hillman (1982) found that dosages of propranolol in excess of 0.01 mg/kg produced a significant reduction in maximal heart rate in Scaphiopus intermontanus. Lillo (1979) reported that 2 mg/kg propranolol caused a long-lasting bradycardia in Rana catesbeiana. Yokota and Hillman (1984) used 2.5 mg/kg propranolol and 12.5 mg/kg phenoxybenzamine in dehydrated Bufo cognatus, although no effect of the α -blocker could be seen in their results. Phenoxybenzamine has been shown to block vasoconstriction by catecholamines in vitro in isolated artery strips from Bufo marinus (Kirby

and Burnstock, 1969a) and to block the in vivo pressor response to catecholamines in Rana pipiens (Erlj et al., 1965) and Bufo marinus (at 2 mg/kg; Kirby and Burnstock, 1969b). In trials prior to this study, 1 mg/kg propranolol was found to depress maximal heart rate in Xenopus, completely abolishing exercise tachycardia in some cases. Both degree and duration of effects were variable, however. No attempt was made to confirm the effectiveness of α -blockade during these experiments.

The relative importance of cardiac (β) and peripheral (α) factors in prolonged hypovolemia and circulatory shock remains controversial, even in mammals. Although heart rate and force of contraction certainly increase under sympathetic stimulation (Chien, 1967), the actual effect on cardiac output may be minor. Ushioda et al. (1983) found no significant difference in cardiac output between β -adrenergically blocked and β -intact sheep with hemorrhage. Apparently the increase in heart rate is offset by a decline in ventricular filling time and stroke volume, despite the positive inotropic effect of adrenergic stimulation. Peripherally, sympathetic vasoconstriction, while maintaining blood pressure and flow to the heart and brain, may lead to widespread tissue ischemia and the release of toxic products, including a myocardial depressant factor (MDF; Lefer, 1978). For this reason, α -adrenergic antagonists are often used in addition to volume replacement

in the treatment of circulatory shock (Chien, 1967; Guyton, 1981). Thus, in the present experiments with Xenopus, β -blockade may have had no effect on cardiac output and α -blockade without volume replacement may have had both beneficial and detrimental effects.

Assuming the adrenergic blockers did interfere significantly with sympathetic reflex compensation in hypovolemia, it is quite likely that this would have little apparent impact on mean water loss at CAP. Hillman (1978b) showed that maximal oxygen consumption rate (VO₂ max) declines with dehydration in Xenopus, and that CAP is the point at which VO₂ max falls below resting metabolic demand. At this point, circulatory oxygen delivery is no longer adequate to satisfy minimal requirements and a generalized tissue anoxia ensues. However, the decline in VO₂ max is not linear. At 20% dehydration, VO₂ max is still 70% of the value for hydrated Xenopus, reflecting a considerable aerobic scope. Water loss in excess of 20% original body mass causes a rapid fall in VO₂ max to CAP. Thus, measured as the curtailment of oxygen delivery to metabolizing tissues, well over half the cardiovascular debilitation caused by dehydration occurs with the last 10-15% water loss. In this range, the slope of the relationship between VO₂ max and water loss is so steep that factors which significantly affect circulatory oxygen transport may have no measurable effect on mean water loss

at CAP. Therefore, the present experimental design may not be sufficiently sensitive to detect the effects of sympathetic blockade on adjustments to hypovolemia.

There is evidence that the sympathetic nervous system does indeed play a role in the response to hypovolemia in anurans. Sympathetic activation in mammals is characterized by tachycardia and an increase in the force of contraction of the heart and by selective vasoconstriction to maintain blood pressure and redistribute blood to vital areas (Chien, 1967). Dehydrated Xenopus show both an increase in resting heart rate (Hillman, 1978b) and a redistribution of systemic blood flow to the head at the expense of other regions (Hillman and Sommerfeldt, 1981). Mean arterial pressure in Rana catesbeiana is unaffected by two days under desiccating conditions but is significantly lower in dehydrated bullfrogs treated with the α -blocker phentolamine (Sham et al., 1984). Each of these responses to dehydration in anurans is consistent with the results of sympathetic activation in mammals. In addition, Yokota and Hillman (1984) demonstrated β -adrenergic involvement in the cutaneous water balance response of Bufo cognatus, which is important in rehydration following hypovolemia.

CHAPTER II

AN ASSAY FOR CATHEPSIN ACTIVITY IN TOAD PLASMA

INTRODUCTION

The term "cathepsin" (from the Greek meaning "to digest") was first applied in 1929 to tissue proteinase activity at acid pH (Mycek, 1970). Work in the 1930s was conducted under the assumption that this activity was due to a single enzyme. However, the use of synthetic peptide substrates clearly demonstrated more than one type of proteolytic activity in "cathepsin" preparations, including activity under less acidic and even neutral conditions. Since 1941, at least 19 different enzymes have been designated "cathepsins" (see Table II). The term is now essentially synonymous with "intracellular proteinase" (Barrett, 1980). With few exceptions (cathepsins F and R), these have proven to be lysosomal enzymes.

Classification

At present, proteinases in general are classified on the basis of catalytic mechanism, and this is the scheme used in Table II to organize the cathepsins (Barrett, 1975; 1980). Endopeptidases hydrolyze proteins to produce shorter peptides, primarily by cleaving bonds away from the ends of

TABLE II
CLASSIFICATION OF THE CATHEPSINS

Endopeptidases

Aspartic (=Carboxyl)	pH Optimum	Source
D	3-4	Kirschke <i>et al.</i> , 1977
E	2-3	Kirschke <i>et al.</i> , 1977
Serine		
G	7.5-8	Kirschke <i>et al.</i> , 1977
R	7+	Levyant <i>et al.</i> , 1976
Cysteine (=Thiol)		
B1	4-6	Barrett and Kirschke, 1981
H	5-7	Barrett and Kirschke, 1981
J	6-7	Liao and Lenney, 1984
K	6-7	Liao and Lenney, 1984
L	5-5.5	Barrett and Kirschke, 1981
M	5-7	Pontremoli <i>et al.</i> , 1982
N	3-6	Evans and Etherington, 1978
P	5-6	Docherty <i>et al.</i> , 1982
S	3	Turnsek <i>et al.</i> , 1975
T	6-7	Gohda and Pitot, 1981
Unknown		
F	4.5	Barrett, 1975

Exopeptidases

Carboxypeptidases

A	5-6	Taylor and Tappel, 1974
IV	3-4	Greenbaum and Sherman, 1962
B2	6	Lones and Singh, 1980

Dipeptidylpeptidase

C	7	McDonald <i>et al.</i> , 1969

the polypeptide chains. Exopeptidases act at the free amino or carboxyl termini of proteins or peptides and remove one or two amino acid residues sequentially. Because the action of endopeptidases would free more termini for attack by exopeptidases, the two types of enzymes are believed to act in concert in the lysosomal hydrolysis of proteins, as shown by Goettlich-Riemann et al. (1971) using hemoglobin as substrate. The endopeptidases generally have lower specific activities and are regarded as rate limiting in this process (Bohley et al., 1971). However, purified exopeptidases show little or no activity with protein substrates and are often not considered true proteinases.

The endopeptidases are further divided into four classes according to their mechanisms of hydrolysis: the aspartic, serine, cysteine, and metalloproteinases (Barrett, 1980). Peptide bond cleavage by proteolytic enzymes involves polarization of the bond by nucleophilic attack on the carbonyl carbon atom together with proton donation to the peptide nitrogen. Each class of endopeptidases is characterized by the amino acid residues that perform the nucleophile and proton donor functions (Neurath, 1984). In addition, some authorities recognize a fifth group which presently includes proteinases of unknown catalytic mechanism.

The aspartic proteinases were formerly called "acid" or "carboxyl" proteinases, since this class includes many

enzymes active at low pH. Two carboxyl groups of aspartic acid residues are responsible for the activity of these enzymes. Pepsin and renin are examples of aspartic proteinases, as are cathepsins D and E.

