THE NATRIURETIC ACTION OF DT-327

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ABSTRACT

THE NATRIURETIC ACTION OF DT-327
(N-[2', 6' - dimethyl piperidyl - (1')]
3 - sulfamyl - 4 - chlorobenzamide)

by Bernard Terry

DT-327 is a new natriuretic-diuretic agent whose chemical structure is related to the benzothiadiazines and furosemide. The nature of the chemical structure of DT-327 was the stimulus to compare its natriuretic action with that of furosemide and the thiazides. The natriuretic activity of this drug was studied following intravenous administration in the anesthetized dog.

DT-327 produced a prompt increase in urine flow and excretion of sodium, potassium and chloride following intravenous administration with no significant alteration in inulin or PAH clearance. Marked diuresis continued for at least 90 minutes following a single intravenous injection of 1 mg/Kg. When increasing doses of DT-327 were administered during mannitol diuresis, the natriuretic response observed was dose-related between 0.01 and 1 mg/Kg.

Infusion of DT-327 into the left renal artery at 0.05 ug/Kg/min. resulted in a significant ipsilateral increase in the excretion of sodium within 5 minutes without affecting the rate of glomerular filtration. A slight but delayed contralateral response was also noted, occurring 10-15 minutes after the ipsilateral response. Systemic administration of probenecid inhibited both the ipsilateral

and contralateral responses. The data demonstrated that DT-327-induced natriuresis was the result of a direct action on the kidney. The inhibition of this response by probenecid indicated that either cellular and/or luminal concentration, rather than plasma concentration, was a prerequisite to the natriuretic activity of this compound.

At a dose capable of producing a significant natriuresis, DT-327 did not alter renal blood flow and by implication did not affect renal vascular resistance. This is in contrast to furosemide which has been shown to decrease resistance and subsequently increase renal blood flow.

Alterations of plasma pH did not appear to affect the natriuretic response to DT-327 in the dog. The small increase in bicarbonate excretion produced by DT-327 in normal animals was enhanced during metabolic alkalosis, thus indicating some carbonic anhydrase inhibition by the drug. There appeared to be no alteration in the chloruretic response following DT-327 administration in rats or dogs pretreated with saline or bicarbonate. This weak carbonic anhydrase inhibitory activity observed with DT-327 appeared to be similar to that observed by others with the more lipid soluble thiazide derivatives.

DT-327, like the thiazides, decreased free water clearance without altering negative free water clearance indicating an action on the distal nephron. This is in contrast to furosemide which has been reported to decrease

negative free water clearance. In stop-flow experiments, DT-327 produced a significant increase in the distal sodium minimum, thereby confirming a distal locus of action.

During maximal natriuresis produced by hydrochlorothiazide, DT-327 had no effect on sodium excretion. Conversely, following a maximally effective dose of DT-327, hydrochlorothiazide was without effect. However, these animals were still capable of responding to furosemide. Thus, it appeared that DT-327, although chemically not a thiazide, could produce a relatively specific blockade of the action of a thiazide diuretic. It is concluded that the natriuretic action of DT-327 is not unique but closely resembles the thiazides.

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by

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INTRODUCTION

It has been about fifty years since Vogl observed the diuretic effect of a new organic mercurial compound, novasurol, in a patient who had received it as an anti-syphilitic agent (Vogl, 1950). Since that fortunate incident, the development of diuretic therapy has proceeded rapidly. Today, there are a large number of drugs available whose administration results in the increased excretion of water by the kidneys. It has been traditional to define a diuretic as a drug that increases the flow of urine. In current pharmacological thinking however, the primary purpose of a diuretic is to rid the body of electrolytes, primarily sodium. Increased electrolyte excretion reduces extracellular fluid volume and results in the elimination of edema. Thus, the most effective agents in the removal of fluid from the body would be those drugs that increase the excretion of sodium. Such drugs are referred to as natriuretic agents. The term saluretic often appears in the literature referring to the action of furosemide or that of the thiazides. Saluresis refers to the excretion of sodium chloride. Kaluretic and chloruretic agents refer to drugs that increase the excretion of potassium and chloride respectively. Thus, as in other disciplines, advancement in renal pharmacology has brought with it a more precise nomenclature to explain certain phenomena.

A new experimental natriuretic agent, DT-327 (N-[2', 6' - dimethyl piperidyl - (1')] 3 - sulfamyl - 4 - chlorobenzamide) was shown to have a chemical structure similar to other natriuretics but different enough to warrant more specific studies. Investigations were carried out in an attempt to more carefully define the natriuretic activity of this compound. This report describes these investigations and the results obtained.

To provide background information concerning this study, a review of the available information concerning other natriuretic-diuretic agents is in order. The following pages serve as a brief review of the literature pertaining to the major classes of diuretics. Much of the information presented and techniques described were utilized as background information for this investigation.

Inorganic mercury compounds (e.g., calomel) were utilized as diuretics as early as the 16th century in the treatment of "dropsy." It was not until Vogl's demonstration in 1919 of the diuretic action of the antisyphilitic organomercurial, novasurol, that organic mercurials found their place in clinical medicine (Vogl, 1950).

Following Vogl's discovery many attempts were made to elucidate the nature of the mercurial induced diuretic activity. The classical renal transplant experiments of Govaerts (1928) were responsible for determining that the target organ for organic mercurials is the kidney. He administered an organic mercurial to a dog and during

maximal diuresis removed the kidney and placed it in the neck of another dog which had received no drug. The transplanted kidney continued to produce large amounts of urine. No diuresis occurred, however, from a kidney which was transplanted from a dog which did not receive drug treatment into the neck of a dog which had been pretreated with an organic mercurial. Therefore, he concluded the target organ of the organic mercurials was the kidney.

Govaerts' findings were later confirmed by Bartram (1932). By injecting mercurial diuretics directly into the renal circulation of one kidney he established the renal action of the mercurial. With low doses he obtained only an ipsilateral diuretic response. Higher doses produced a response from the contralateral kidney. It was concluded that mercury in low doses was removed from the blood and bound in the kidney where it exerted its effect. When the dose of the drug was increased the capacity of the kidney to bind the drug was at a maximum. The excess drug reached the systemic circulation and exerted an effect on the contralateral kidney. It was also noted that the contralateral kidney was unable to produce urine at these high doses. This was due to the fact that at high doses, mercury is nephrotoxic.

Pitts (1958) conducted an experiment similar to that of Bartram. He injected Hg^{203} labeled chlormerodrin into one renal artery and observed a diuretic effect first on the ipsilateral kidney and later an increased flow from the contralateral kidney.

Once it had been established that the organic mercurials acted directly on the kidney, much work was done in trying to elucidate the site and mode of action. The literature is extensive in this area but even at present relatively little is known regarding the mechanism of the natriuretic activity of these compounds. It was demonstrated that a mercurial diuresis was not dependent upon changes in filtration rate or renal plasma flow but was the result of inhibiting the reabsorption of certain ions (Blumgart et al., 1935; Farnsworth, 1946). Some investigators believed that the saluresis produced by mercury was due to a decreased distal reabsorption of chloride (Farnsworth, 1946; Pitts, 1958). Mudge and Weiner (1958) demonstrated, in salt retaining patients, that the increase in chloride excretion often exceeds that of the increase in sodium excretion and that a hypochloremic alkalosis results with prolonged mercury therapy. This was interpreted as an action of the organic mercurial on the reabsorption of the chloride ion. However, micropuncture studies conducted by Giebisch (1958) have shown that the intratubular potential produced by active sodium transport is diminished by a mercurial. Such findings indicate that mercury inhibits active sodium reabsorption and the increased chloride excretion is merely secondary to the sodium effect. Giebisch's findings tend to substantiate a theory set forth by Berliner (1958). Berliner stated that mercury inhibits sodium transport

proximally. The excess sodium chloride delivered to the distal area would be handled in the following fashion. Some of the excess sodium would be exchanged with potassium or hydrogen through a distal exchange mechanism. The chloride ion, on the other hand, would remain in the lumen, thus explaining the greater increase in chloride ion excretion over that of sodium ion excretion. With prolonged administration, the use of mercury can lead to a hypochloremic alkalosis (Axelrod and Pitts, 1952). It has also been reported that acidosis potentiates the action of mercurials (Ethridge et al., 1936) and alkalosis renders the mercurials ineffective. Thus, the mercurials are self-limiting as they produce metabolic alkalosis with prolonged use.

With the advent of more sophisticated techniques others have attempted to explain the site of action of the organic mercurials. The literature contains much conflicting data. Vander et al. (1958) and White and Rolf (1963) have stop-flow data that indicates a proximal site of action. Recently, however, Orloff (1966) has pointed out some of the pitfalls in the interpretation of stop-flow results especially if the diuretic produced a pattern that would indicate a proximal action.

Other techniques have been utilized to determine site of action of diuretics. One such method is that of the free water clearance technique. The concept of free water clearance is best explained in terms of the functional

anatomy of the kidney and has been reviewed by Gottschalk (1964). In the proximal portion of the nephron, reabsorption of solutes is isosmotic with respect to plasma. Thus, the concentration of fluid passing through the proximal nephron does not change and is isosmotic with plasma when it reaches the loop of Henle. The cells lining the descending limb of the loop of Henle are freely permeable to both solutes and water. However, in the ascending limb, the cells of the tubule are impermeable to water. Sodium is actively transported out of the ascending limb resulting in the delivery of a hyposmotic fluid into the distal portion of the nephron. In the distal areas, certain distinct processes can occur (reabsorption, sodium-potassium exchange, hydrogen ion secretion, etc.) resulting in a decreased concentration of total solutes but with relatively little change in volume occurring until the fluid enters the collecting duct. In the presence of antidiuretic hormone (ADH), water in the collecting duct diffuses back into the medullary interstitium due to the high sodium content (resulting from sodium transport out of the ascending limb of the loop of Henle) producing a concentrated or hyperosmotic urine. Without antidiuretic hormone, the collecting duct is relatively impermeable to water resulting in the excretion of a dilute or hyposmotic urine. Thus dilution or concentration of the urine is determined by events occurring within the loop of Henle and in the collecting duct.

Free water clearance may be defined as osmotically unobligated water or the amount of water excreted in excess of the volume necessary to maintain the solutes of the urine isosmotic with respect to plasma. Free water clearance is expressed in ml/min. and is obtained by subtracting the osmolar clearance (the number of milliliters of plasma cleared of osmotically active solutes per minute) from the volume (ml/min.) of urine excreted.

Agents that act as a single anatomical site in the nephron can be expected to alter free water clearance in a predictable manner. Once a maximum water diuresis is obtained by the infusion of hyposmotic saline or dextrose, the drug in question is administered and the free water clearance is determined and compared to that obtained under control conditions. One other point should be emphasized. Normally, the two major sites of water reabsorption within the nephron are in the proximal area and the collecting duct. By creating a water diuresis, it can be assumed that the amount of ADH acting at the collecting duct is negligible. Thus, there is only one major site of water reabsorption, that being the proximal portion of the nephron where reabsorption is isosmotic with respect to plasma. Now, what is the effect of natriuretic agents that act at different sites along the nephron on free water clearance? If a drug were to act solely on the proximal convoluted tubule to prevent the reabsorption of sodium, one would observe an increased delivery of filtrate to the

loop of Henle. Some of this increased filtrate would be reabsorbed in the distal portions of the nephron but the excess water that accompanied this increased solute out of the proximal nephron would be excreted since water reabsorption in the collecting duct is negligible (no ADH). Thus, one would observe an increase in free water clearance following such a drug. In contrast, drugs that act solely in the distal tubule to inhibit sodium reabsorption would tend to increase the amount of solute excreted in the urine and thus decrease free water clearance. If a drug were to act solely on the ascending limb of the loop of Henle to inhibit sodium reabsorption, one would expect to see an increase in electrolyte excretion and therefore a decrease in free water clearance. It should be emphasized that the free water clearance technique is only able to differentiate agents that act proximally as compared to distally. With this technique one cannot differentiate between an effect on the distal nephron and the ascending loop of Henle. Thus, if a drug were to decrease free water clearance, it then would be necessary to perform a negative free water clearance study.

Negative free water clearance experiments have also been utilized to elucidate the site of action of diuretics. During hydropenia, there is maximum ADH activity, and the urine is more concentrated than the plasma. The amount of water needed to render the urine isosmotic with respect to plasma is referred to as the

negative free water clearance. It actually is a reflection of water reabsorption out of the collecting duct. Negative free water clearance is expressed in ml/min. and is obtained by subtracting the urinary volume (ml/min.) from the osmolar clearance (ml/min.).

During hydropenia, one measures the negative free water clearance before and after drug administration to see if a significant change has occurred. As noted before, this technique is primarily used to differentiate between a site of action on the ascending limb of the loop of Henle and the distal nephron. The elaboration of negative free water is dependent on the reabsorption of sodium in the medullary portion of the ascending limb. To test the effects of diuretics on negative free water clearance, a normal relationship between negative free water and osmolar clearance is established. Negative free water clearance increases with increasing osmolar clearance under control conditions. Since the rate at which solute free water is removed from the collecting duct must be dependent to a major extent on the rate at which sodium is transported from the ascending limb of the loop of Henle into the medullary interstitium, it follows that impairment of solute transport in this segment diminishes the ability of the urine to become concentrated. Thus, negative free water clearance would decrease. Seldin (1966) has reported such a decrease in negative free water clearance follows furosemide and ethacrynic acid, agents

that are believed to act on the ascending limb. If, on the other hand, an agent acted distally to prevent reabsorption of sodium, no change in negative free water clearance would be observed compared to that of control. Since there is no alteration of the concentration within the medullary interstitium, the urine is still able to become concentrated and the effect observed on negative free water clearance is merely that which occurs when the osmolar clearance increases.