The serine proteinases make up the largest class of endopeptidases, numbering over 50 in mammals. Enzymes such as trypsin, chymotrypsin, thrombin, and cathepsins G and R all contain a reactive serine residue in their catalytic site. Serine proteinases are active only under neutral or slightly alkaline conditions.

Most of the cathepsins identified to date have been classified as cysteine or "thiol" endopeptidases (see Table II). These enzymes require the presence of sulfhydryl compounds (e.g., cysteine, mercaptoethylamine, or glutathione) for maximal activity, indicating the importance of the thiol group of a cysteine residue in the active site. Mammalian cathepsins B1 and H show a remarkable degree of sequence homology with papain, a plant cysteine endopeptidase, particularly in the region of the catalytic center (Takio *et al.*, 1983). Because of their relatively broad activity against peptide bonds, Barrett and Kirschke (1981) consider the lysosomal cysteine proteinases as a group to be the most active proteinases in higher animals.

None of the cathepsins is currently classified as a metalloproteinase, and only cathepsin F is considered a proteinase of unknown catalytic mechanism (Barrett, 1980).

Physiological and Pathological Functions

Cathepsins perform a number of important physiological functions, ranging from the complete degradation of proteins to enzyme activation by the hydrolysis of specific peptide bonds. In discussing the evolution of proteases, Neurath (1984) proposed that such enzymes evolved from relatively non-specific digestive enzymes and have adapted to more complex regulatory tasks. Thus, the fundamental role of cathepsins is thought to be the breakdown of nonfunctional proteins (Lebez and Kopitar, 1970), the other functions having arisen in more advanced cells.

Protein degradation by cathepsins occurs intracellularly. The substrates include both endogenous cell proteins and exogenous proteins, such as collagen, fibrin, and several globulins, brought into the cell by some type of endocytosis (Barrett, 1978). Within the cell, cathepsins are generally confined to the lysosomal system, known to be the site of extensive protein hydrolysis (Coffey and DeDuve, 1968; Bohley *et al.*, 1971). The wide range of pH optima among the cathepsins (pH 2 to 8, see Table II) suggests that the intralysosomal pH may change sequentially from acidic to neutral or slightly alkaline to acidic, and so on. Such changes in pH have been shown to facilitate protein degradation by cathepsin mixtures in vitro (Goettlich-Riemann *et al.*, 1971). The importance of these enzymes is demonstrated by their widespread distribution in

organs and cells having phagocytic or proteolytic activity. Cathepsins have been identified in preparations of liver, spleen, kidney, pancreas, lung, thymus, placenta, skeletal muscle, bone marrow, and lymph nodes from several mammalian species and have been further localized in macrophages, lymphocytes, and leukocytes.

Some of the cathepsins are also known to participate in enzyme activation by the limited hydrolysis of precursor molecules. For example, bovine spleen cathepsin B1 catalyzes the production of trypsin from trypsinogen at pH 3.6 (Greenbaum and Sherman, 1962). Cathepsin G from human neutrophil lysosomes has been shown to convert angiotensin I to angiotensin II and also to cleave angiotensin II directly from a plasma protein precursor, both in the absence of renin, as part of a local pathway to regulate blood flow (Wintroub *et al.*, 1984). Docherty *et al.* (1982) isolated an enzyme from the islets of Langerhans of rat pancreas that converts proinsulin to insulin at pH 5-6, and named it cathepsin P.

Recently, a serine endopeptidase has been found associated with ribosomes in both prokaryotes and eukaryotes. It has been named cathepsin R and is tightly bound to the 40S subunit in eukaryotes (Levyant *et al.*, 1979), mostly in a latent or "cryptic" state. Cathepsin R is believed to be responsible for the modification and

cleavage of newly synthesized proteins and the excision of chains with translational errors (Levyant et al., 1976).

Because the uncontrolled release of proteolytic enzymes would be extremely dangerous to cells and tissues, the production and activity of cellular proteinases are tightly regulated. Only the inactive, higher molecular weight precursors of cathepsins are normally found in plasma and other extracellular fluids (Zuehlisdorf et al., 1983). Activation of these precursors is regulated by limited proteolysis, and a variety of proteinase inhibitors exist in plasma to block or eliminate the active forms (Heimbürger, 1975).

As a consequence of some pathological conditions, however, this regulation of cathepsin activity may break down. Many serious diseases involve excessive protein degradation. The wasting of muscle tissue in muscular dystrophy is thought to be caused by an unexplained increase in cathepsin A and D content in dystrophic muscle (Iodice et al., 1966). Pulmonary emphysema is apparently the result of proteolysis of the lung by cathepsin G and elastase when the natural inhibitor of these enzymes is inactivated by smoke condensate (Nakajima et al., 1979). Cathepsins have also been linked to the production of a myocardial depressant factor (MDF) in circulatory shock, as described in the General Introduction.

The vast majority of cathepsin research has dealt with the characteristics and functions of these enzymes in mammals. Even so, there is evidence that cathepsins are present within lysosomes of all typical animal cells (Mellors, 1971). Cathepsin D, in particular, has been found in lysosomes of cells from protozoans to higher vertebrates (Barrett, 1970). Very little work has been done on cathepsins in amphibians, however. Weber (1957) was able to link an unspecified "cathepsin" to the resorption of the tail in metamorphosing Xenopus larvae. This is apparently the only aspect of cathepsin activity, physiological or pathological, to have been examined in amphibians.

Assay Techniques

Many different assays have been developed for the determination of cathepsin activity in animal tissues. In general, an extract of the tissue in question is allowed to digest some substrate solution and the products of the reaction (amino acids or short peptides) are measured spectrophotometrically. The extent of the digestion is thus proportional to the amount of cathepsin activity in the tissue. Each cathepsin is usually assayed using a substrate toward which it is particularly reactive. With the development of synthetic substrates, it has become possible to design an assay for a single cathepsin.

Plasma cathepsin D activity has been used previously to assess the role of lysosomal proteinases in circulatory shock. Curtis and Lefer (1980) measured cathepsin D activity in plasma of cats in hemorrhagic shock and were able to detect a ten-fold increase in activity over time. Cathepsin D activity has been shown to parallel myocardial depressant factor activity in subcellular fractions of incubated pancreas (Litvin et al., 1973). Cathepsin B1 may also be involved in the production of MDF. Litvin et al. (1973) found the optimal pH for MDF formation by pancreatic homogenates to be in the same range (pH 5-6) as the optimum for cathepsin B1 activity. In addition, the same researchers showed that MDF production could be significantly reduced by the addition of p-chloro-mercuribenzoate (PCMB) to the incubation medium. PCMB is a sulfhydryl reagent known to inhibit cathepsin B1 activity, but it is without effect against cathepsin D.

Cathepsin D is relatively easy to assay. Although many different procedures have been devised, most researchers use a modified version of the method of Anson (1936). The assay used in the present work is essentially that of Mycek (1970), using hemoglobin as substrate. Published procedures, however, require purified or partially purified enzyme preparations. Thus, it was necessary to adapt the standard assay for use with unpurified toad plasma.

Cathepsin B1 is usually assayed using a synthetic peptide substrate such as benzoyl-L-argininamide, but it may be assayed using hemoglobin if cysteine is added to the substrate solution (Mycek, 1970). Although not as specific for cathepsin B1, this method has the advantage of allowing direct comparison with cathepsin D activity on the same protein substrate.

The objectives of this chapter are: (1) to characterize the cathepsins present in shock plasma of toads by determining the relationship between plasma cathepsin activity and pH (with and without sulfhydryl activation), and (2) to develop a specific assay procedure for cathepsin activity in toad plasma.

MATERIALS AND METHODS

"Shock" Plasma

In order to develop an assay for cathepsin activity in plasma, it was necessary to obtain blood from toads in circulatory distress or shock. Since the splanchnic region appears to be the major site of lysosomal damage and cathepsin release in circulatory shock (Glenn and Lefer, 1971), restriction of the blood supply to this region was used to induce a "shock" state. Toads (Bufo marinus) were anesthetized by partial immersion in a solution of tricaine methanesulfonate (MS-222), and the body cavity was opened by

a lateral incision. Two ligatures were placed around the celiaco-mesenteric artery, thus blocking splanchnic blood flow, and the muscle and skin sutured closed. These toads were maintained with access to tap water for 1-3 weeks, at which point they were doubly pithed, and as much blood as possible was withdrawn from the exposed ventricle by heparinized syringe. The plasma was separated by centrifugation ($1000 \times g$) and kept refrigerated or frozen until used. Plasma so obtained was consistently high in proteolytic activity.

Hemoglobin Solution

A 2.5% (w/v) hemoglobin solution was prepared by slowly dissolving 2.5 g bovine hemoglobin substrate powder (Sigma, Type II) per 100 ml distilled water. To prevent bacterial growth, 2.5 ml Merthiolate was added (Mycek, 1970). The solution was kept refrigerated until needed. Any volume not used within two weeks was discarded, since longer storage causes an increase in control absorbance (Takahashi and Tang, 1981).

Chemical Determinations

The relationship between plasma cathepsin activity and pH was determined over a broad pH range, both in the presence and absence of cysteine, a sulfhydryl activator.

Sulfhydryl-independent activity was assayed with a substrate solution consisting of 4 ml of the 2.5% hemoglobin

solution, plus 2 ml of a McIlvaine citrate-phosphate buffer at the appropriate pH. This solution was allowed to equilibrate in a water bath at 37°C for 30 minutes. To initiate the reaction, 0.05 ml shock plasma was added to 0.3 ml of the substrate solution. The mixture was incubated at 37°C for 24 hours, at which point the reaction was terminated by the addition of 0.45 ml of a 5% trichloroacetic acid (TCA) solution. After standing for 10 minutes at room temperature, the undigested hemoglobin precipitated by TCA was separated by centrifugation (1000 x g), and the clear supernatant was removed and centrifuged again. The absorbance at 280 nm of this second supernatant was determined spectrophotometrically. Duplicates were carried out for each sample, and the average value compared to the average of two controls in which TCA was added to the same substrate solution prior to the addition of the plasma. The net absorbance at 280 nm (experimental minus control) is thus a measure of plasma cathepsin activity. The effect of pH on this activity was determined by changing the pH of the buffer and therefore the final pH of the substrate solution as measured at 37°C.

Total activity, including sulfhydryl-dependent activity, was assayed in the same manner, except that the substrate solution consisted of 4 ml of the 2.5% hemoglobin solution, 1 ml citrate-phosphate buffer, and 1 ml of 0.07 M

cysteine hydrochloride (adjusted to the pH of the buffer with NaOH).

Because the addition of cysteine to the assay appeared to promote proteolysis even in the controls, it was suspected that results obtained using cysteine might overestimate actual plasma cathepsin activity. To account for this "background" activity, the sulfhydryl assay was repeated, using tap water in place of the shock plasma. The difference between the net absorbance at 280 nm using plasma and that using tap water is the actual plasma-dependent activity.

To confirm this, and to confirm the existence of cathepsin B-type activity in shock plasma, a more direct comparison was made over the pH range 4.0-7.0. In this experiment, plasma was added to the substrate solution with cysteine, as before, while in the controls an equal volume of 0.9% NaCl was substituted for the plasma. No TCA was added to either set until the reaction was terminated 24 hours later.

To verify cathepsin D-type activity in shock plasma, both the tap water assay and the comparison of plasma and saline activities were then performed in the absence of cysteine over the pH range 3.0-6.0.

The sulfhydryl-free assay was used to assess the effects of time and volume of plasma used on the extent of the reaction. The relationship between the net absorbance

at 280 nm and the time of incubation at 37°C was examined at pH 3.0 in the absence of cysteine. The reaction was terminated after 6, 12, 18, 24, and 30 hours. Because this proved to be non-linear at the longer incubation times, the amounts of substrate solution and TCA used were doubled (to 0.6 ml and 0.9 ml, respectively) in all further experiments. The relationship between the net absorbance at 280 nm and the volume of plasma used in the assay was determined (at pH 3.0) for plasma samples from 0-0.5 ml, in 0.1 ml increments. Least squares linear regressions were performed on the data from these two experiments.

RESULTS

The pH dependence of toad plasma cathepsin activity without cysteine is shown in Figure 1. The optimum pH for hemoglobin degradation was in the range of 2.5-3.2. Activity decreased sharply at higher pH, and no activity at all was observed when the pH of the substrate solution exceeded 4.5. A final pH of 2.5 is the most acidic possible using this buffer.

There was a relatively small amount of "background" proteolytic activity, as shown by the results using tap water (Fig. 1), and this also disappeared above pH 4.5. If the difference between the plasma and tap water curves is plotted as in Figure 2, a slightly different pH relationship

Figure 1. The relationship between net absorbance at 280 nm and the final pH of the assay mixture without cysteine in experiments conducted with toad plasma (closed circles) and tap water (open circles).

Figure 2. The estimated difference in net absorbance at 280 nm between results using toad plasma and tap water in the cysteine-free assay (closed triangles) as compared to actual net absorbance at 280 nm in the plasma vs. saline assay (open triangles). The line was drawn for ease of comparison only.

is obtained. Maximum hydrolysis occurs below pH 3.0, and no activity is seen above pH 3.9. This same narrow pH range was apparent in the assay using saline as a control (Fig. 2).

The pH dependence of toad plasma cathepsin activity in the presence of cysteine is shown in Figure 3. Two peaks in net absorbance at 280 nm are apparent, one under acidic conditions and one nearer neutral pH. The former is very similar to the results seen without the sulfhydryl activator. The optimum pH for hemoglobin digestion was below 3.0, and activity decreased rapidly between pH 3.0 and 4.0. The second peak in plasma cathepsin activity with cysteine showed a pH optimum of 6.0 and was much broader than the first, even overlapping the more acidic peak at pH 4.0-4.5. Assays conducted above pH 7.0 gave erratic results.

When tap water was substituted for toad plasma in the sulfhydryl assay, the background proteolytic activity also appeared as two peaks (Fig. 3). The more acidic peak is virtually identical to the tap water curve seen without cysteine, except that the sulfhydryl activity was about 35% higher at its maximum (pH 3.5). The second peak in background activity is much larger, with an optimum pH of 6.5. Thus, most of the "plasma cathepsin activity" seen in this pH range is not plasma-dependent. However, if the difference between the plasma and tap water curves for

Figure 3. The relationship between net absorbance at 280 nm and the final pH of the assay mixture with cysteine in experiments conducted with toad plasma (closed circles) and tap water (open circles).

pH 4.0-7.0 is plotted as in Figure 4, some activity remains and a very different pH relationship is obtained. The optimum pH is in the range 5.0-5.4, with little or no activity above pH 6.0. The sulfhydryl assay using saline as a control gave similar results (Fig. 4).

Some limited comparisons were made using the same plasma in different assays. Plasma-dependent proteolytic activity near pH 3.0 was increased 10-20% by the addition of cysteine to the substrate solution (Table III). The relationship between activity with cysteine at pH 3.0 and pH 5.0 was too variable to draw any conclusions.

The relationship between net absorbance at 280nm and time of incubation at 37°C is shown in Figure 5, for assays conducted at pH 3.0 without cysteine. Activity increased linearly with reaction time to approximately 18 hours. Least squares linear regression using the data at 6, 12, and 18 hours produced a line ($r=0.99$) having a y-intercept (net absorbance at zero time) of 0.084. This agrees almost exactly with the result obtained when saline was substituted for plasma in the same assay at 24 hours (net absorbance=0.083), and therefore is a reasonable estimate of "zero activity."

Because the relationship between activity and reaction time was not linear at 24 hours, the volume of substrate solution used was doubled. With this adjustment, net absorbance at 280 nm was found to be positively correlated

Figure 4. The estimated difference in net absorbance at 280 nm between results using toad plasma and tap water in the sulfhydryl assay (closed triangles) as compared to actual net absorbance at 280 nm in the plasma vs. saline assay (open triangles). The line was drawn for ease of comparison only.