Results from free water clearance studies on the organomercurials had to be reinterpreted when it was realized that theophylline which is found in combination with many organic mercurial preparations was, in part, the reason for the increased free water clearance observed (Farah and Miller, 1962). It has been shown that theophylline is a proximally acting diuretic (Walker et al., 1937) and this could explain the increase in free water clearance that was observed. Miller and Riggs (1961) using organomercurials without theophylline found no change in free water clearance. This would tend to indicate a site of action on the loop of Henle or the distal tubule in addition to an effect on the proximal tubule. Utilizing negative free water clearance technique Lambie and Robson (1960) found that under conditions of acidosis the mercurials could decrease negative free water thus acting to reduce the concentrating capacity of the kidney. This points to a site of

action on the ascending limb of the loop of Henle. It now appears that, under the proper conditions, the organic mercurials might act along the entire tubule (Seldin et al., 1966). This latter theory would help explain much of the conflicting data in the literature.

Carbonic Anhydrase Inhibitors

The development of carbonic anhydrase inhibitors was an outgrowth of investigations with sulfanilamide. Pitts et al. (1949) traced the metabolic acidosis produced by sulfanilamide to an inhibition of the acidification of the urine. This observation combined with the knowledge that this compound was an inhibitor of carbonic anhydrase led to the idea that other inhibitors of carbonic anhydrase might be effective diuretics.

According to the most generally accepted concept, bicarbonate reabsorption is mediated through an exchange of intracellulary produced hydrogen ions for reabsorbed sodium ions. Carbonic anhydrase catalyzes the intracellular reaction of carbon dioxide and water to form carbonic acid which breaks down into hydrogen ions and bicarbonate ions. An adequate supply of hydrogen ions is thus generated to take part in this exchange process. The hydrogen ion is secreted into the lumen and can react with filtered bicarbonate to form water and carbon dioxide. The bicarbonate formed intracellulary from the breakdown of carbonic acid diffuses back into the blood stream (Pitts, 1963).

The importance of the enzyme carbonic anhydrase in the process of bicarbonate reabsorption has been established in the rat, dog and man by the use of carbonic anhydrase inhibitors. Berlinger (1952) has shown in the dog as much as 50% of the filtered bicarbonate can be excreted in the urine after carbonic anhydrase inhibition by acetazolamide. Since the major part of the filtered bicarbonate is reabsorbed in the proximal tubule (Clapp et al., 1963) it seems reasonable that inhibition of carbonic anhydrase would result in a decreased bicarbonate reabsorption by the proximal tubule.

Unlike the mercurials, which result in a hypochloremic alkalosis, the carbonic anhydrase inhibitors lead to a hyperchloremic acidosis due to the enchanced bicarbonate excretion (Berliner et al., 1951). Like the mercurials, the carbonic anhydrase inhibitors are self-limiting. They result in the production of a metabolic acidosis which decreases the amount of bicarbonate filtered thus reducing the effectiveness of these agents as diuretics (Pitts et al., 1949). The use of carbonic anhydrase inhibitors in intermittent therapy will probably potentiate the action of the mercurials due to the production of metabolic acidosis.

The Thiazides

The thiazides were discovered during the search for improved diuretics similar in structure to the sulfonamide, acetazolamide. Novello and Sprague (1957) synthesized chlorothiazide and Beyer (1958) reported on the diuretic

activity of this compound. Since then chlorothiazide has become the prototype of a series of heterocyclic sulfonamides possessing diuretic properties. The discovery of chlorothiazide was the result of an attempt to increase carbonic anhydrase activity by the addition of two functional sulfonamide groups. However, it was noted by Beyer (1958) that this benzothiadiazine compound increased chloride excretion, an action not attributed to former carbonic anhydrase inhibitors. Newer compounds chemically related to chlorothiazide have demonstrated a significant chloruretic activity but reduced effectiveness as carbonic anhydrase inhibitors. Longemann et al. (1959) reported that benzothiadiazine compounds substituted on the sulfonamide nitrogen were completely devoid of carbonic anhydrase inhibitory activity yet still retained the capacity to produce a significant chloruresis as well as a saluresis. It seems clear that this increase in sodium and chloride excretion is not entirely due to the carbonic anhydrase inhibitory activity of these compounds.

In fairly large doses, chlorothiazide has been shown to increase the excretion of sodium, chloride, potassium, and bicarbonate (Beyer, 1958). A maximally effective dose of 10 mg/Kg affects approximately 10% of filtered sodium and chloride (Pitts et al., 1958). The new derivatives are more potent on a milligram basis but they do not increase the maximum response observed with chlorothiazide (Beyer and Baer, 1961).

Beyer (1958) also noted that alterations in plasma pH did not affect the natriuretic response to chlorothiazide. This was indeed a distinct advantage over the mercurials and carbonic anhydrase inhibitors. Beyer also observed that during conditions of acidosis, the excretion of chloride was greatly enhanced compared to that of bicarbonate, whereas during conditions of alkalosis, bicarbonate was more affected. One might conclude from the above observations that carbonic anhydrase inhibition occurs when the bicarbonate levels in the plasma are elevated, but this in itself is not a primary requisite for the establishment of a natriuresis.

The thiazides, as a group, are rapidly absorbed from the gastrointestinal tract and excreted in the urine by a process involving active tubular secretion (Beyer, 1958). Chlorothiazide is secreted by a renal mechanism similar to the one used by paraaminohippurate, (PAH), phenol red and other organic acids. Beyer (1958) was able to block the diuretic action of a minimal effective dose of chlorothiazide by pretreatment with probenecid. Chlorothiazide can also inhibit the accumulation of PAH in renal slices of the rabbit (Essig, 1961). Thus, it would appear that an active secretory process is responsible, in part, for the entrance of the drugs into the renal cells.

Kessler and his associates (1959) and Vander et al. (1958), using the stop-flow method in dogs, concluded that

the major effect of chlorothiazide was on the proximal tubule. However, Earley (1961) and others utilizing free water clearance have reported no alteration in negative free water clearance following thiazide administration. These results would indicate a distal action of the drug. Orloff (1966) pointed out certain problems of interpretation of a stop-flow pattern especially if the results indicate that the diuretic is acting on the proximal tubule. Inasmuch as the proximal samples move past the distal areas of the nephron prior to collection, small alterations in distal function could be reflected in proximal samples. A second difficulty has to do with continuing filtration during the period of stasis. Filtration cannot cease entirely during the period of obstruction since if fluid is reabsorbed to any extent, it should be replaced by new filtrate. A third difficulty is known as the so-called "smearing" of the concentration profile since tubules of different length contribute to specific samples. Any given sample of urine will contain fluid from more than one nephron area. The first samples may be an admixture of urine from the pelvis and collecting ducts, the next from the collecting ducts and distal tubules and so on. The technique of stop-flow is certainly not without serious fault and its use in the localization of the diuretic action of a specific agent leaves much to be desired. Thus, considering the disadvantages in the stop-flow technique and also the data obtained utilizing free water and negative free water clearances, it would appear that

the major site of action of the thiazides is the distal tubule.

In an attempt to distinguish the diuretic action of the thiazides from the mercurials Pitts et al. (1958) performed an additive study. It was observed that chlormerodrin was capable of producing its natriuretic response during maximal natriuresis produced by chlorothiazide. Conversely, chlorothiazide produced its effect during maximal mercurial natriuresis. Since either agent was capable of eliciting an additive response during maximal natriuresis produced by the other, it was reasoned that they must be acting by different mechanisms or at different sites along the nephron. Pitts et al. also observed an additive response between chlorothiazide and acetazolamide, thus again indicating that the mechanism of action of the thiazides is not entirely due to its carbonic anhydrase inhibitory activity.

Ethacrynic Acid

In 1962 Schultz et al. (1962) reported a class of diuretic compounds, the α, β, unsaturated ketone derivatives of aryloxyacetic acids. One of the compounds, ethacrynic acid, has been the subject of extensive experimentation and clinical evaluation. Chemically, it is quite different than previously know diuretics. Baer et al. (1964) and Beyer (1965) have described the saluretic activity of this unique compound. Like the organomercurials, ethacrynic acid results in an enhanced

excretion of sodium and chloride. Bicarbonate excretion is unchanged or may actually decrease in contrast to the carbonic anhydrase inhibitors and some of the thiazides which increase the excretion of bicarbonate. Ethacrynic acid is quite potent and the magnitude of natriuresis is equal to or greater than the mercurials, affecting approximately 18% of filtered sodium. Unlike carbonic anhydrase inhibitors, ethacrynic acid is effective in the presence of acidosis. It has a rapid onset of action and peak effects occur within 20 minutes in contrast to the 1-2 hours with mercurials. Finally, during maximal effects of either organomercurial or thiazides, ethacrynic acid produces a further increase in sodium excretion. However, during maximal mercurial diuresis, the increase in sodium excretion that occurred when ethacrynic acid was administered was less than additive. Also, during an acid potentiated mercurial diuresis, ethacrynic acid produced no further response. Such data indicates that perhaps the mode of action or the site of action or both of ethacrynic acid is similar to the mercurials under acidotic conditions.

Beyer (1965) reported that ethacrynic acid abolishes the renal medullary sodium gradient, which would imply an action on the ascending limb of the loop of Henle. His stop-flow data suggests that this agent acts all along the nephron. Goldberg et al. (1964) and Earley and Friedler (1964) utilized free water and negative free water

clearance techniques to demonstrate inhibition of sodium reabsorption on the ascending limb of the loop of Henle. Both the free water and negative free water clearance decreased. However, since these techniques represent the algebraic sum of events that occur along the entire nephron, a site of action of ethacrynic acid on the proximal nephron cannot be ignored (Seldin, 1966). This proximal action would certainly help to explain the results of Beyer (1965) which indicate that ethacrynic acid is capable of increasing urine flow to nearly 40% of GFR. Giebisch and Wendhaser (1964) demonstrated that approximately 70% of filtrate is normally reabsorbed in the proximal nephron. Excretion of 40% of the filtrate by ethacrynic acid would tend to indicate that the proximal tubule is being affected. Thus, it appears that ethacrynic acid, like the mercurials, acts along the entire tubule with a substantial effect on the loop of Henle.

Furosemide

Furosemide, a new and extremely potent diuretic-saluretic agent, has been the subject of much investigation in the last few years. Structurally, it is similar to the benzothiadiazines but compared to hydrochlorothiazide, furosemide is capable of eliciting a greater saluretic response (Timmerman et al., 1964). Their studies in both rat and man indicate that not only did furosemide produce a greater maximal response than the thiazides but also was more rapid in onset and shorter in duration.

Studies conducted in the attempt to elucidate the localization of the site of action of furosemide have proved to be quite interesting. Suki et al. (1964) reported that furosemide impairs the concentrating ability of the kidney which implies an inhibition of sodium reabsorption along the ascending limb of the loop of Henle. Suki et al. also observed that furosemide increased urine flow to approximately 40% of GFR. This clearly points to an action on the proximal tubule. However, free water clearance decreased which would not be expected if an agent acted solely on the proximal tubule. This decrease in free water clearance could be explained however, if furosemide acted to inhibit sodium reabsorption in an area distal to the proximal tubule. Negative free water clearances were performed and a decrease was observed indicating an action on the ascending limb of the loop of Henle. It was therefore concluded that furosemide inhibited sodium reabsorption primarily in the proximal tubule and ascending limb of the loop of Henle. Hook and Williamson (1965a) reported the abolishment of the renal medullary sodium gradient with furosemide indicating an action on the ascending limb.

Additive studies have also been conducted to compare the action of furosemide with other known diuretics. Hook and Williamson (1965b) showed that the natriuretic action of furosemide and ethacrynic acid were not additive. They also observed that the administration of furosemide during

maximal mercurial natriuresis resulted in an increase in sodium excreted. However, during an acid-potentiated mercurial natriuresis, furosemide did not produce an increase in sodium excretion (Hook and Williamson, 1966b). Since the natriuretic activity of furosemide is not altered during acidosis, (Hook and Williamson, 1965c), it would appear that during the acidotic state of the action of the mercurials might be extended to the ascending limb of the loop of Henle.

The effect of furosemide on renal hemodynamics has important clinical significance. The organic mercurials have been shown to increase renal vascular resistance (Vargas and Cafruny, 1962) and produce a transient fall in GFR (Farah, 1952). Studies with furosemide indicate a rapid response in patients with severely impaired renal function (Jachnecke et al., 1964). They observed that furosemide increased the clearance of PAH and inulin, particularly in patients with depressed renal function. Hook and Williamson (1966a), using an electromagnetic flowmeter, reported that furosemide and ethacrynic acid reduced renal vascular resistance in contrast to the thiazides and the organomercurials which increased renal vascular resistance. Since one of the major clinical uses of saluretic agents is in the management of edema secondary to congestive heart failure, a condition often characterized by an increased renal vascular resistance, the ability of furosemide and ethacrynic to decrease renal vascular

resistance and thus increase renal blood flow might be the reason why these agents are effective in patients which do not respond to either mercurial or thiazide therapy.

Other natriuretic agents are in use today like the spironolactones, chlorthalidone, and others, each possessing certain advantages and disadvantages. Due to the disadvantages, none of these natriuretic agents are considered to be ideal. Inasmuch as diuretic therapy is becoming more widespread in the treatment of various pathological conditions, the search is continuing for new and better agents.