TABLE III
PLASMA-DEPENDENT CATHEPSIN ACTIVITY
WITH AND WITHOUT CYSTEINE
NEAR pH 3.0

pH	Net Absorbance at 280 nm		% Change
	Without Cysteine	With Cysteine	
2.8	.321	.382	+19%
2.7	.405	.452	+12%
2.6	.546	.608	+11%

Figure 5. Net absorbance at 280 nm as a function of time of incubation of the assay mixture at 37°C. The points represent the results of individual assays; the line was produced by least-squares linear regression using the data at 6, 12, and 18 hours.

with the volume of plasma used in the assay ($r=0.99$, Figure 6). The y-intercept of the regression line for this data is 0.113, representing zero plasma or "zero activity."

DISCUSSION

As stated previously, the assay used in this investigation is essentially that of Mycek (1970). Two modifications of this procedure were necessary. First, the McIlvaine citrate-phosphate buffer was used because of its wide range of buffering capacity (pH 2.2-8.0). This buffer has been used before in cathepsin assays (Taylor and Tappel, 1974; Shcherbak and Zhloba, 1979). Second, because no attempt was made to purify or concentrate the cathepsins in toad plasma, the time of incubation at 37°C was increased from 10 minutes to 24 hours. Increasing the reaction time is equivalent to increasing the amount of enzyme (Anson, 1936). Incubation times as long as 24 hours were common prior to Anson's work and have been used since in cathepsin assays (Goettlich-Riemann et al., 1971).

Certain cathepsins would not be detected by this assay and may be excluded from consideration. The exopeptidases do not readily digest protein substrates, and cathepsin F has been shown to be without activity against hemoglobin (Lebez et al., 1971). The serine proteinases, cathepsins G and R, have pH optima (7.0 and above) that are outside the

Figure 6. Net absorbance at 280 nm as a function of the volume of toad plasma used in the assay. The points represent the results of individual assays; the line was produced by least-squares linear regression.

pH range of the assay. Therefore, of all the known cathepsins, only the aspartic and cysteine endopeptidases (if present) should be detected by this procedure.

The comparison of the results obtained with this assay to those published in the literature is complicated by the fact that the pH optima of the various cathepsins are not absolute. The relationship between activity and pH for a given cathepsin may depend on the source of the enzyme, the nature of the substrate (Barrett, 1978), the buffer used in the assay (Otto, 1971), and even the incubation time (Woessner, 1971). Thus, published results for mammalian cathepsins, especially using substrates other than hemoglobin or buffers other than citrate-phosphate, would not be expected to agree precisely with the results of this investigation.

When toad plasma was assayed for cathepsin activity in the absence of cysteine, the sulfhydryl-dependent endopeptidases were presumably excluded from the reaction. Therefore, Figure 2 shows only sulfhydryl-independent activity corrected for the "background" activity seen in Figure 1. The pH relationship of this net activity is consistent with data in the literature for cathepsins D and E. Published values for the pH optimum of hemoglobin digestion by cathepsin D range from 2.8 to 4.0, with many reports of peak activity at or near pH 3.0 (Press *et al.*, 1960; Iodice *et al.*, 1966; Barrett, 1971). The decrease in

activity with increasing pH was much more rapid in the present study than is usually reported for cathepsin D, however. Cathepsin D typically retains some activity above pH 5.0, contrary to the results presented here. Cathepsin E hydrolyzes serum albumins more readily than hemoglobin (Lebez et al., 1971), but has been reported to digest hemoglobin optimally at pH 2.5-3.5 (Barrett, 1969).

The "background" activity seen at low pH may be the result of acid denaturation or degradation of the substrate. Hemoglobin begins to dissociate into subunits at pH 4.5 (Barrett, 1969) and may be further degraded at lower pH under the conditions of this assay. This degradation could be a direct result of the acidic conditions or the result of some other reaction facilitated by the dissociation of the hemoglobin molecule (Bohley et al., 1971).

Activity measured in the presence of cysteine should reflect total plasma cathepsin activity, including both sulfhydryl-dependent and sulfhydryl-independent enzymes. Accordingly, the more acidic peak observed in the sulfhydryl assay (Figs. 3 and 4) is very similar to the results obtained without cysteine and probably represents cathepsins D and E also. Cathepsin S, a cysteine endopeptidase with maximum activity against hemoglobin at pH 3.0 (Turnsek et al., 1975), may also be involved, although it is found in very small quantities in typical lysosomes (Takio et al., 1983). The addition of cysteine to the substrate solution

caused a slight increase in "background" proteolytic activity at low pH, either by facilitating the acid denaturation of hemoglobin or by introducing or promoting some other biological reaction (see below).

While no activity was observed above pH 4.5 in the sulfhydryl-free assay, a very large peak appeared with the addition of cysteine (Fig. 3). However, most of this "plasma cathepsin activity" is spurious, since it also appeared when shock plasma was omitted from the assay. The actual plasma-dependent cathepsin activity had an optimum pH in the range 5.0-5.4 (Fig. 4). Cathepsin B1 is one of the major thiol endopeptidases in lysosomes (Taylor and Tappel, 1974) and was probably the source of much of this activity. The pH optimum for hemoglobin hydrolysis by cathepsin B1 varies, depending on the source of the enzyme or conditions of the assay, from as low as pH 4.0-4.5 (Otto, 1971) to as high as pH 6.0 (Barrett, 1973). Cathepsin L (pH optimum 5.0-5.5) may also be involved. Cathepsin L has much greater activity than cathepsin B1 against most proteins (Kirschke *et al.*, 1977), but has relatively low activity against hemoglobin (Barrett and Kirschke, 1981). Cathepsin H varies between species in its proteolytic activity and thus may or may not have contributed in this instance (Barrett and Kirschke, 1981). Of the the remaining cysteine endopeptidases, cathepsin S is not active at this pH (Turnsek *et al.*, 1975), cathepsin N appears to hydrolyze

only collagen (Evans and Etherington, 1978), cathepsin M is more or less specific for aldolases (Pontremoli *et al.*, 1982), and cathepsins P and T are present only in small quantities in most lysosomes (Takio *et al.*, 1983). Cathepsins J and K were isolated only recently (Liao and Lenney, 1984) and have not been fully characterized.

The "background" activity observed above pH 4.5 was clearly related to the presence of cysteine. Some type of biological contamination was suspected, since the conditions of the assay (warm temperature, relatively mild pH, and long incubation time) seem ideal for the growth of bacteria or other microorganisms using the hemoglobin as medium. Because no such growth was evident when cysteine was not included in the substrate solution, it is likely that the cysteine itself was contaminated or that it was an essential nutrient of the microorganisms.

Using this information, two assays may be described for cathepsin activity in toad plasma. The first, for cathepsin D-type activity, would be performed at pH 3.0 in the absence of cysteine; the second, for cathepsin B1-type activity, would be performed with cysteine at a pH between 5.0 and 5.4.

Although there is some evidence that intralysosomal pH may drop as low as 3.0 (Rous, 1925; Goettlich-Riemann *et al.*, 1971), it is unlikely that this degree of acidity is widespread in the ischemic pancreas, even in severe shock.

Thus, the activity of cathepsin D must be extremely localized at best. Glenn and Lefer (1971) proposed that enzymes active at pH 5.5 (the intracellular pH of splanchnic tissues) would be far more important in the production of MDF by the ischemic pancreas. On this basis alone, it would seem that the assay for cathepsin B1-type activity would be a better measure of the shock state.

However, the assay for cathepsin D-type activity is simpler and suffers from fewer complications. In particular, the "background" proteolytic activity inherent in the D assay is far less than that associated with the B1 assay. Working at low pH and omitting cysteine reduces the likelihood of bacterial contamination.

Therefore, I chose to use cathepsin D-type activity as an indicator of overall plasma cathepsin activity and thus the degree of circulatory shock. This was apparently the assumption made by Curtis and Lefer (1980) in their study of hemorrhagic shock in cats. If pancreatic lysosomes are disrupted as a consequence of hypoxia, then all the enzymes therein will be released and any one may be used as an indicator of lysosomal damage. This assumption is valid so long as the lysosomes are homogeneous, containing the same cathepsins in like quantities. There is recent evidence, however, that this is not true. Crie *et al.* (1983) found that cardiac cathepsin D activity did not necessarily correlate with cathepsin B1 activity or with the rate of

protein degradation in rat hearts subjected to various treatments. Even so, cathepsin D activity has been shown to be a reliable indicator of MDF production (Litvin et al., 1973) and will be so used in this investigation.