DT-327 has been shown to be an orally effective natriuretic-diuretic in man and laboratory animals (Gold, 1965). Although chemically related, in part to diuretics like hydrochlorothiazide, furosemide and chlorthalidone by virture of its substituted benzyl sulfamyl group, the structure of DT-327 on the whole is different because of its side chain (figure 1). Even though the chemical structure of furosemide closely resembles the thiazides it is a much more efficacious natriuretic agent (Hook and Williamson, 1965b). It was, therefore, of interest to determine if this side chain modification in the structure of DT-327 resulted in an agent whose natriuretic action more closely resembles furosemide or, like chlorthalidone, more closely resembles the thiazides.

METHODS

Mongrel dogs of either sex weighing from 8-18 Kg were anesthetized with pentobarbital sodium (30 mg/Kg, intravenously) and tracheotomized. Carotid blood pressure was monitored using a Statham arterial pressure transducer and an Offner direct writing oscillograph. A femoral artery and vein were cannulated with polyethylene tubing to facilitate withdrawal of blood samples and infusion of fluids respectively. Both ureters were exposed by a small midline incision and were cannulated with polyethylene tubing. Unless otherwise indicated urine from both kidneys was collected in single graduated cylinders. All collection periods were of 10 minute duration. Blood samples were obtained at 20 minute intervals. Inulin (0.2%) was added to all infusions to monitor the rate of glomerular filtration. In some experiments para-aminohippurate (0.4%) was also added to the infusion so that estimations of renal plasma flow could be made.

Preliminary experiments were performed to determine both a dose response curve and duration of action of DT-327. Control diuresis was produced by infusing 5% mannitol into a femoral vein at 0.3 ml/Kg/min. A dose response curve was obtained by intravenously injecting increasing doses of DT-327, each successive dose being given when the diuretic response from the previous dose had reached a plateau. To determine the duration of action, a single dose

of DT-327 (1 mg/Kg) was administered, intravenously. Urine was collected for a total of 90 minutes at 10 minute intervals.

The Effect of Intrarenal Infusion of DT-327

The following investigation was performed for a variety of purposes. A series of experiments were conducted to verify the assumption that the target organ of DT-327 is the kidney. The second purpose was to observe if the natriuretic activity was due to the drug per se or the result of some metabolic alteration. Finally, it was of interest to determine whether probenecid inhibited the natriuretic activity of DT-327, thus providing information as to whether the plasma, tubular or luminal concentration of this drug was responsible for its action.

The left renal artery was isolated retroperitoneally through a flank incision and a 21-gauge needle was introduced as close to the aorta as possible. A slow (1 ml/min.) saline infusion insured patency of this system. In order to insure an adequate rate of urine flow, these experiments were conducted during an osmotic diuresis produced by the intravenous infusion of 5% mannitol (0.5 ml/Kg/min.). Urine was collected every 5 minutes and blood samples withdrawn from the femoral artery every 10 minutes. After the rate of urine flow had stabilized, several control urine samples were collected. The saline infusion into the renal artery was then replaced with a solution containing sufficient DT-327 to deliver 0.5 μg/Kg/min. Two

animals were treated with 30 mg/Kg of probenecid intravenously and then the above procedure was repeated.

Effects of DT-327 on Renal Hemodynamics

Since certain natriuretic agents like furosemide and ethacrynic acid have been reported to increase renal blood flow without any significant alteration in the clearance of inulin, (Hook and Williamson, 1966a), it was of interest to determine the effects of DT-327 on renal blood flow.

The left kidney was approached retroperitoneally through a flank incision and the ureter was cannulated. Systemic hydration was attained by infusing 0.9% sodium chloride (0.5 mg/Kg/min.) throughout the experiment. The left renal artery was isolated and flow was measured directly using an electromagnetic flow meter (Carolina Medical Electronics) with a probe placed on the renal artery. DT-327 (1 mg/Kg) was administered intravenously and the effects on renal blood flow were monitored on an oscillograph. At the end of the experiment, the renal artery was cannulated and blood was collected in a graduated cylinder for the purpose of calibration.

Effects of Acid-Base Balance on the Natriuretic Activity of DT-327

The natriuretic activity of the mercurials and the carbonic anhydrase inhibitors have been reported to be affected by changes in acid-base balance (Ethridge et al.,

1936; Pitts et al., 1949). Since such changes occur in pathological conditions, it was of interest to determine if the natriuretic activity of DT-327 was likewise altered by changes in plasma pH.

In order to determine the effect of DT-327 during metabolic acidosis and alkalosis, acid-base balance was altered by a slight modification of the method of Farah et al. (1959). To induce systemic acidosis, mongrel dogs were pretreated with ammonium chloride (100 mEq/day) for 3 days prior to the experiment. Hydrochloric acid (0.08N) was infused intravenously at the rate of 5 ml/min/square meter of body surface (in 0.9% sodium chloride) prior to and throughout the experiment. When plasma pH reached 7.2, DT-327 (1 mg/Kg) was administered intravenously. Blood samples were drawn under oil and pH was recorded anaerobically. To induce systemic alkalosis, animals were pretreated with sodium bicarbonate (100 mEq/day) for 3 days prior to the experiment. A 0.25N sodium bicarbonate infusion replaced the 0.08N hydrochloric acid infusion. The animals were considered to be alkalotic when plasma pH reached 7.5.

The influence of metabolic alkalosis on the electrolyte excretion of DT-327 was determined in another species of animals using the assay procedure of Lipschitz et al. (1943). Male Sprague-Dowley rats, 250-300 g, were given an oral load of either 0.9% sodium chloride or 1.0% sodium bicarbonate, 25 ml/Kg, at the beginning of the experiment.

Various concentrations of DT-327 (0.01 mg/Kg, 0.1 mg/Kg, or 1.0 mg/Kg) were dissolved in the oral load. For urine collection the rats were placed in metabolism cages and urine was collected over a 3 hour period. At the end of the collection period, the animals were killed by cervical dislocation, the bladders removed and their contents combined with the previously collected urine. Urine samples were analyzed for sodium, potassium and chloride.

Effects of DT-327 on Free Water and Negative Free Water Clearance

To more carefully delineate the tubular areas affected by DT-327, studies were conducted utilizing the free water clearance techniques. The following procedure was employed in our free water clearance studies. Dogs were hydrated by the infusion of 500 ml of 6% dextran in 0.9% sodium chloride at a rate of 1 ml/Kg/min. This served the purpose of expanding blood volume and limiting the endogenous release of antidiuretic hormone (ADH). A 2.5% glucose solution was then infused at 1 ml/Kg/min. When the urine osmolality was below 100 milliosmoles, and the urine volume relatively constant, the diuretic (1 mg/Kg) was administered intravenously and urine collected for 40 minutes.

To observe the effect of DT-327 on negative free water clearance, the following procedure was employed. Mongrel dogs were deprived of food and water 24 hours prior to the experiment. An injection of vasopressin in oil (Pitressin

tannate, 5 units) was given intramusculary the night before surgery. The animals were anesthetized and prepared surgically as in the preceding experiments. An infusion of 2% sodium chloride containing aqueous Pitressin (100 mU/Kg/hr.) was administered at 0.5 ml/Kg/min. and maintained throughout the experiment. After urine flow had stablized, DT-327 (1 mg/Kg) was injected intravenously and urine was collected for 40 minutes.

Stop-Flow Analysis of the Tubular Site of Action of DT-327

The technique of stop-flow analysis (Malvin et al., 1958) with a slight modification (Sullivan and Pirch, 1965) was also employed to elucidate the site of action of DT-327. Although the stop-flow technique has certain limitations as mentioned in the introduction, it can serve as a useful tool especially if the drug under investigation acts primarily on the distal tubule.

The left ureter was exposed by a flank incision and catheterized. Twenty percent mannitol in a 1% sodium chloride solution containing 0.8% inulin and 0.08% PAH was infused at a rate of 10 ml/min. to establish a diuresis. Two grams of sodium chloride were injected intravenously approximately 10 minutes prior to the occlusion of the left ureter. When urine flow was adequate, two 5-minute control urine samples were collected, blood samples being taken for each control urine period. The ureter was then occluded for 4 minutes, a blood sample being taken at the end of the first 2-minutes of occlusion. At the end of the

4 minute occlusion, the urine was collected serially in small graduated tubes. The same procedure was repeated after the administration of DT-327 (1 mg/Kg, intravenously) and the two stop-flow patterns were then compared.

Effect of DT-327 During Maximal Thiazide and Furosemide Natriuresis

To further classify DT-327 as to site of action, an additive study with hydrochlorothiazide and furosemide was employed using the method described by Pitts et al. (1958). Such a technique, as described in the introduction, has proved to be quite useful in determining the site or mechanism of action of various diuretic agents.

Saline (0.9%) was infused intravenously at 0.8 ml/Kg/min. for about 1 1/2 hours prior to and throughout the experiment. After equilibration, maximal thiazide saluresis was produced by the administration of 3 mg/Kg of hydrochlorothiazide intravenously; a maintenance dose of 3 mg/Kg/hr. was then added to the infusion. When urine flow had plateaued, 1 mg/Kg of DT-327 was injected intravenously. Urine was then collected for 40 minutes. In another series of animals, the order of drugs administered was reversed in an attempt to determine if hydrochlorothiazide was additive in animals undergoing a DT-327 saluresis. Maximal DT-327 saluresis was produced by the administration of 5 mg/Kg and a maintenance infusion of 5 mg/Kg/hr. When urine flow had reached a plateau, 3 mg/Kg of hydrochlorothiazide was injected intravenously.

The above procedure was repeated substituting 5 mg/Kg of furosemide for hydrochlorothiazide.

Inulin was determined by the method of Schreiner (1950) and PAH by the method of Smith et al. (1945). Sodium and potassium were determined by flame photometry (Instrumentation Laboratories, internal standard flame photometer) and chloride was determined with a Buchler-Cotlove Chloridometer. Bicarbonate was determined by simple titration. Osmolalities were determined with an Advanced Osmometer. The data obtained were analyzed statistically using the student "t" test (Snedecor, 1956). The 0.05 level of probability was used as the criterion of significance.

RESULTS

The diuretic response to DT-327 is dose related. The protocol of an individual experiment is illustrated in table 1 and the results of 4 animals are summarized in figure 2. When electrolyte excretion was plotted against increasing doses of DT-327, increased sodium, chloride, and potassium excretion was observed. No significant alteration in the clearance of inulin or PAH occurred. The increased cation excretion was not completely balanced by increased chloride excretion indicating that another anion was probably being affected. The greater effect of DT-327 on sodium excretion relative to potassium excretion is illustrated by the slopes of their respective dose response curve (figure 2).

The natriuretic effect of a single intravenous injection of 1 mg/Kg of DT-327 is illustrated in figure 3. Maximum sodium excretion, as well as the other variables measured, occurred in the first 10-minute interval following DT-327 injection. A substantial effect was still present 90 minutes after drug administration.

The Effect of Intrarenal Infusion of DT-327

Infusion of DT-327 (0.5 ug/Kg/min.) into the left renal artery increased the rate of urine flow and sodium excretion from the left kidney. A slight contralateral response from the right kidney was also noted appearing approximately 10-15 minutes after the response observed

on the ipsilateral side. The protocol of a typical experiment is shown in table 2 and the results from 4 animals in figure 4. Urine flow and sodium excretion from the left kidney increased during the first 5-minutes of DT-327 infusion and continued to rise during the remaining drug periods. Upon withdrawal of the DT-327 infusion, both urine flow and sodium excretion declined. A slight increase in urine flow and sodium excretion appeared from the right kidney after approximately 15 minutes of drug infusion but appeared to be markedly less in magnitude than the increase observed from the left kidney. The response noted from the right kidney did not decrease as quickly as did that from the left kidney once the drug infusion was discontinued.

The effect of 30 mg/Kg of probenecid on the natriuresis induced by DT-327 is illustrated in figure 4. Following probenecid administration, the natriuresis produced by DT-327 was greatly reduced especially from the left kidney.

Effects of DT-327 on Renal Hemodynamics

Studies concerning the effect of DT-327 on renal blood flow were conducted in 3 animals and the results of a typical blood flow experiment are illustrated in figure 5. Mean blood pressure (BP) before DT-327 administration was 100 mm/Hg and renal blood flow (RBF) was approximately 210 ml/min. Following the intravenous injection of 1 mg/Kg of DT-327, there was no demonstrable alteration in either renal blood flow or mean blood pressure. However, urinary

volume increased from control values of 0.30 ml/min. to 1.17 ml/min. following drug administration. Sodium excretion likewise increased from control values of 20 μEq/min. to 196 μEq/min. This increased sodium excretion is comparable to changes observed in other experiments (figure 2, 3). There was no alteration in the clearance of inulin.

Effects of Acid-Base Balance on the Natriuretic Activity of DT-327

When injected intravenously into dogs receiving a saline infusion, DT-327 (1 mg/Kg) produced a marked natriuretic-saluretic response (figure 2, table 3, 4). An increase in potassium and bicarbonate excretion was also observed, the magnitude of the latter, compared to the sodium and chloride increase, being quite small. As illustrated in table 5, 1 mg/Kg of DT-327 administered during metabolic acidosis produced an increase in sodium, chloride, and potassium excretion. The increase in chloride excretion was slightly greater than that of the increase in sodium excretion. No apparent change in the clearance of inulin occurred. During metabolic alkalosis, 1 mg/Kg of DT-327 resulted in an increased excretion of sodium chloride, potassium and bicarbonate (table 6). The increase in bicarbonate excretion appeared to be greater than that observed in the dogs receiving saline infusion. However, due to the small number of animals studied, no statistical comparison could be made.