The relationship between plasma cathepsin D-type activity and time of incubation at 37°C was linear for reaction times up to 18 hours (Fig. 5). The net absorbance at 280 nm for 18 hours of incubation was 0.800, or approximately 0.7 when corrected for the spurious activity at zero time. This latter value probably represents an upper limit to net absorbance over the linear range of this assay; i.e., any value for net absorbance at 280 nm that exceeds 0.7 must be regarded as suspect, since absorbance is no longer proportional to reaction time. Previous studies have found a more limited range of linearity (up to 0.3 net absorbance at 280 nm), but these assays were performed on purified enzyme preparations over much shorter incubation times (Barrett, 1970; Takahashi and Tang, 1981). This departure from linearity may be caused by the build-up of inhibitory products, by the self-digestion of the enzyme, or by depletion of the substrate.

In order to use a 24-hour incubation time, it was necessary to double the volume of substrate solution used, effectively halving the concentration of enzyme in the assay. Net absorbance at 280 nm after 24 hours was then positively correlated to the amount of toad plasma assayed

for volumes from 0-0.05 ml (Fig. 6). The y-intercept of the regression line for this data (0.113) is "background" activity not associated with the plasma. The relationship between activity and volume of plasma is quite linear up to a corrected net absorbance of 0.5. Since increasing the reaction time is equivalent to increasing the amount of enzyme (or plasma), this relationship should also be linear up to 0.7 net absorbance (see above).

The final form of the cathepsin assay to be used for toad plasma was determined by these results. It is based on the sulfhydryl-free assay described in the Materials and Methods section of this chapter. The hemoglobin substrate solution will have a final pH of 3.0 and will not contain cysteine. A volume of toad plasma between 0.01 ml and 0.04 ml will be used in each assay, the volume to be adjusted such that the net absorbance at 280 nm does not exceed 0.7. The assay mixture will be incubated at 37°C for 24 hours and analyzed as before. The net absorbance at 280 nm so obtained will be corrected for "background" activity by subtracting 0.113. In this way, assays using different volumes of toad plasma may be compared directly.

One limitation of this assay procedure is that it is relatively non-specific. Although the activity it measures is characteristic of cathepsin D, other proteolytic enzymes may also be involved. Hemoglobin is a suitable substrate for many proteinases, particularly when denatured by acidic

conditions over 24 hours (Bohley et al., 1971). But this assay has the virtues of simplicity and practicality, and did give repeatable results in preliminary work with toad plasma.

CHAPTER III

DEHYDRATION AND CIRCULATORY SHOCK IN BUFO MARINUS

INTRODUCTION

The physiological consequences of dehydration in the toad Bufo marinus have been described in detail by Shoemaker (1964). This study provides evidence that cardiovascular degeneration is the proximate cause of death in severely dehydrated toads. When water loss exceeds 20-25% in this species, both circulating plasma volume and arterial pressure fall off sharply, indicative of circulatory collapse.

The importance of prolonged hypovolemia in the cardiovascular debilitation leading to this collapse is unknown. In particular, it is not known whether Bufo marinus -- or indeed any anuran -- suffers from circulatory shock as a result of dehydration. If hypovolemia is directly responsible for the circulatory failure in dehydrational death in anurans, it should be possible to demonstrate the existence of a shock state in these animals, even if only in the terminal collapse of the circulation.

As discussed in the General Introduction, progressive circulatory shock is characterized by a number of positive-

feedback systems. Among these, the production of a myocardial depressant factor (MDF) by the ischemic pancreas has been the subject of many recent investigations (see Lefer, 1978). MDF has been found in the plasma of mammals suffering from many types of shock, including hemorrhagic, septic, cardiogenic, and splanchnic ischemic shock (Lefer, 1978). At present, plasma MDF activity must be determined with a complicated bioassay using isolated cat papillary muscles (Lefer *et al.*, 1967). However, certain cathepsins appear to be essential in the biosynthesis of MDF (Glenn and Lefer, 1971; Ferguson *et al.*, 1972; Lefer and Barenholz, 1972; Letfler *et al.*, 1973; Litvin *et al.*, 1973). In particular, cathepsin D has been shown to be a reliable indicator of MDF production (Litvin *et al.*, 1973; Curtis and Lefer, 1980).

In mammals, circulatory shock may also be induced by splanchnic artery occlusion (SAO), resulting in ischemia and hypoxia in the visceral organs, including the pancreas (Lefer and Barenholz, 1972). SAO shock has been shown to involve lysosomal disruption and increased plasma proteinase and MDF activity (Lefer and Barenholz, 1972; Leffler *et al.*, 1973; Curtis and Lefer, 1981). Occlusion is accomplished by ligating the celiac, superior mesenteric, and inferior mesenteric arteries (Curtis and Lefer, 1981). In anurans, the celiac and superior (anterior) mesenteric arteries are branches of the celiaco-mesenteric artery which arises from

the ventral aspect of the dorsal aorta immediately posterior to the junction of the two systemic arches and supplies blood to the abdominal viscera (Bohensky, 1982). The inferior (posterior) mesenteric artery arises independently from the dorsal aorta and supplies the rectum. SAO shock has never been attempted in anurans, but ligation of the celiaco-mesenteric artery should occlude splanchnic blood flow sufficiently to determine whether or not SAO shock can be induced in these animals.

I propose to use plasma cathepsin D activity as an indicator of circulatory shock. To my knowledge, this has never before been done in anurans. Two experimental groups will be examined: one group of toads will be dehydrated to produce a hypovolemic condition; the second group will be subjected to ligation to produce splanchnic ischemia. Plasma cathepsin activity in the former will be used to evaluate the degree of shock caused by dehydration. The extent of hypovolemia will be evaluated by changes in plasma sodium, potassium, and protein concentrations and hematocrit with dehydration. Ligation should also produce shock enzymes if the splanchnic hypothesis for cathepsin release applies to anurans.

MATERIALS AND METHODS

Animals

Bufo marinus were purchased from commercial suppliers. The toads were maintained on soil at room temperature with access to water in shallow pans but were not fed. No attempt was made to control photoperiod.

Cannulation and Blood Sampling

The ventral abdominal vein of each toad used in this experiment was cannulated occlusively to permit periodic blood sampling. Toads were anesthetized by partial immersion in a solution of tricaine methanesulfonate (MS-222). A small incision was made in the abdominal skin and muscle to expose the ventral abdominal vein. A short section (1-2 cm) of this vessel was isolated and the heat-flared end of a polyethylene cannula (PE-90) filled with heparinized saline (0.9% NaCl) was inserted caudally through a nick in the vessel wall. The cannula was secured in place with silk thread tied around the vein and the muscle and skin were sutured closed. The patency of the cannula was verified and 0.5 ml heparinized saline infused into the toad. The free end of the cannula was then sealed with a metal plug. Blood loss during this procedure was minimal.

Sampling was usually accomplished simply by removing the metal plug and allowing the toad's blood pressure to

force the saline out of the cannula. It was sometimes necessary to flush clotted blood from the cannula using a syringe of heparinized saline, then allow the saline to drain as before. The first few drops of blood were discarded, since they were diluted with saline. Blood was collected in 0.2 ml heparinized glass tubes which were then sealed at one end. A total of approximately one milliliter of blood was taken from each toad per sampling period. The cannula was then flushed with just enough heparinized saline to force all blood back into the animal and the metal plug was replaced. Hematocrit was determined following centrifugation at $1000 \times g$ for 15 minutes and is reported as the average value obtained for each sampling period. The plasma was separated and frozen for later analysis.

Dehydration

Toads selected for dehydration were allowed to recover from cannulation for 24 hours in individual chambers with access to tap water. At this point, an initial blood sample was taken as described above. The hydrated mass of each toad was determined (to the nearest 0.1 g) after it was blotted dry and its bladder drained by inserting a glass tube into the cloaca and applying pressure to the lower abdomen. The toads were dehydrated in individual plastic chambers covered by wire screens, and were kept at room temperature and weighed periodically. Blood samples were

taken at or near losses of 10% and 20% initial (hydrated) body mass, if possible. Frequently, blood flow through the cannula became very slow or stopped as dehydration proceeded and no blood could be obtained. A final sample was taken at the critical activity point (CAP; Hillman, 1978b). At CAP, the toad was doubly pithed, weighed, and blood withdrawn directly from the exposed ventricle by heparinized syringe. Several toads died before the CAP sample could be taken. A total of nine animals were used to obtain at least five blood samples at 10% loss, 20% loss, and CAP.

Ligation

In toads to be subjected to ligation, the initial blood sample was taken immediately after cannulation. While still under anesthesia, the body cavity was opened by a lateral incision. Two ligatures were tied around the celiaco-mesenteric artery to block splanchnic blood flow and the muscle and skin were sutured closed. The toads were weighed with bladders drained as before and placed in individual chambers with access to tap water. Blood samples were collected from these animals via the ventral cannula one day and four days after surgery. One week after ligation, each toad was doubly pithed and a large blood sample was withdrawn from the exposed ventricle by heparinized syringe. The lateral incision and internal

organs were examined post mortem and placement of the ligatures was verified.

Controls

Control toads were cannulated as described above but were otherwise untreated. Blood samples were taken immediately after cannulation and following one, four, and five days in individual chambers with access to tap water.

Plasma Analyses

Sodium and potassium concentrations were determined with an Instrumentation Laboratories Model 143 flame photometer. Plasma was diluted by adding 0.02 ml of the sample to 4.0 ml lithium diluent. The instrument was calibrated with standard solutions of sodium and potassium encompassing the range of concentrations in the samples. The calibration and reliability of the instrument were verified by frequent use of a standard solution during the course of analysis.

Total protein was determined by the Biuret method, using human serum protein (8.0 g/100ml, Sigma) as the standard. Each plasma sample was assayed in duplicate and the mean value reported for protein concentration.

Plasma cathepsin activity was assayed as described in Chapter II, using a volume of plasma between 0.01 ml and 0.04 ml in duplicate control and experimental assays. If the net absorbance at 280 nm was in excess of 0.7, the

results were discarded and the analysis repeated using a smaller volume of plasma. One unit of cathepsin activity (cathepsin unit, C.U.) is here defined arbitrarily as that amount of enzyme which produces a net absorbance at 280 nm of 0.01 over 24 hours. Specific activity is expressed as units of cathepsin activity per milligram total protein in plasma (C.U./mg). The precision of this assay was determined to be $\pm 4\%$ of net absorbance at 280 nm based on repeated assays of the same plasma sample.

Statistics

Data for plasma sodium, potassium, and protein concentrations and hematocrit were subjected to analysis of variance, using the Student-Newman-Keuls test to compare individual means within and between treatment groups. Relative plasma cathepsin activities were compared using the nonparametric Kruskal-Wallis test (Zar, 1984). A $P < 0.05$ was considered significant.

Calculations

Predicted plasma concentrations for dehydrating toads were calculated by the method of Shoemaker (1964). This method assumes an initial whole animal water content of 79.4%, that plasma is 100% water, and that the plasma volume loses an amount of water proportional to the loss from the whole animal.

RESULTS

Mean water loss at CAP for dehydrated Bufo marinus was 31.6% initial body mass (SE = $\pm 1.1\%$, N=6). The mean time between surgery and the initiation of dehydration (0% water loss) and the mean dehydration times required to reach 10% loss, 20% loss, and CAP are given in Table IV. This allows direct comparison of the results of dehydration and those from the control and ligation groups based on elapsed time after cannulation.

Plasma sodium concentration increases with dehydration (Table V, Figure 7). Mean plasma sodium concentration at each increment of dehydration is significantly greater than the previous value ($P < 0.05$). There is close agreement between predicted and actual sodium concentrations (Table V). There were no significant differences in the control and ligation groups.

Plasma potassium concentration was significantly elevated only in toads dehydrated to CAP ($P < 0.05$; Table VI), although there is also the suggestion of a slight post-operative increase.

Hematocrit increased with dehydration, but not as rapidly as predicted up to 20% water loss (Table VII). Hematocrit at CAP was significantly greater than at lower water deficits ($P < 0.05$) and slightly greater than predicted. There was a significant fall in hematocrit in ligated toads

TABLE IV
TIME REQUIRED TO REACH VARIOUS
STAGES OF DEHYDRATION

Water Loss	Days Post-Surgery mean (2 SE)	N
0%	1.03 (0.03)	9
10%	2.25 (0.33)	5
20%	3.55 (0.51)	5
CAP	4.90 (0.30)	6

TABLE V
PLASMA SODIUM CONCENTRATIONS*

Water Loss	Dehydration		Time	Control		Ligation
	Actual Na ⁺	Predicted Na ⁺		Na ⁺	Na ⁺	
0%	111 (4) ^a	--	post-op	105 (7)	111 (5)	
10%	127 (10)	127	+1 day	104 (8)	109 (4)	
20%	147 (9)	148	+2			
CAP	181 (8) ^b	184	+4	103 (4)	112 (6)	
			+5	106 (8)		
			+7		106 (7)	

* Expressed as mean (2 SE), units are mequiv./l.
N=5, except as noted: (a) N=9, (b) N=6.

Figure 7. Mean plasma sodium concentrations for all three treatment groups as related to elapsed time following cannulation. The lines represent $\pm$ two standard errors of the mean. Dehydration times are also mean values (see Table IV).

TABLE VI
PLASMA POTASSIUM CONCENTRATIONS*

Water Loss	Dehydration		Time	Control		Ligation
	Actual K ⁺	Predicted K ⁺		K ⁺	K ⁺	
0%	3.4 (0.3) ^a	--	post-op	2.3 (0.3)	2.2 (0.6)	
10%	3.2 (0.5)	3.9	+1 day	3.0 (0.6)	2.9 (0.6)	
20%	4.0 (0.5)	4.5	+2			
CAP	7.6 (2.7) ^b	5.6	+4	2.9 (0.6)	2.8 (0.3)	
			+5	3.2 (0.7)		
			+7		3.0 (0.5)	

* Expressed as mean (2 SE), units are mequiv./l.
N=5, except as noted: (a) N=9, (b) N=6.

TABLE VII
HEMATOCRIT*

Water Loss	Dehydration		Time	Control		Ligation	
	Actual Hct	Predicted Hct		Hct		Hct	
0%	33 (2) ^a	--	post-op	36 (5)		32 (5)	
10%	33 (1)	38	+1 day	26 (3)		19 (4)	
20%	38 (7)	44	+2				
CAP	57 (10)	55	+4	24 (3)		22 (5)	
			+5	25 (4)			
			+7			23 (4)	

* Expressed as mean (2 SE), units are %cells by volume.
N=5, except as noted: (a) N=9.

one day after surgery and in control toads four days after surgery ($P < 0.05$). Ligated toads also had a lower mean hematocrit than dehydrated toads on the first day after surgery ($P < 0.05$), but there was no statistical difference between ligated and control toads on the first day.

Plasma protein concentration rose with dehydration, as predicted, to 20% water loss (Table VIII). Mean protein concentration at 20% loss was significantly greater than for hydrated toads ($P < 0.05$). The mean plasma protein concentration at CAP was greater than the concentration at 20% water loss ($P < 0.05$), but is also greater than predicted. There were no significant differences in the control and ligation groups.

Initial post-operative or hydrated plasma cathepsin activities were extremely variable (range = 4.7 - 83.8 C.U./mg). Mean activities for the first sample from each treatment group are presented in Table IX. Analysis of variance indicated a significant difference between these means ($P < 0.05$), although the Student-Newman-Keuls test did not. The only other initial parameter found to differ significantly between groups was body mass, which showed a similar statistical difference (Table IX). Because of the variability in plasma cathepsin activity, it is expressed below only as relative activity.