The effect of increasing doses of DT-327 on sodium and chloride excretion in rats orally loaded with saline or sodium bicarbonate is illustrated in figure 6. The increase in chloride excretion seemed to parallel quite closely the increase in sodium excretion in both the saline and bicarbonate treated rats.

Effects of DT-327 on Free Water and Negative Free Water Clearance

A significant decrease in free water clearance was observed with a dose of 1 mg/Kg of DT-327. Figure 7 illustrates the results of plotting free water clearance against urinary volume. In a total of 5 animals, free water clearance decreased from a control value of 9.4 $\pm$ 0.6 (S.E.) ml/min. to 6.1 $\pm$ 0.5 ml/min. It is apparent that the diuretic reduced the maximal capacity to form free water to about 70% of that obtained in control animals at comparable rates of delivery.

The effect of DT-327 on negative free water clearance is illustrated in figure 8. Negative free water clearance is plotted against osmolar clearance. No significant difference in negative free water clearance was observed between control values and values obtained with DT-327.

Stop-Flow Analysis of the Tubular Site of Action of DT-327

The concentrating pattern of a typical stop-flow experiment before and after DT-327 administration is illustrated in figure 9. The urine/plasma (U/P) ratios of sodium and potassium, each factored by the corresponding

U/P ratio of inulin (to account for water reabsorption) are plotted against the accumulated urine volumes. In the control, or non-drug stop-flow pattern, the U/P ratios of sodium during free flow were approximately 0.35. After the release of occlusion there was a fall in these ratios, the lowest value reached being 0.05. This ratio represented that sample acted upon by a maximum number of distal tubules and is referred to as the distal minimum. Those samples that represented the loop of Henle and proximal tubules showed a gradual increase in the U/P ratios of sodium eventually establishing a plateau at the original free flow ratio of 0.35. The U/P ratios of potassium obtained from samples after the occlusion did not seem to vary significantly from the ratios obtained during free flow, an average value being 0.10.

Following administration of DT-327, there appeared to be a significant alteration in the stop-flow pattern. The low U/P ratio of sodium achieved by the distal tubule during the control stop-flow procedure was not present, the lowest ration being 0.14 as compared to the control ratio of 0.05. In a total of 4 animals, this increase in the distal minimum following DT-327 administration proved to be statistically significant. A mean increase in the U/P ratio for sodium of 0.056 $\pm$ 0.021 (S.E.) was significant (p 0.05 "one tailed"). The remaining portion of the stop-flow pattern with DT-327 appeared to be identical with the control stop-flow pattern concerning the U/P ratios of sodium. The U/P ratios for potassium following DT-327

administration increased in the distal area of the tubule, a maximum value of 0.30 being obtained as compared to 0.12 from the control stop-flow pattern.

Effects of DT-327 During Maximal Thiazide and Furosemide Natriuresis

During administration of a maximally effective dose of hydrochlorothiazide, DT-327 (1 mg/Kg) did not produce saluretic and diuretic responses significantly greater than those produced by hydrochlorothiazide alone (figure 10). A small but significant decrease in potassium excretion occurred. It should be noted that this decrease in potassium excretion was quite small in most cases only amounting to a few uEq/min. thus creating doubt as to its practical significance. During maximal DT-327 diuresis intravenous administration of hydrochlorothiazide (3 mg/Kg) did not significantly affect any of the variables measured (figure 11). However, animals were capable of responding to other agents. In 4 animals receiving a maximally effective dose of DT-327, furosemide (5 mg/Kg) produced a further increase in sodium and chloride excretion (table 7). This indicates that the blockade of hydrochlorothiazide by DT-327 was, most probably, a specific effect and not some non-specific blockade of all natriuretic agents.

DISCUSSION

Intravenous administration of DT-327 resulted in a rapid onset of action, producing a significant increase in urine volume and the excretion of sodium, potassium, and chloride (figure 2, 3, table 1). The magnitude of response was related to the dose, and an effect was present with a dose as low as 0.01 mg/Kg (figure 2, table 1). With a supramaximal dose of DT-327 (5 mg/Kg), the drug appeared to be equi-effective with hydrochlorothiazide (figure 7). The duration of action of DT-327 (figure 3) appeared to be similar to that of the thiazides, a substantial effect being present 90 minutes after drug administration.

DT-327 infusion (0.5 ug/Kg/min.) into the renal artery resulted in an ipsilateral increase in sodium excretion (figure 4, table 2). The contralateral natriuresis observed was delayed in onset and only a small fraction of the response of the ipsilateral kidney. This observation verified the assumption that the kidney is the target organ for the natriuretic effect of DT-327. Inasmuch as the ipsilateral increase in sodium excretion occurred within the first 5-minutes of drug infusion, it can be concluded that the drug per se acted directly on the kidney and that transport into the systemic circulation and metabolism of the parent compound was not necessary for natriuresis to occur. The lipid solubility of DT-327 offers a possible explanation for the contralateral response that occurred. Most likely some of the drug was reabsorbed from the urine

of the ipsilateral kidney and reached the contralateral kidney via the systemic circulation. Such an explanation would account for the time lag noted before observing this contralateral response. A tubular site of action within the kidney is also indicated by these results as DT-327 did not have any significant effect of the glomerular filtration rate.

Since DT-327 is an organic acid, one could speculate that DT-327 would be actively secreted by the tubular cells. Probenecid, a competitive inhibitor of the secretory transport for organic acids (Weiner et al., 1960), was used to block the tubular transport of DT-327 in vivo. A large dose (30 mg/Kg) was administered intravenously to insure a significant blockade of this transport system. This dose has been shown by others to have no demonstrable effect on renal function other than to block organic anion transport (Weiner et al., 1960; Hook and Williamson, 1965c). As illustrated in figure 4, the natriuretic response to DT-327 after probenecid administration was greatly reduced. Probenecid has been reported to have a similar effect on the natriuresis produced by chlorothiazide (Beyer and Baer, 1961). Thus, it appears that DT-327 is transported to a great extent by tubular secretion and that cellular or luminal concentration rather than plasma concentration of the drug appears to be critical for the natriuretic response to occur.

The organomercurials have been shown to increase renal vascular resistance (Vargas and Cafruny, 1962) and produce

a transient fall in glomerular filtration rate (Farah, 1952). It has been shown that patients with severely impaired renal function do not respond well to the organomercurials (Reubi and Cottier, 1961). In contrast, Hook and Williamson (1966a) have reported that saluretic doses of furosemide significantly decrease renal vascular resistance. They also indicated that hydrochlorothiazide has no significant effect on renal vascular resistance although it tended to increase it. If renal blood flow is diminished due to some pathological state, it is conceivable that increased vascular resistance produced by the mercurials, for example, would be great enough to limit blood flow to the point of masking the saluretic response. Therefore, it was of interest to observe the response of DT-327 on renal vascular resistance and compare this to the response of other natriuretic agents.

The effect of DT-327 on renal hemodynamics is illustrated in figure 5. It was apparent that the drug at a dose capable of producing a significant natriuresis did not alter renal blood flow and consequently did not affect renal vascular resistance. The clearance of inulin was likewise unaltered following DT-327 administration. Thus, it appeared that an increase in blood flow or glomerular filtration rate was not necessary for a natriuretic response to occur. DT-327, like the thiazides and the organomercurials, would therefore appear to have definite limitations in the management of edema secondary to congestive heart failure which is usually characterized by an

increase vascular resistance.

When compared to normal animals (table 4) the natriuretic response to DT-327 did not seem to be altered by the existance of metabolic acidosis or alkalosis. However, there did appear to be a slight alteration in the nature of the anion excreted with changes in the acid-base balance. During systemic acidosis, administration of DT-327 produced a greater increase in chloride excretion than sodium excretion (table 5). In our preliminary experiments with normal animals, it was noted that following DT-327 administration, the increase in sodium excretion was not completely balanced by the increase in chloride excretion suggesting that DT-327 might have an effect on another anion, perhaps bicarbonate. Some in vitro carbonic anhydrase inhibitory activity has been observed (Trapold, 1967) and our results tend to confirm this in the intact animal. In dogs undergoing a saline diuresis, administration of DT-327 produced a small but significant increase in bicarbonate excretion (table 3, 4). During alkalosis, an injection of DT-327 resulted in an even greater increase in bicarbonate excretion (table 6) which would be expected if a diuretic possessed carbonic anhydrase inhibitory activity. However, it should be noted that the magnitude of this increase in bicarbonate excretion did not appear to decrease the effect of the drug on chloride excretion. Additional studies conducted in rats orally loaded with saline or sodium bicarbonate tend to confirm the above finding as illustrated

in figure 6. There appeared to be no significant alteration in the chloruretic response following DT-327 administration between rats pretreated with saline or bicarbonate. It has been reported by Baer et al. (1959) that the thiazides possess carbonic anhydrase inhibitory activity. It was also noted that the enhanced bicarbonate excretion produced by hydrochlorothiazide during alkalosis was of a lesser magnitude than that produced by an equivalent dose of chlorothiazide. Such differences appear to be related to the greater inhibitory effect of chlorothiazide on carbonic anhydrase. Beyer and Baer (1961) reported that as the lipid solubility of a thiazide increases, its carbonic anhydrase inhibitory activity decreases. Likewise, DT-327 being extremely lipid soluble, possesses some carbonic anhydrase activity but not to such a degree as to significantly alter the nature of the major anion affected.

In an attempt to delineate the tubular areas affected by DT-327, the free water clearance technique was utilized. Free water clearance has been defined as osmotically unobligated water (Pitts, 1963). In the absence of antidiuretic hormone, the collecting duct is relatively impermeable to water resulting in the excretion of a dilute urine. If a drug were to act only on the proximal tubule to inhibit isosmotic reabsorption, the excess solute retained within the nephron would be accompanied by an osmotically equivalent amount of water. The excess solute would be partially reabsorbed in the distal nephron, resulting in an increase free water clearance. If, on the

other hand, a drug acted distally to the site of isosmotic reabsorption of water to prevent reabsorption of electrolytes, the excess solute delivered to the collecting duct and later excreted would result in a decreased free water clearance. The thiazides decrease free water clearance, and this has been attributed to a distal site of action (Earley et al., 1961; Seldin et al., 1966). Results with DT-327 also show a significant depression of free water clearance suggesting a distal locus of action for this compound (figure 7). Negative free water clearance ($T^{c}H_{2}0$), measured during maximal ADH activity is a measure of renal concentrating capacity and results from sodium reabsorption out of the ascending limb of the loop of Henle (Seldin et al., 1966). Inasmuch as DT-327 had no demonstrable effect on negative free water clearance (figure 8), an effect on the loop of Henle was considered unlikely.

In an attempt to confirm the data obtained from free water and negative free water clearance studies which indicated a distal site of action for DT-327, experiments were performed utilizing the stop-flow technique. As discussed in the introduction, the interpretation of a stop-flow pattern is highly questionable if the data indicate an effect on the proximal tubule because urine from this tubular segment has to pass through the distal tubule after the occlusion period is terminated. Since this is not the case with stop-flow samples collected from

the distal nephron, stop-flow patterns which suggest that a drug inhibits reabsorption of sodium in the distal tubule are not considered as questionable as those which suggest drug inhibition of sodium in the proximal tubule.

Examination of the stop-flow pattern before and after DT-327 administration clearly indicated that a major site of action is the distal nephron. The results of an experiment in which an animal was subjected to a slight modification of the stop-flow technique are illustrated in figure 9. This animal was given 2 g of sodium chloride prior to occlusion thus preventing electrolyte depletion during the mannitol diuresis. In 4 animals the distal minimum of sodium, or the U/P ratio that represents the tubular area in the distal nephron where maximum reabsorption of sodium normally occurs, was significantly increased following DT-327 administration. Since sodium is not secreted along the tubular nephron, such data suggested that inhibition of sodium reabsorption had occurred. Likewise, the U/P ratio of potassium also increased in the distal nephron after an injection of DT-327. This would be expected especially if sodium reabsorption was inhibited in this tubular area. More sodium would now be available to exchange with intracellular potassium, this being proposed as the major mechanism of potassium secretion (Pitts, 1963). A similar stop-flow pattern to that of DT-327 has been reported to occur with the thiazides (Cafruny and Ross, 1962). Thus, the effect of DT-327 on free water and negative free water clearances

relate quite well with the stop-flow data indicating that a major site of action of this drug is the distal nephron.

Pitts et al. (1958) demonstrated that chlormerodrin was capable of producing its natriuretic response during maximal natriuresis produced by chlorothiazide. Conversely, chlorothiazide produced its effect during maximal mercurial natriuresis. Since either agent was capable of eliciting an additive response during maximal natriuresis produced by the other, it was reasoned that they must be acting by different mechanisms or at different sites along the nephron. By the same reasoning, lack of a response to a given agent during maximal natriuresis produced by another has been interpreted to indicate that the two agents act by the same mechanism or at the same site (Hook and Williamson, 1965b). During maximal hydrochlorothiazide natriuresis (figure 10) DT-327 was without effect except for a significant depression of potassium excretion which was probably due to a general deterioration of the animals. During maximal DT-327 natriuresis, hydrochlorothiazide was without effect (figure 11). Using the reasoning outlined above, it could be assumed that the two agents were acting by the same mechanism and at the same tubular site. However, Small and Cafruny (1967) demonstrated the dangers of concluding that lack of addition of maximally effective doses of two agents implies that two drugs are acting by a similar mechanism. It is possible

that two drugs would not be additive if they acted at different steps in the same biochemical sequence or if they differed in the affinity for the same biochemical receptor. Nevertheless, the ability of DT-327 to decrease free water clearance without altering negative free water clearance in addition to the lack of addition with hydrochlorothiazide gives ample support to the proposition that both act at the same site along the nephron.

In maximally effective doses furosemide is more efficacious than the thiazides (Hook and Williamson, 1965b) and by implication more efficacious than DT-327. Furthermore, in four animals furosemide was capable of increasing sodium excretion during maximal DT-327 natriuresis whereas hydrochlorothiazide was not (table 5). Thus, even though the precise biochemical site cannot be defined, it is apparent that DT-327 produced a specific blockade of the action of the thiazide. Furosemide also has the ability to decrease negative free water clearance (Seldin et al., 1966) and to decrease renal vascular resistance (Hook and Williamson, 1966a) both properties not shared by DT-327 or the thiazides. It is concluded, therefore, that the natriuretic activity of DT-327 more closely resembles the thiazides than furosemide.

SUMMARY

The diuretic response to DT-327 is dose related. The intravenous administration of DT-327 resulted in the increased excretion of sodium, chloride, and potassium, the responses observed being dose related. Maximum sodium excretion, as well as other variables measured, occurred in the first 10-minute interval following the administration of a maximal effective dose of DT-327 (1 mg/Kg).

Infusion of DT-327 into the left renal artery resulted in a significant ipsilateral increase in the excretion of sodium within 5 minutes without affecting the rate of glomerular filtration. A slight contralateral response was also noted occurring 10-15 minutes after the ipsilateral response. At a dose capable of producing a significant natriuresis, DT-327 did not alter renal blood flow and thus did not affect renal vascular resistance.

Alterations of plasma pH with hydrochloric acid and sodium bicarbonate did not appear to affect the natriuretic response to DT-327. Increased bicarbonate excretion did occur during metabolic alkalosis following drug administration indicating some carbonic anhydrase inhibitory activity, but this did not seem to affect the increase in chloride excretion observed.

DT-327 decreased free water clearance without altering negative free water clearance indicating an action on the distal nephron. The diuretic produced a significant increase in the distal minimum of the stop-flow pattern

again indicating a distal site of action. During maximal DT-327 natriuresis, hydrochlorothiazide was without effect, and likewise during maximal hydrochlorothiazide natriuresis, DT-327 was without effect. However, during maximal DT-327 natriuresis, furosemide was capable of producing an increased effect. Thus, results from free water and negative free water clearance studies along with the stop-flow, renal hemodynamics, and additive studies all seem to indicate that the natriuretic activity of DT-327 more closely resembles the thiazides than furosemide.

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TABLE 1

Protocol of a typical experiment demonstrating the dose-response relationship of DT-327

Intravenous infusion: 5% mannitol (0.3 ml/Kg/min.) 15 Kg male dog

period (10 min.)	urine flow	inulin clearance	PAH clearance	electrolyte excretion sodium	potassium	chloride
		ml/min.			μEq/min.	
1	2.4	44	222	72	22	7
2	2.7	50	186	94	27	14
		DT-327, 0.01 mg/Kg, i.v.				
3	2.9	56	204	145	32	38
4	3.2	54	163	205	35	86
5	3.0	61	187	168	33	57
		DT-327, 0.1 mg/Kg, i.v.				
6	3.8	55	176	342	57	243
7	4.1	56	184	348	70	271
8	4.0	46	158	324	64	252
		DT-327, 1.0 mg/Kg, i.v.				
9	4.3	44	155	383	82	292
10	4.4	43	172	378	70	304
		DT-327, 2 mg/Kg, i.v.				
11	4.2	40	134	374	67	269
12	4.2	41	146	370	76	265

TABLE 2

Protocol of a typical experiment demonstrating the renal actions of DT-327[a]

Intravenous infusion: 5% mannitol (0.5 ml/Kg/min.) started at 60 minutes

Collection Period (5 min.)	Left Renal Artery Infusions	Urine Flow (ml/min.)		Inulin Clearance (ml/min.)		Sodium Excretion (uEq/min.)	
		L[b]	R[b]	L	R	L	R
1.	Saline 1 ml/min.	1.86	1.80	28	31	125	104
2.		1.96	1.92	27	31	137	113
3.	DT-327 0.5 ug/Kg/min.	2.04	2.08	27	33	128	131
4.		2.64	2.36	30	35	206	139
5.		3.16	2.68	31	35	256	169
6.		3.40	2.88	25	25	275	181
7.	Saline 1 ml/min.	3.42	3.16	25	27	260	180
8.		3.16	3.12	22	27	215	165
9.		2.88	3.04	25	31	156	152
10.		2.80	3.00	27	35	137	153

[a] 14.0 Kg male dog

[b] L = left kidney
R = right kidney

TABLE 3

Effects of DT-327 (1 mg/Kg, i.v.) on bicarbonate excretion[a]

Intravenous infusion: 0.9% NaCl (6 ml/min./m^2)

	Electrolyte Excretion µEq/min.				pH	
	Na	K		HCO_3	plasma	urine
Control	132	62	95	24	7.40	7.12
DT-327	508	113	483	43	7.42	7.20
Mean Difference	376[b]	51[b]	388[b]	19[b]	0.02	0.08
± S.E.	(6)	(10)	(65)	(9)	(0.03)	(0.06)

[a]Electrolyte excretion and pH values during the two 10-minute collection periods prior to DT-327 were averaged as were the data from the two 10-minute collection periods following DT-327. Values in the table represent the means of 4 animals.

[b]Significant difference ($P < .05$).

TABLE 5

Effects of DT-327 (1 mg/Kg, i.v.) during metabolic acidosis[a]

Intravenous infusion: 0.08N HCl in 0.9% sodium chloride (6 ml/min./m^2)

	Electrolyte Excretion ⁄uEq/min.				Plasma mEq/liter				pH		GFR[b]	V (ml/min.)
	Na	K	Cl	HCO_3	Na	K	Cl	HCO_3	urine	plasma		
1 Control[c]	133	106	159		125	4.4	111		6.85	7.20	51	4.2
2 DT-327[d]	338	193	444		115	4.0	106		6.82	7.20	48	6.1
1 Control	128	32	152		121	2.8	100		5.70	7.25	66	4.9
2 DT-327	654	87	739		110	2.9	97		4.86	7.20	55	8.1

[a]Ammonium chloride was administered at an oral dose of 100 mEq/day for 3 days prior to the infusion of 0.08N HCl.

[b]GFR = glomerular filtration rate; V = urinary volume

[c]Control values represent the mean values of two 10-minute collection periods prior to drug administration.

[d]DT-327 values represent the mean values of four 10-minute collection periods following administration of the drug.

TABLE 7

Effects of furosemide (5 mg/Kg, i.v.) during maximal DT-327 natriuresis[a]

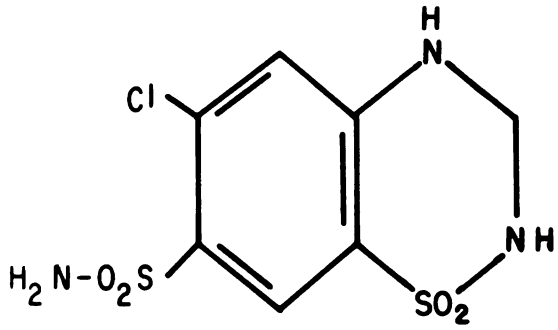
	Electrolyte Excretion ʌuEq/min.		
	Na	K	Cl
DT-327	1200	112	1179
DT-327 plus furosemide	1978	154	2093
Mean Difference (± S.E.)	778[b] (48)	42[b] (17)	914[b] (15)

[a]Electrolyte excretion during the two 10-minute collection periods prior to furosemide were averaged as were the data from the two 10-minute collection periods following furosemide. Values in the table represent the means of 4 animals.

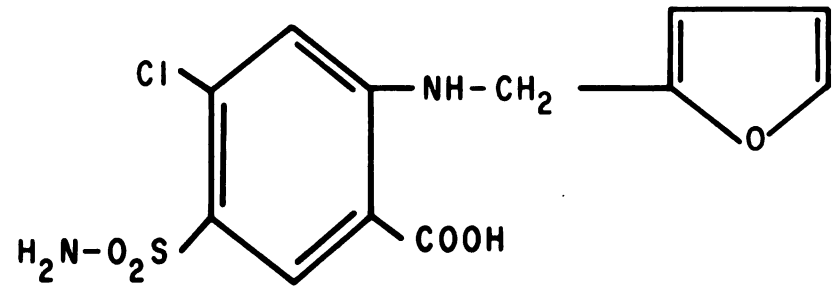
[b]Significant difference (P .05).

Figure 1: Chemical structures of hydrochlorothiazide, furosemide, DT-327, and chlorthalidone.

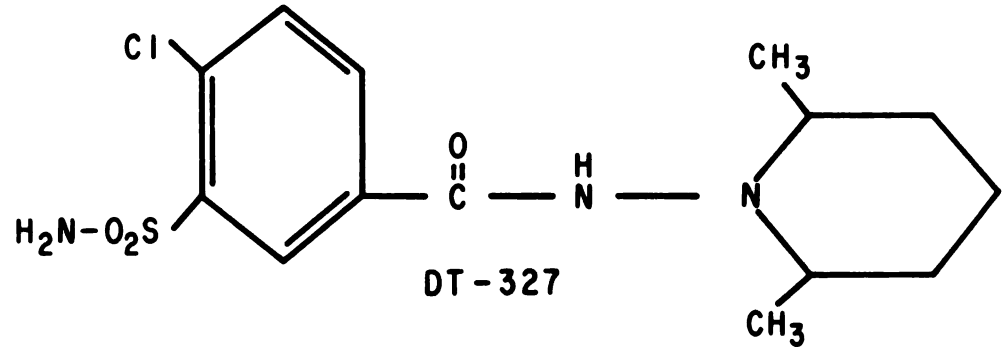
FIGURE 1



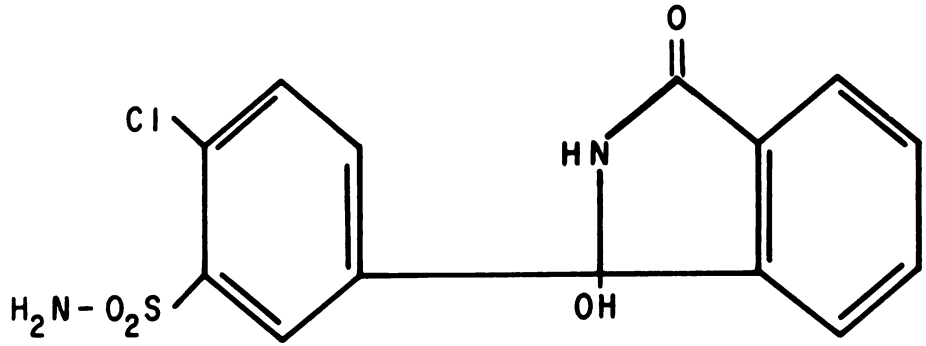
HYDROCHLOROTHIAZIDE



FUROSEMIDE



DT-327



CHLORTHALIDONE

Figure 2: Effect of increasing doses of DT-327 on sodium excretion. Increasing doses were administered intravenously when the diuretic response from the previous dose had plateaued. Bars indicate means (± standard error) of comparable clearance periods from four experiments.