There is no significant difference between the mean plasma cathepsin activities within or between any of the

TABLE VIII
PLASMA PROTEIN CONCENTRATIONS*

Water Loss	Dehydration		Time	Control		Ligation
	Actual Pr	Predicted Pr		Pr	Pr	
0%	3.6 (0.3) ^a	--	post-op	3.2 (0.4)	3.3 (0.5)	
10%	4.1 (0.3)	4.1	+1 day	3.1 (0.3)	3.1 (0.6)	
20%	4.1 (0.7)	4.8	+2			
CAP	6.7 (1.0) ^b	6.0	+4	3.0 (0.2)	3.1 (0.4)	
			+5	3.0 (0.2)		
			+7		3.0 (0.6)	

* Expressed as mean (2 SE), units are mg/100ml plasma.
N=5, except as noted: (a) N=9, (b) N=6.

TABLE IX
INITIAL PLASMA CATHEPSIN ACTIVITY
AND BODY MASS*

	Plasma Cathepsin Activity (C.U./mg)	Body Mass (g)	N
Dehydration (hydrated)	15.7 (9.3)	297.4 (24.4)	9
Control (post-op)	52.2 (25.3)	189.7 (38.9)	5
Ligation (post-op)	20.3 (13.0)	246.7 (87.2)	5

* Expressed as mean (2 SE).

three groups when activities are expressed relative to one day after surgery (the only sampling time common to all three groups; Table X). The changes in cathepsin activity over time in the control toads was quite variable, ranging from a slight decrease to a two-fold increase on the fourth day (Figure 8). All values for plasma cathepsin activity in dehydrated toads fell within the range of values for control animals. Two of the ligation toads showed the highest relative activity measured (see below), although the other three were within or below the control range.

If plasma cathepsin activity is expressed not as specific activity (C.U./mg plasma protein) but as absolute activity (C.U./ml plasma), the effects of dehydration can be better appreciated (Table XI). Absolute plasma cathepsin activity increases about as predicted to 20% water loss but rises dramatically at CAP ($P < 0.05$). Absolute plasma activity at CAP is twice as high as would be expected from simple concentration effects but is not significantly different from the 4-day control value.

The results of ligation on plasma cathepsin activity should really be related to activity immediately after cannulation and before ligation, as in Table XII. Once again there is no significant difference within or between groups. However, there is a tendency for cathepsin activity to peak on the fourth day and then decline in both groups (see also Figure 8). Relative to post-operative values, two

TABLE X

PLASMA CATHEPSIN ACTIVITY
RELATIVE TO 24 HOURS
POST-SURGERY *

Water Loss	Dehydration Relative C.A.	Time	Control Relative C.A.	Ligation Relative C.A.
		post-op	1.16 (0.41)	0.90 (0.47)
0%	(1)	+1 day	(1)	(1)
10%	0.94 (0.20)	+2		
20%	0.96 (0.49)	+4	1.71 (0.61)	1.75 (1.24)
CAP	1.66 (0.68) ^a	+5	1.43 (0.50)	
		+7		1.25 (0.66)

* Relative activity calculated for individual toads as:

$$\text{C.U./mg} \div \text{C.U./mg 24 hours post-op.}$$

Expressed as mean (2 SE). N=5, except as noted: (a) N=6.

Figure 8. Plasma cathepsin activity relative to 24 hours after cannulation. Lines connect results from the same individual. Single values for dehydrated toads are represented as unconnected triangles.

TABLE XI
ABSOLUTE PLASMA CATHEPSIN ACTIVITY
RELATIVE TO 24 HOURS
POST-SURGERY*

Water Loss	Dehydration		Control	
	Actual Rel. C.A.	Predicted Rel. C.A.	Time	Rel. C.A.
			post-op	1.20 (0.46)
0%	(1)	--	+1 day	(1)
10%	1.02 (0.17)	--	+2	
20%	1.23 (0.64)	1.19	+4	1.69 (0.62)
CAP	3.01 (1.17) ^a	1.48	+5	1.39 (0.54)
			+7	

*Relative activity calculated for individual toads as:

$$\text{C.U./ml} \div \text{C.U./ml 24 hours post-op.}$$

Predictions based on relative C.A. at 10% loss.
Expressed as mean (2 SE). N=5, except as noted: (a) N=6.

TABLE XII

PLASMA CATHEPSIN ACTIVITY
RELATIVE TO IMMEDIATELY
POST-SURGERY*

Time -----	Controls		Ligation	
	-----		-----	
	Rel. C.A.		Rel. C.A.	
	-----		-----	
post-op	(1)	(1)		
+1 day	0.94 (0.29)		1.42 (0.82)	
+2				
+4	1.58 (0.67)		2.12 (1.74)	
+5	1.35 (0.65)			
+7			1.52 (0.83)	

* Relative activity calculated for individual toads as:

$$\text{C.U./mg} \div \text{C.U./mg post-op.}$$

Expressed as mean (2 SE), N=5.

ligation toads showed very high levels of plasma cathepsin activity: one peaked on the first day at 2.66 times initial activity, the other peaked on the fourth day at 5.12 times initial activity.

Post mortem examination of the latter toad revealed a large gelatinous mass associated with the ventral abdominal vein cannula, probably indicating a serious infection. However, no such infection was found in the former. The ligated toad showing the lowest increase in plasma cathepsin activity had enlarged, blood engorged vessels in the splanchnic region. Except for these few observations, all ligated toads appeared relatively free of infection and internal abnormalities. The ligatures were in place and occlusion was verified in all animals.

DISCUSSION

The mean water loss at CAP reported here for Bufo marinus (31.6% initial body mass) is considerably lower than expected. Dehydration tolerance in this species has been estimated previously at 38% water loss using the same criterion of death (Hillman, personal communication). It is likely that stress or sepsis caused by the cannulation procedure or occlusion of the ventral abdominal vein contributed to the reduction in tolerance. If cardiovascular debilitation is the mechanism of

dehydrational death in anurans, then any factor which exacerbates that debilitation must reduce dehydration tolerance.

Plasma sodium concentration increased with dehydration exactly as predicted by the method of Shoemaker (1964). This calculation assumes that loss of water from any fluid compartment in a toad is proportional to the total evaporative water loss from the animal. The close agreement between actual and predicted sodium concentrations would appear to validate this assumption. However, there is other evidence suggesting that this is not so (see below). In any event, plasma sodium concentration is considered a reliable indicator of dehydration in toads (Shoemaker, 1964). Thus, no significant dehydration or hypovolemia occurred in either control or ligated toads.

Hematocrit was unaffected by 10% water loss and only slightly increased by 20% loss. Since red blood cell volume does not change with dehydration (Shoemaker, 1964), this suggests that plasma volume is maintained during dehydration at the expense of some other fluid compartment. Shoemaker (1964) reported that toads can maintain plasma volume to 20-25% water deficit, although this was after an initial 35% fall in plasma volume over the first 5% water loss. This initial decline was thought to result from increased capillary hydrostatic pressure and fluid filtration due to renal vasoconstriction. However, mean arterial pressure in

toads is unaffected by water deficits of 10% or more (Shoemaker, 1964). It seems highly unlikely that an animal which can maintain plasma volume until it has lost one-fourth of its body mass as water would suffer a 35% reduction in plasma volume when dehydrated by only 5%. There is evidence that this initial fall in plasma volume is an artifact of Shoemaker's dilution techniques (Hillman, personal communication). Long equilibration time in a hydrated toad appears to allow the marker to pass through leaky capillaries, leading to an over-estimate of plasma volume. Capillaries become less leaky with the onset of dehydration and the artifact disappears. Thus, plasma volume in toads may be completely maintained until the water deficit reaches 20-25%.

If plasma volume is maintained and yet the sodium concentration increases as expected, then the fluid taken up by the circulatory system must contain sodium. Similarly, since plasma protein concentration rises as predicted with dehydration to 20% water loss, the diluting fluid must also contain protein. Hydrated toads typically contain a large volume of extracellular fluid in the lymphatic system, coelomic cavity, and interstitial spaces which could act as a reservoir in dehydration (Shoemaker, 1964). Anuran extracellular fluids contain both sodium and protein (Deyrup, 1964) and could be easily transferred to the circulation via the lymphatic system. Extracellular fluid

volume in toads decreases with dehydration by an amount almost equal to the overall water deficit (Shoemaker, 1964). If plasma volume does not change, then fluid shifts must occur from the other extracellular compartments (lymphatic, coelomic, and interstitial fluids) into the plasma. This is consistent with observations on Rana pipiens indicating that up to 80% of lymphatic fluid is lost with severe dehydration (Smith and Jackson, 1931).