FIGURE 2

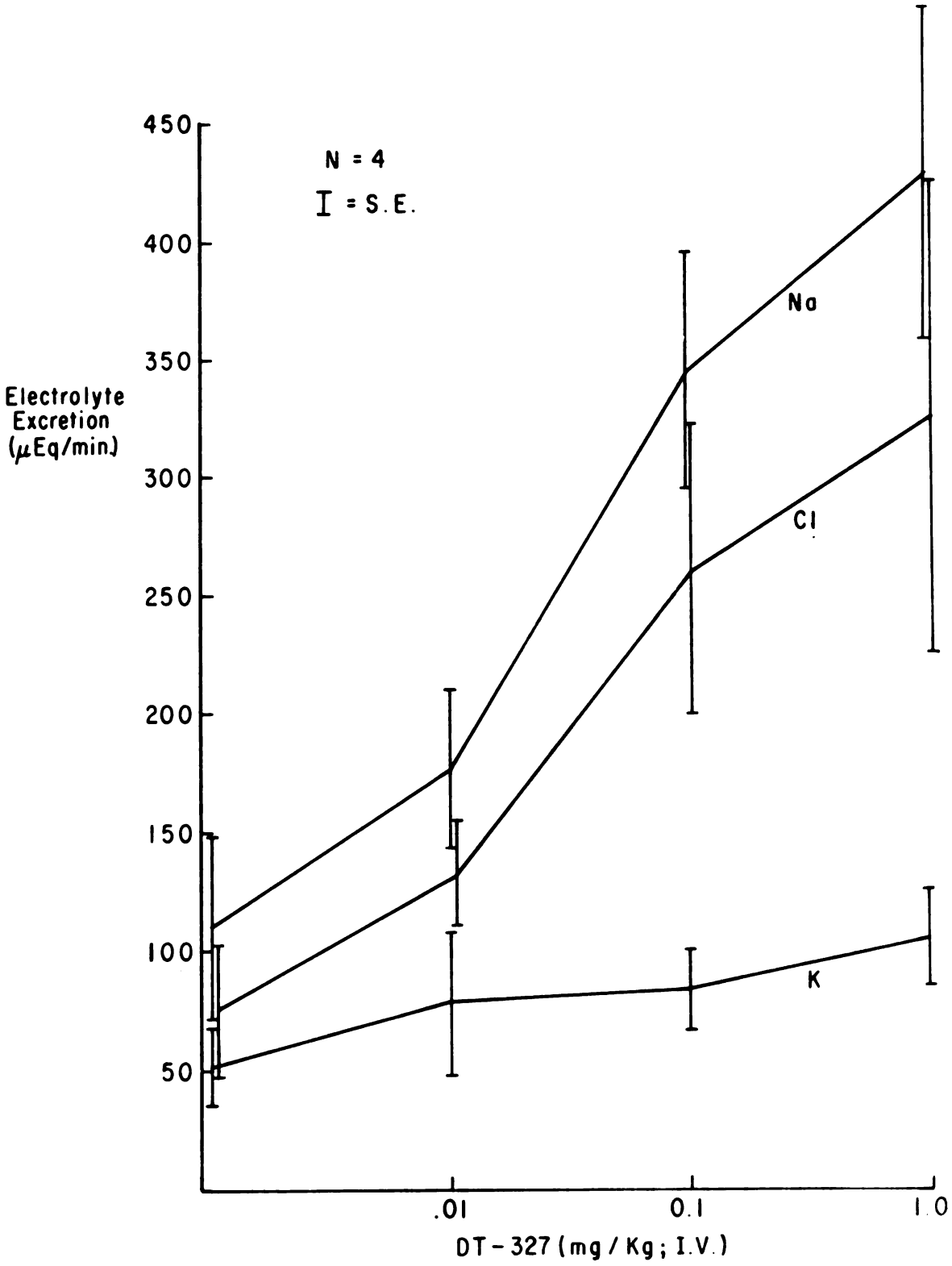

N = 4
I = S.E.
Electrolyte Excretion (μEq/min)
450
400
350
300
250
200
150
100
50
Na
Cl
K
.01
0.1
1.0
DT-327 (mg/Kg; I.V.)

Figure 3: Effects of DT-327 (1 mg/kg, intravenously) on sodium excretion in the dog during mannitol diuresis. Bars indicate means (± standard error) of comparable clearance periods from five experiments.

FIGURE 3

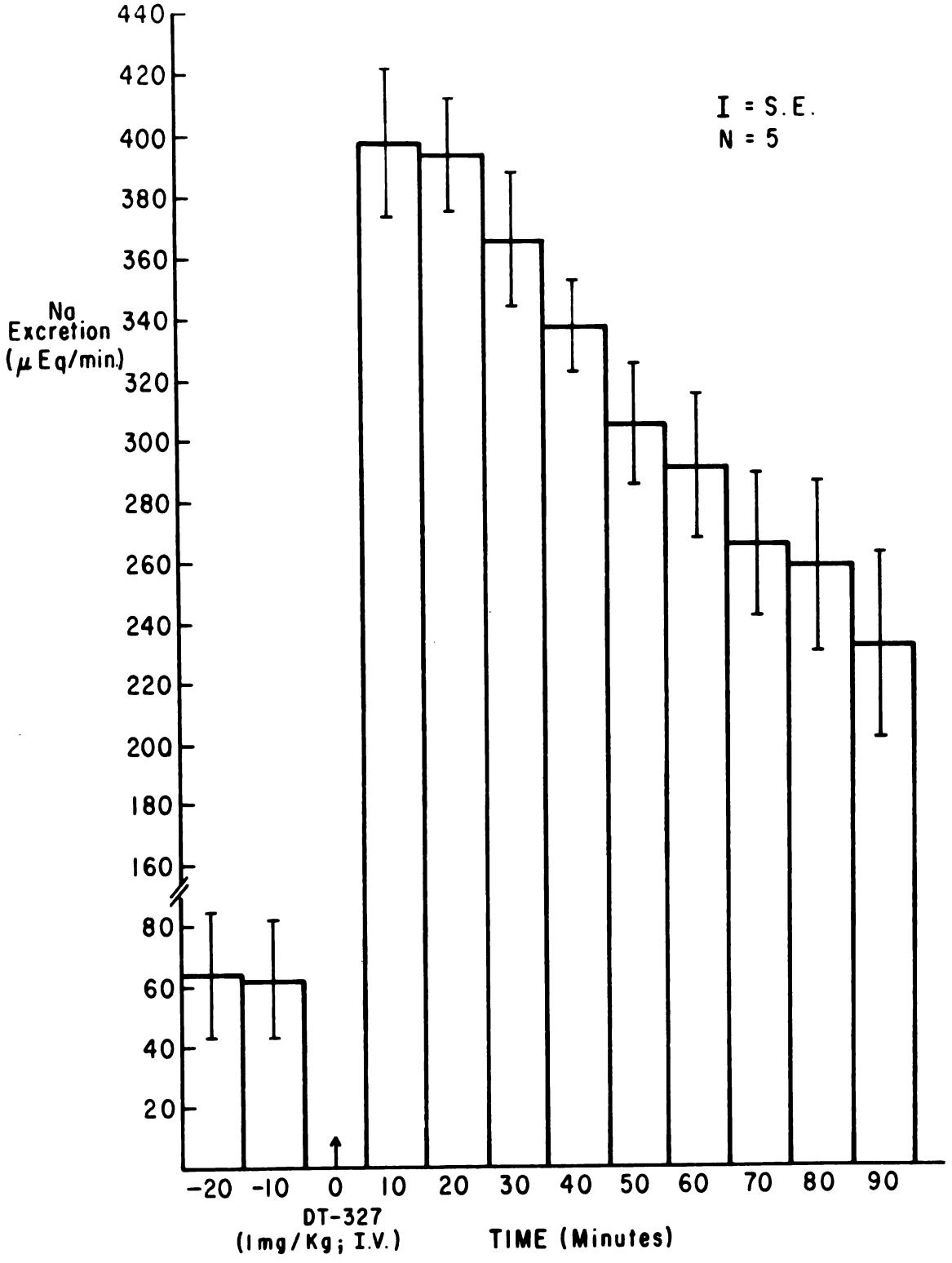

I = S.E.
N = 5
Na Excretion (μ Eq/min.)
440
420
400
380
360
340
320
300
280
260
240
220
200
180
160
80
60
40
20
-20
-10
0
10
20
30
40
50
60
70
80
90
DT-327
(1 mg/Kg; I.V.)
TIME (Minutes)

Figure 4: The effect of probenecid on the natriuretic action of DT-327. An infusion of DT-327 (0.5 μG/Kg/min.) was administered into the left renal artery of four mongrel dogs. In two other animals, probenecid (30 mg/Kg) was injected intravenously before the above DT-327 infusion was administered. Bars represent standard errors.

FIGURE 4

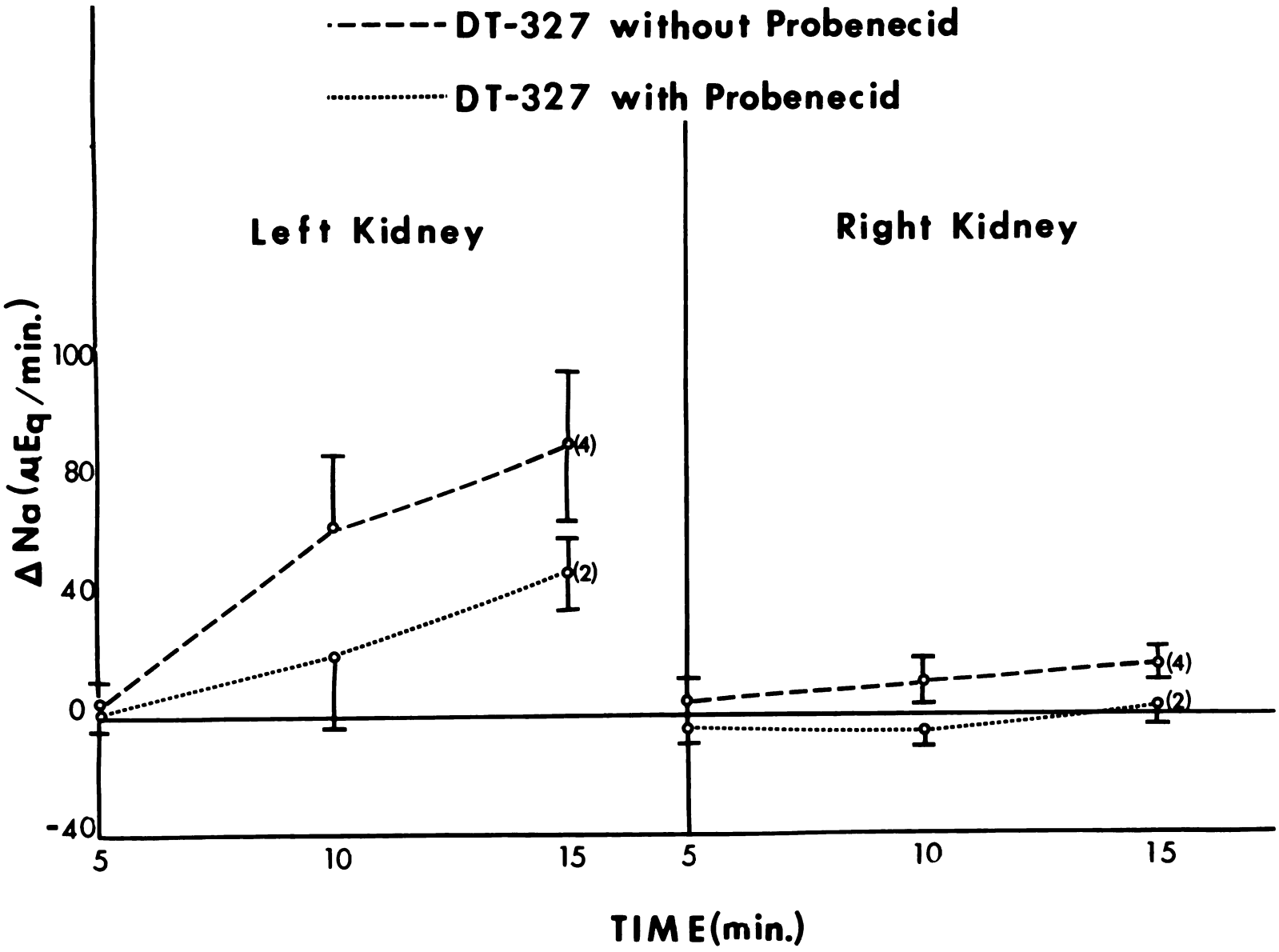

DT-327 without Probenecid
DT-327 with Probenecid
Left Kidney
Right Kidney
ΔNa (μEq/min.)
100
80
40
0
-40
(4)
(2)
(4)
(2)
5
10
15
5
10
15
TIME(min.)

Figure 5: Effect of DT-327 on renal blood flow in the anesthetized dog. The top tracing represents systemic blood pressure and the bottom tracing renal blood flow measured with an electromagnetic flowmeter. DT-327 (1 mg/Kg) was injected intravenously after stable control readings were obtained.

FIGURE 5

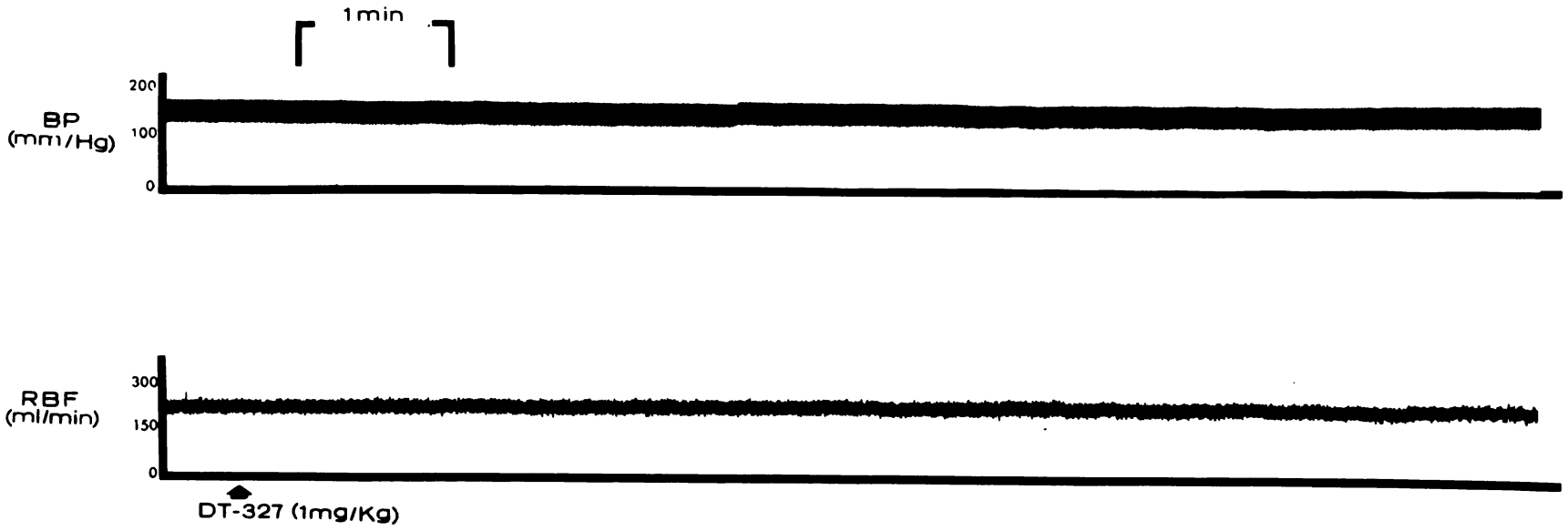


Figure 6: Effect of increasing doses of DT-327 on sodium and chloride excretion in rats orally loaded with sodium chloride or sodium bicarbonate (25 ml/Kg). The solid line represents those rats orally loaded with sodium chloride while the broken line represents those rats orally loaded with sodium bicarbonate. One of three doses of DT-327 (0.01 mg/Kg, 0.1 mg/Kg, or 1.0 mg/Kg) was administered to either the sodium chloride or sodium bicarbonate oral load. The excretion data obtained from twelve rats were averaged. The verticle bars represent standard errors.

FIGURE 6

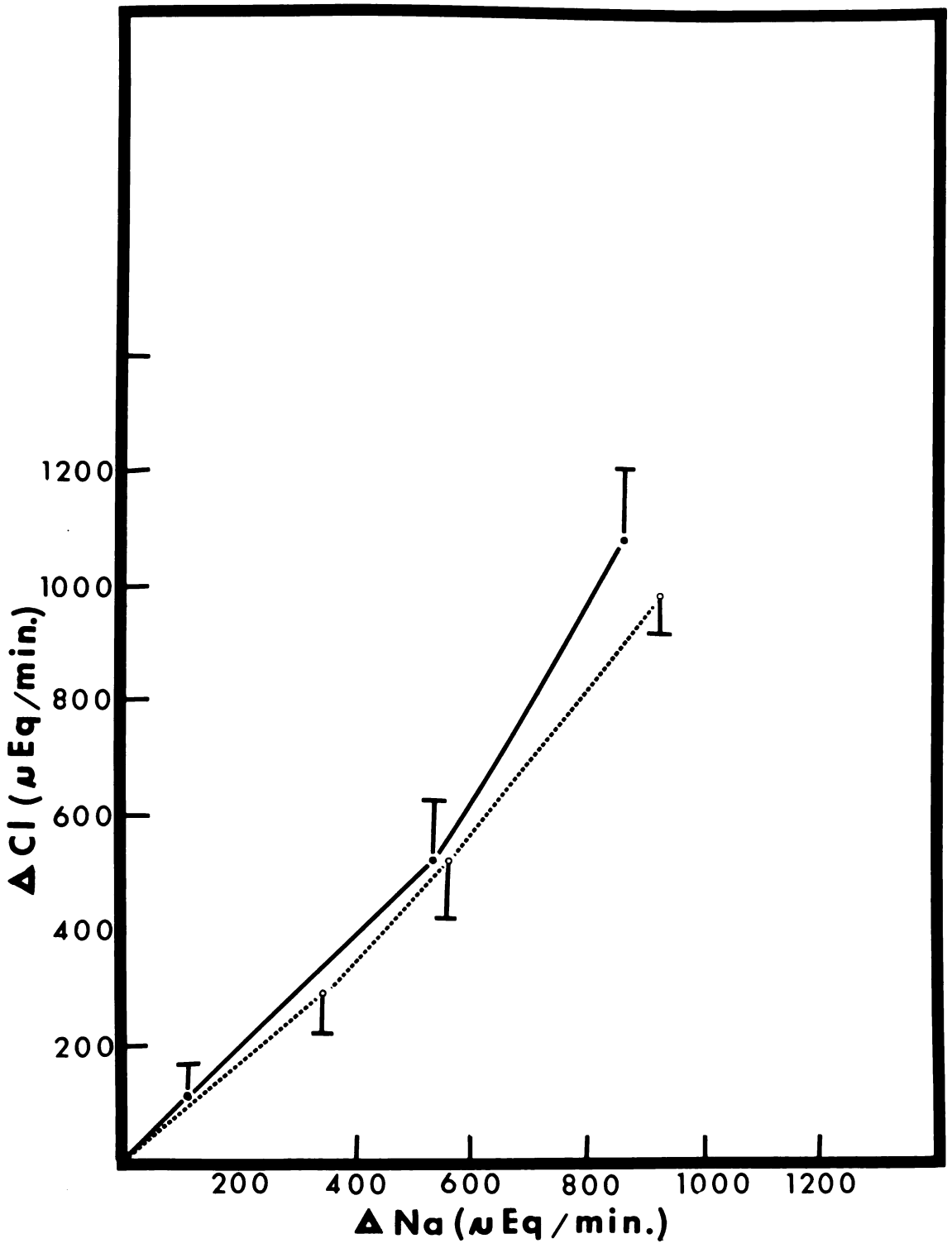

1200
1000
800
600
400
200
Δ Cl (μEq/min.)
200
400
600
800
1000
1200
Δ Na (μEq / min.)

Figure 7: The relationship of free water clearance (C_{H2O}) to urine volume during hypotonic saline infusion (control) compared to that following administration of DT-327 (1 mg/Kg, intravenously) in five dogs.

FIGURE 7

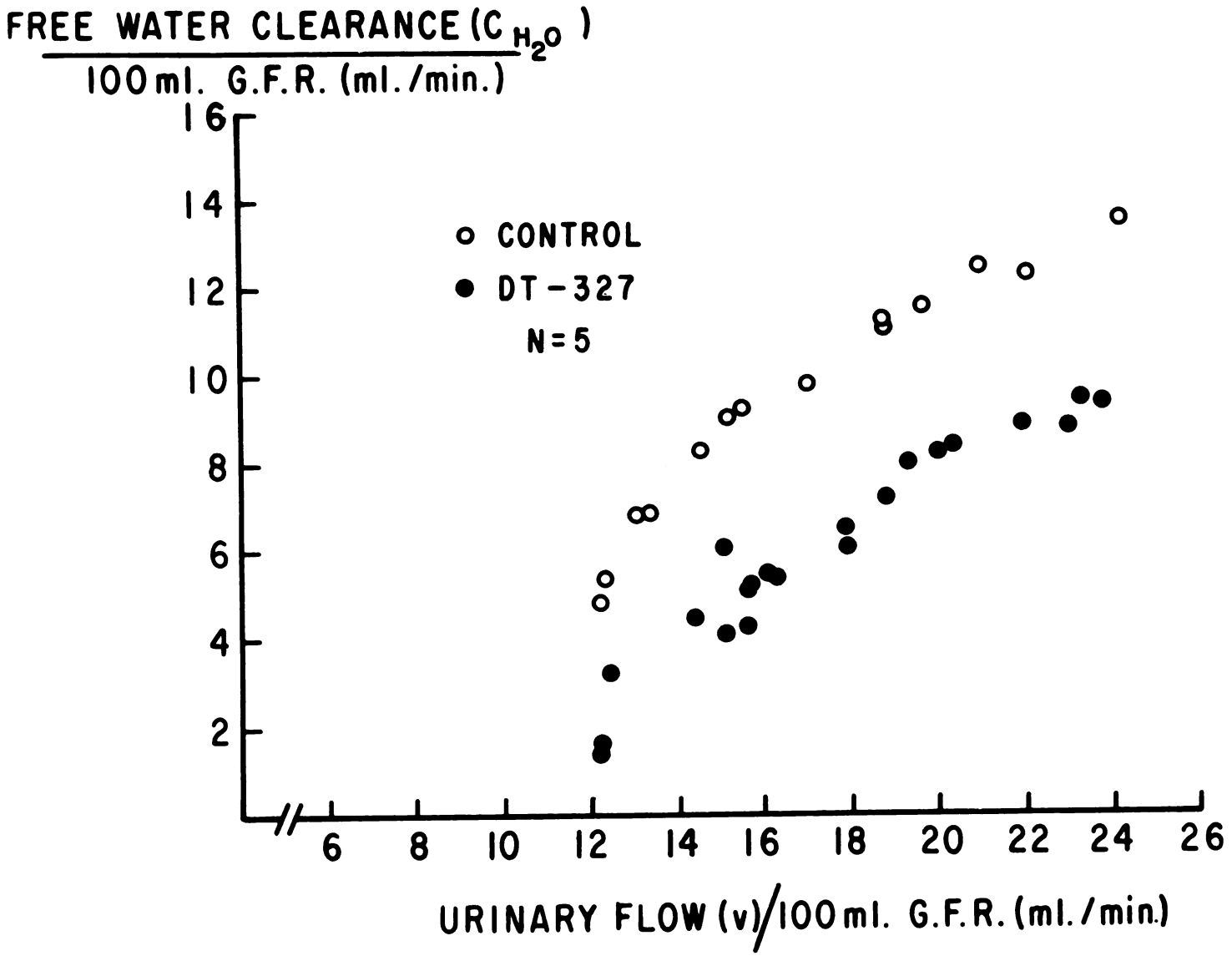

FREE WATER CLEARANCE (C_{H_2O})
100 ml. G.F.R. (ml./min.)
16
14
12
10
8
6
4
2
CONTROL
DT-327
N=5
6
8
10
12
14
16
18
20
22
24
26
URINARY FLOW (v)/100 ml. G.F.R. (ml./min.)

Figure 8: The relationship of negative free water clearance (T^{C}_{H2O}) to osmolar clearance (Cosm) during hypertonic saline infusion (control) compared to that following the administration of DT-327 (1 mg/Kg, intravenously) in four dogs.

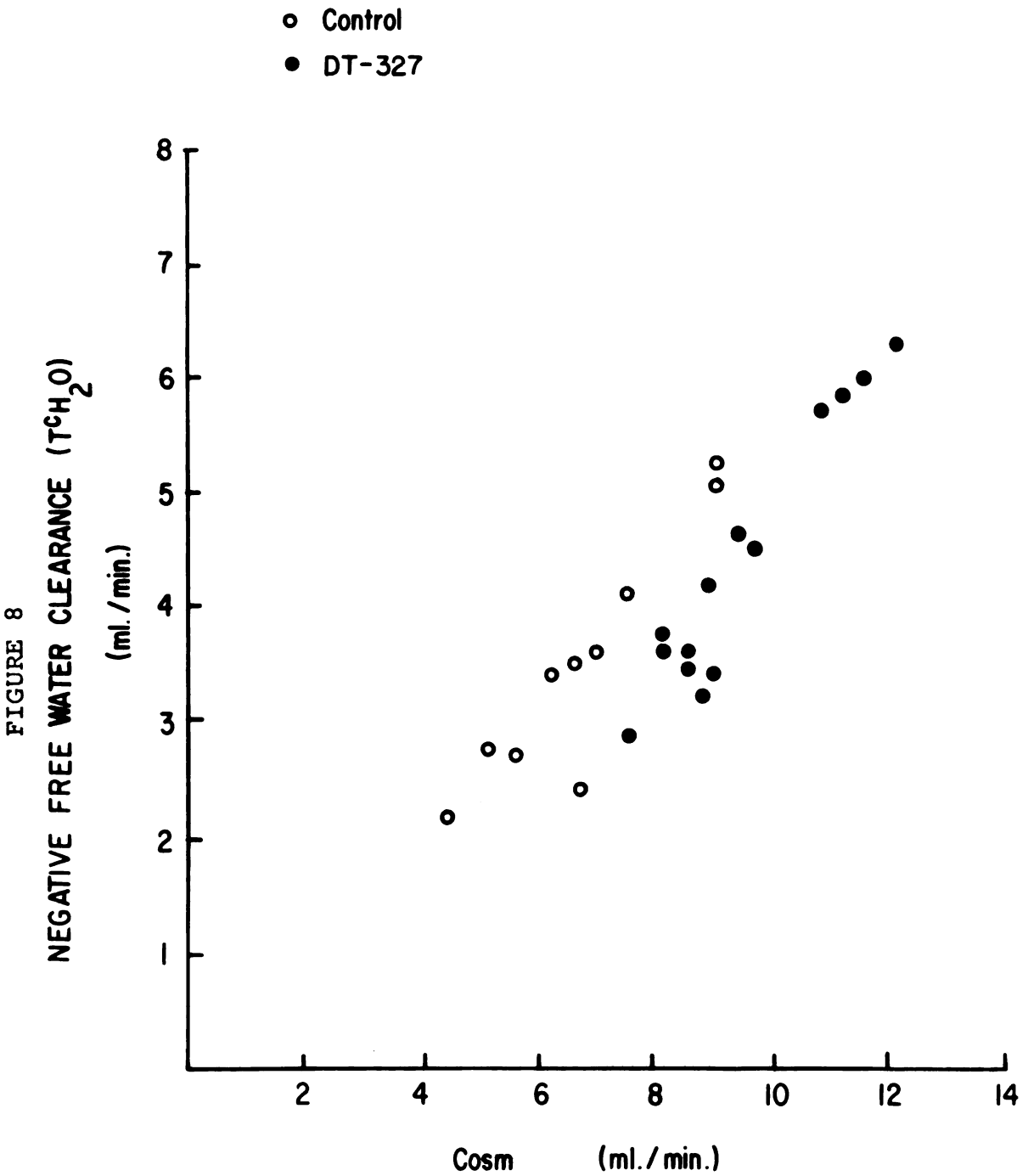

Control
DT-327
NEGATIVE FREE WATER CLEARANCE (T^cH_2O) (ml./min.)
8
7
6
5
4
3
2
1
2
4
6
8
10
12
14
Cosm (ml./min.)