The relatively large increase in hematocrit and plasma protein concentration as toads approach CAP is probably due to the falling plasma volume that occurs at water deficits greater than 20-25% (Shoemaker, 1964). Increasing hematocrit and the resulting exponential increase in blood viscosity have been implicated as contributing factors in dehydrational death in anurans (Hillman, 1978a; 1978b; 1980). However, hematocrit would not be expected to have a significant impact on cardiovascular function until after the onset of circulatory collapse, when plasma volume begins to fall. Hyperviscosity may worsen the situation in toads but it cannot be the cause.

Plasma sodium concentrations continued to increase as predicted to CAP and therefore do not reflect the fall in plasma volume at this stage of dehydration. Sodium is removed from the extracellular space in dehydrating Xenopus (Hillman, 1978a) and in Bufo marinus muscle and liver tissue have been shown to take up sodium and potassium as a means

of retaining water (Shoemaker, 1964). Plasma sodium concentration near CAP would thus represent a dynamic relationship between hemoconcentration and tissue uptake and not a static indicator of plasma volume changes in toads as Shoemaker has proposed.

Changes in plasma potassium concentration with dehydration are complicated by the fact that lysis of red blood cells can lead to highly variable potassium levels (Hillman, 1978a). There is no evidence of lysis in the present study, however, except at CAP. The standard error of this mean is 4-5 times greater than any other, suggesting that lysis may have exaggerated the increase in plasma potassium with dehydration. That plasma potassium concentration does not rise as predicted is evidence for the removal of this ion from the extracellular space, as described previously for Bufo marinus (Shoemaker, 1964) and Ambystoma tigrinum (Alvarado, 1972).

Hematocrit was the only measured parameter to change significantly in control or ligated toads. It fell more rapidly in ligated than control animals which would indicate that stress may be partly responsible. Ligation is clearly not the sole cause, however. Toads isolated in individual chambers with access to water have a tendency to sit for long periods of time in the water, which provides the only available "cover." The decrease in hematocrit is probably just a consequence of passive water uptake. Plasma ionic

and protein concentrations are apparently adjusted with pre-existing stores of these components, but red blood cell production is not fast enough to maintain hematocrit. A similar decrease in hematocrit after surgery has been noted in the bullfrog Rana catesbeiana (Herman and Sandoval, 1983).

The extreme variability in initial plasma cathepsin activity was unexpected, since the first blood samples presumably represented the normal resting state of these animals. The differences in mean initial activity between groups can probably be explained by the fact that the toads in each group came from different shipments from the supplier. The treatment groups were examined consecutively, not concurrently, since no significant difference was expected among initial samples. High levels of plasma cathepsin activity could potentially result from any number of stressful conditions, including infection (see below), injury, or fasting. Animals of low body mass appear to be particularly susceptible (see Table IX).

Overall, relative plasma cathepsin activity increased in 10 of 19 toads studied and remained unchanged or fell in the other nine. Relative activity increased in 3 of 5 control toads, suggesting a stimulus for cathepsin release other than dehydration or ligation. Circulatory problems associated with occlusive cannulation are probably not responsible since this should have affected all animals more

or less equally. However, the degree of infection following surgery could vary considerably. The cathepsin/MDF pathway has been linked previously to septic shock in mammals (Lefer, 1978). Varying levels of post-operative infection and septic shock is the most straightforward explanation for different plasma cathepsin activities in control toads and in dehydrated and ligated toads as well.

There was no measurable effect of dehydration on relative plasma cathepsin activity over and above controls. Activity increased only near CAP and then only in 4 of 9 toads. This is entirely reasonable since it appears that no hypovolemia occurs in toads until the level of dehydration approaches CAP (see above). Even at CAP, however, plasma cathepsin activity is not significantly elevated above controls. The effects of hemoconcentration on relative activity are largely negated since it is specific cathepsin activity (C.U./mg) and is expressed relative to a plasma protein concentration which is also increasing.

Three of the five ligated toads showed increases in relative plasma cathepsin activity but only two were in excess of the control range. It is possible that these two toads suffered from particularly severe infections (one gave this appearance post mortem) but it is also possible that the effectiveness of splanchnic artery occlusion varied in this group. Flow through the celiaco-mesenteric artery was substantially if not totally blocked by the two ligatures,

but plastic casts of the arterial system of Bufo marinus indicate the possibility of collateral circulation to the splanchnic region (Hillman, personal communication). The variation in plasma cathepsin activity could thus be due to differential utilization of collateral vessels. In particular, the inferior mesenteric artery to the rectum was not ligated in the present study, although it is ligated to produce SAO shock in mammals (Curtis and Lefer, 1981).

In any event, Bufo marinus proved to be remarkably resistant to SAO shock. This is a highly lethal form of shock in mammals (Lefer and Barenholz, 1972). In one experiment, occlusion of the three splanchnic arteries for only 2.25 hours caused the death of 6 out of 7 cats (Curtis and Lefer, 1981). The ligatures are usually released after some time to allow blood flow to flush the products of splanchnic ischemia into the circulation. This was not possible in toads. Nevertheless, these toads were subjected to substantial or complete splanchnic ischemia for 7 days and yet survived with no obvious ill effects. Since SAO shock is intended to mimic the splanchnic ischemia present in hypovolemic shock, the conclusion that toads do not suffer from either form of shock is consistent.

These experiments have shown no evidence of hypovolemic shock with dehydration in Bufo marinus. Hypovolemia does not occur in toads until water loss exceeds 20-25% hydrated body mass. It would be interesting to

repeat this work on a species such as Xenopus laevis in which plasma volume is not maintained with dehydration (Hillman, 1978a).

GENERAL DISCUSSION

The results of this study suggest that hypovolemia is relatively unimportant in dehydrational death in anuran amphibians. In Xenopus laevis, a species which experiences hypovolemia from the onset of dehydration (Hillman, 1978a), interference with the physiological responses to falling plasma volume had little if any effect on dehydration tolerance. In Bufo marinus, a species which appears to maintain plasma volume until 20-25% water loss, there is no evidence of hypovolemic shock, even at the point of death. Even so, any conclusions must be constrained by the limitations of these experiments.

Adrenergic blockade in Xenopus produced no significant reduction in dehydration tolerance but, for reasons discussed in Chapter I, this measure may be insensitive to the effects of the treatments. This experiment was conducted on confined frogs and gave no indication whether or not adrenergic blockade significantly reduced aerobic scope with dehydration. Xenopus responds to dehydration by increasing activity, thought to have adaptive significance in escape from desiccating conditions (Putnam and Hillman, 1977). Such activity might be impossible if blood pressure could not be maintained by sympathetic compensation, and

"ability to survive dehydration" may be reduced even if "dehydration tolerance" is not.

There was no detectable increase in plasma cathepsin activity due to dehydration in Bufo marinus. It is possible that the splanchnic hypothesis for cathepsin release does not apply to anurans, in that splanchnic hypoperfusion does not result in lysosomal disruption and only infection causes an increase in plasma cathepsin activity. However, hypovolemia does not appear to develop in toads until the water deficit reaches 20-25% and splanchnic hypoxia may not occur until death is imminent. In any event, plasma cathepsin activity is but one measure of the status of the circulation and may not adequately reflect the consequences of dehydration and hypovolemia in toads.

Regardless of the limitations of these techniques, the results presented here argue against the hypothesis that hypovolemia is a critical factor in dehydrational death in anurans. If hypovolemia were the proximate cause of death in dehydration, one would expect much stronger evidence of the value of sympathetic reflexes or the presence of shock factors. The absence of any convincing evidence for the importance of hypovolemia offers indirect support for the importance of hyperosmolality and increased viscosity in dehydrational death in anurans.

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