FIGURE 8

Figure 9: Stop-flow analysis of the tubular site of action of DT-327. DT-327 (1 mg/Kg) was administered intravenously thirty minutes before the second occlusion. The closed circles represent the control stop-flow pattern and the open circles represent the stop-flow pattern after administration of DT-327. The arrow indicating maximum PAH represents the stop-flow samples where maximum para-aminohippurate (PAH) secretion occurred. U/P = (Urine/Plasma).

FIGURE 9

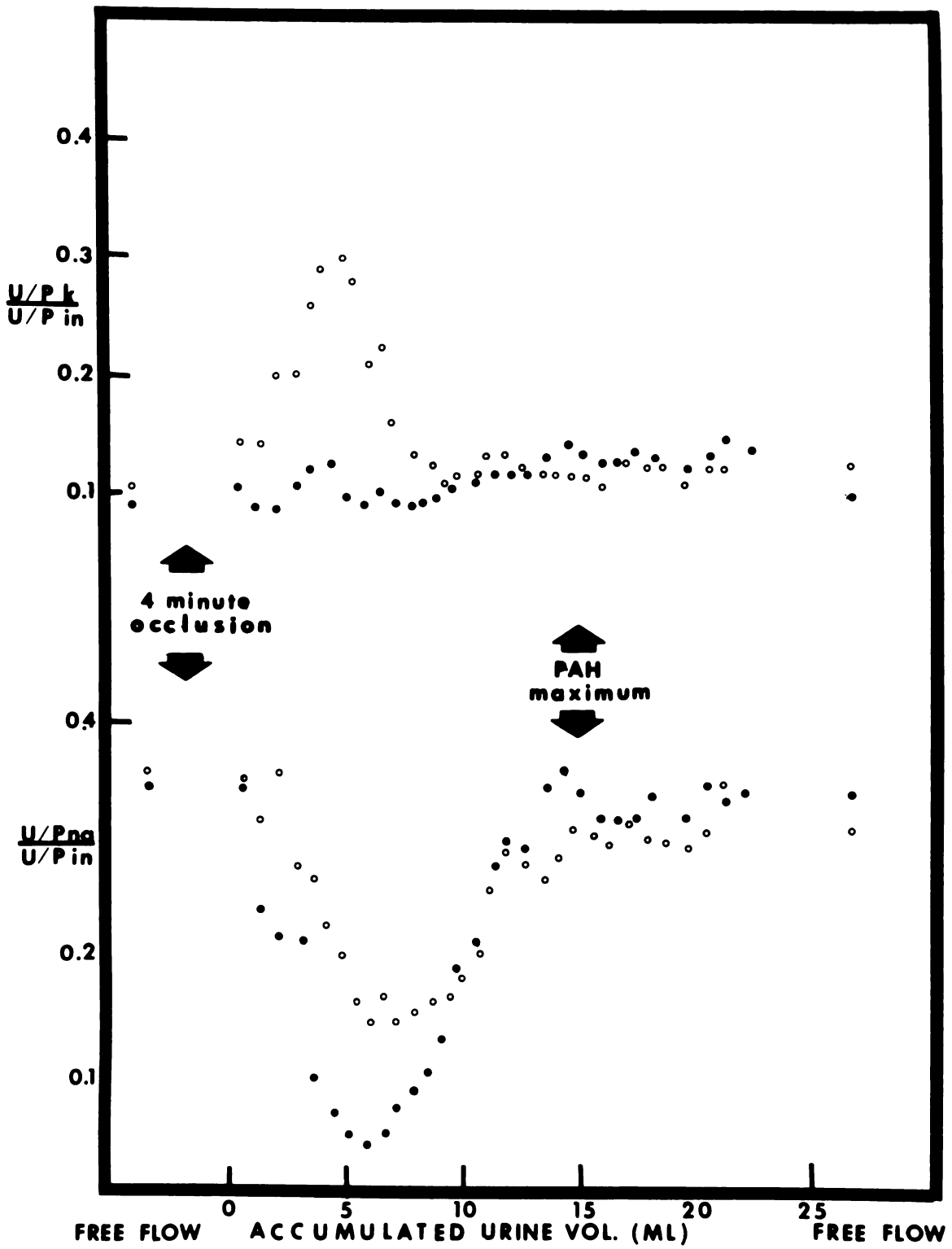

0.4
0.3
U/P k
U/P in
0.2
0.1
4 minute
occlusion
PAH
maximum
0.4
U/Pna
U/P in
0.2
0.1
0
5
10
15
20
25
FREE FLOW
ACCUMULATED URINE VOL. (ML)
FREE FLOW

Figure 10: Effect of DT-327 (1 mg/Kg, intravenously) during maximal thiazide diuresis. The excretion data obtained during two 10-minute periods after DT-327 were averaged. Bars represent means (± standard error) of four experiments. C_{IN} indicates clearance of inulin. The decrease in potassium excretion was statistically significant.

FIGURE 10

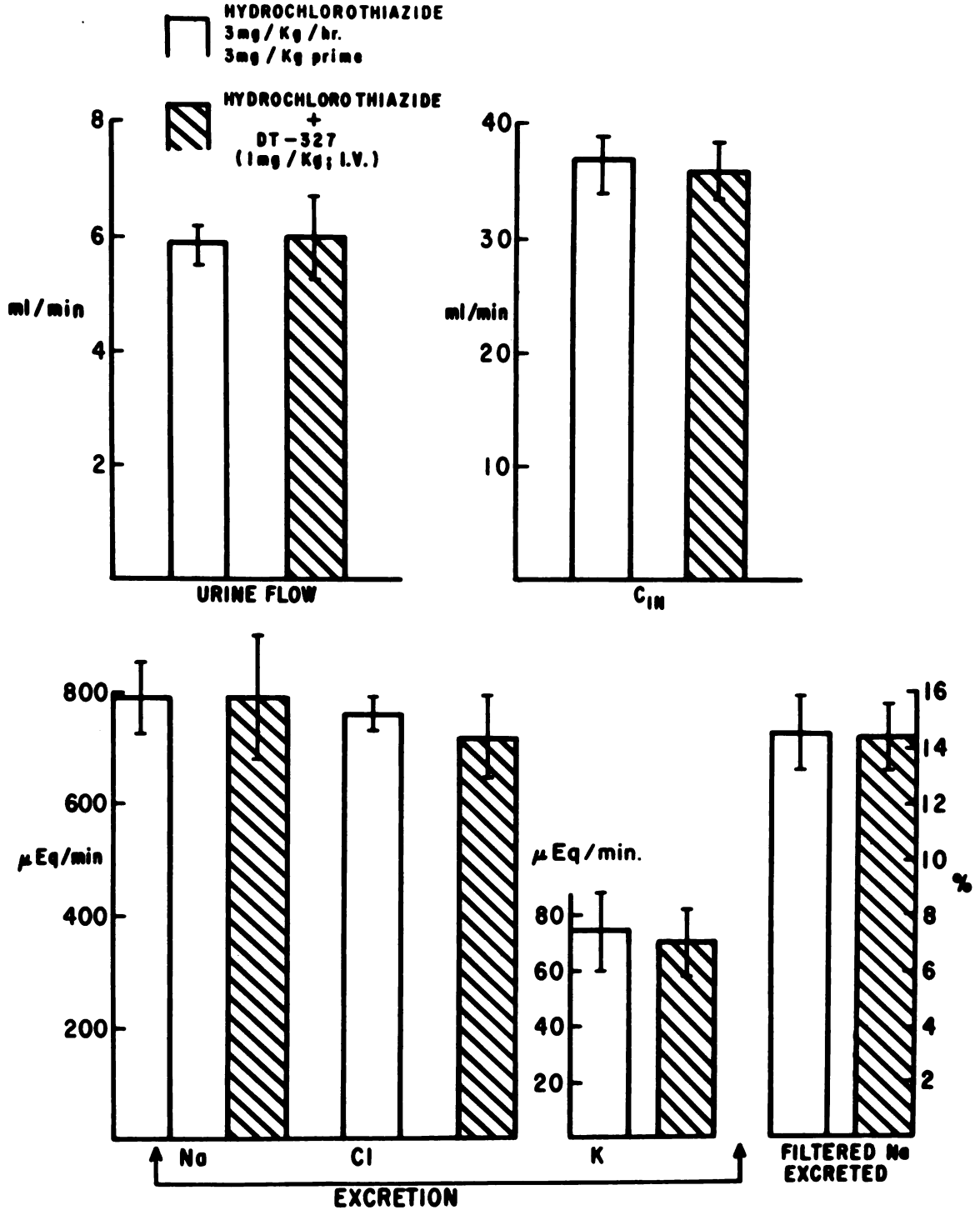
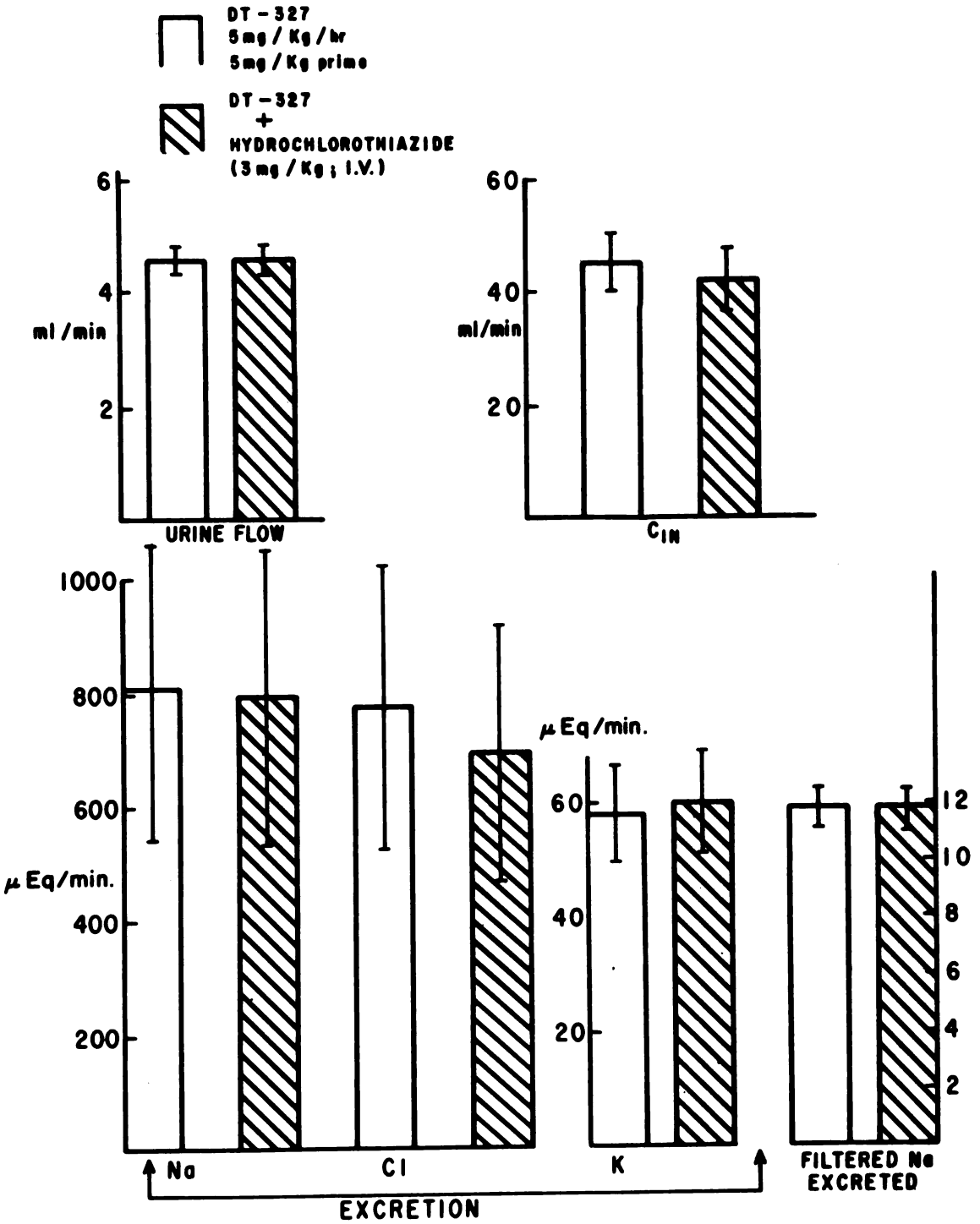

HYDROCHLOROTHIAZIDE
3mg / Kg / hr.
3mg / Kg prime
HYDROCHLOROTHIAZIDE
+
DT-327
(1mg / Kg; I.V.)
8
6
4
2
ml/min
URINE FLOW
40
30
20
10
ml/min
C_{IN}
800
600
400
200
µEq/min
Na
Cl
µEq/min.
80
60
40
20
K
16
14
12
10
8
6
4
2
%
FILTERED Na
EXCRETED
EXCRETION

Figure 11: Effect of hydrochlorothiazide (3 mg/Kg, intravenously) during maximal DT-327 diuresis. The excretion data obtained during two 10-minute periods prior to and three 10-minute periods after DT-327 were averaged. Bars represent means (± standard error) of four experiments. C_{IN} indicates clearance of inulin.

FIGURE 11


DT - 327
5mg / Kg / hr
5mg / Kg prime
DT - 327
+
HYDROCHLOROTHIAZIDE
(3 mg / Kg ; I.V.)
6
4
2
ml / min
URINE FLOW
60
40
20
ml/min
C_{IN}
1000
800
600
400
200
μ Eq/min.
Na
Cl
μ Eq/min.
60
40
20
K
12
10
8
6
4
2
FILTERED Na
EXCRETED
EXCRETION