animals. This trend could be completely reversed in some animals and partially reversed in the remainder of the group by administration of mannitol. The group, receiving mannitol throughout the experiment (Group 3), maintained urine volumes, which in most instances closely approximated the control urine volumes throughout the period of diffusion respiration. Results of these experiments are shown in Fig. 1.

Discussion. One of the more recent and more promising methods for producing acute renal dysfunction in experimental animals is the technic described by Draper and Whitehead(1-3). However, little study has been given to the mechanisms involved in its development. The present work lends some insight into the development of this type of anuria, since the production of urine, even with a diuretic, would have been impossible if some glomerular filtration had not occurred. However, more important than this small insight into the mechanisms of this type of renal dysfunction is the fact that this anuria may prove a valuable tool for probing the role of neurogenic as well as other factors in acute renal dysfunction, and that the ability to reverse it with an osmotic diuretic may prove equally valuable as a means of facilitating such investigations.

Summary. Dogs, well hydrated with saline became anuric or oliguric when their respiration was suspended with overdoses of thiopental sodium. This anuria could either be reversed by administration of an isotonic mannitol solution, or did not occur when mannitol was administered throughout the experiment. The ability to reverse an experimental anuria with an osmotic diuretic presents a potentially valuable tool for the study of this type of renal dysfunction.

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"Collagenase" Activity of Cathepsins.* (21308)

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Collagen is a rather unique protein since it is not digested at neutral pH by any of the common proteinases. Its turnover rate in the normal adult mammalian organism is exceedingly slow(1) and little is known about the enzymes or mechanisms concerned with its synthesis or degradation. The only conclusively demonstrated collagenases have been described in culture filtrates of *Clostridium perfringens* and *C. histolyticum*(2). In the course of studies on collagenase activity, it was observed that the plant cathepsins, papain and ficin, and animal cathepsins were able to digest collagen by a mechanism differing from that of the bacterial collagenases, and involving a preliminary reversible alteration in the state of the substrate. It is the purpose of this report to describe these studies.

Methods. Collagenase activity was measured by the soluble nitrogen or soluble hydroxyproline peptides released from a weighed portion of purified Achilles tendon and is expressed in terms of percent digestion. Fifty mg of dried tendon was suspended in 4 ml of a buffer-enzyme mixture (3:1) and incubated for 3 hours at 37° C. After incubation, 5 ml of 10% NaCl was added and the mixture filtered. Kjeldahl analyses for nitrogen were performed on 1 ml aliquots of the filtrate. The percent digestion was calculated from the

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value obtained for soluble nitrogen, after correction for controls and reagents, and from the total nitrogen present in the collagen preparation. For hydroxyproline analyses, aliquots were hydrolyzed by 6 N HCl for 24 hours at 110°C, and the hydroxyproline determined (3). Purified bovine Achilles tendon was prepared by the method of Neuman and Tytell (4). Proteolysis of native and urea-denatured hemoglobin was determined by the method of Anson(5). 0.1 M buffers were prepared from the following acids: HCl, pH 1.5; H_3PO_4, pH 1.5 - 2.6; lactic acid, pH 3.0 - 4.5; acetic acid, pH 5 - 6; phosphate, pH 6 - 8; boric acid, pH 9 - 10. Commercially available preparations of papain, ficin, and crystalline trypsin were employed. Preparations of beef cathepsins were prepared according to the method of Tallan, Jones and Fruton(6).‡ The animal and plant cathepsins were activated by 0.01 M or 0.005 M cysteine. *C. histolyticum* collagenase was obtained from 18-20 hour cultures of the organism, strain CHT.§ The culture was filtered through a layer of Supercel, and the filtrate brought to 40% saturation with $(NH_4)_2SO_4$. The precipitate was redissolved in water, dialyzed and either used directly or after lyophilization.

Results. In Fig. 1 is shown the collagenase activity of papain, ficin, and the *C. histolyticum* collagenase preparation as a function of pH. The percent digestion is calculated from soluble nitrogen determinations. Hydroxyproline assays yielded identical results. No hydrolysis of collagen was evident with acid alone.

The activity of papain and ficin was optimal in the pH range of 2.0 to 4.5 and practically nonexistent near neutrality. By contrast, the bacterial collagenase had its optimum activity on collagen in the pH range from 6 to 8. The observations with papain and ficin were surprising since the pH optimum of ficin and papain against most protein substrates is near neutrality(7). An explanation for this phenomenon was suggested when the pH optimum of papain and ficin against native and urea-denatured hemoglobin was investigated (Fig. 2).

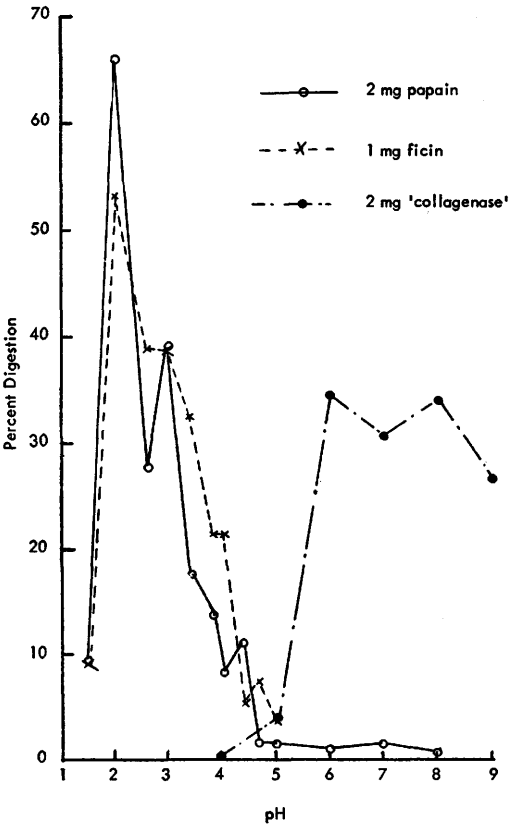

FIG. 1. pH optima of papain, ficin, and *C. histolyticum* collagenase on Achilles tendon.

It can be seen from the data in Fig. 2 that both papain and ficin optimally attacked urea-denatured hemoglobin at pH 7-8, but attacked native hemoglobin optimally at pH 4.0. The data suggested that the enhancement of enzymatic attack on native hemoglobin at pH 4 was due to the state of the substrate rather than a characteristic of the enzyme, since native hemoglobin is no longer in the "native" state at pH 4, undergoing an irreversible acid denaturation. Additional evidence for this view was obtained by investigating the pH optimum of crystalline trypsin on denatured and native hemoglobin. As suspected, the optimum observed with urea-denatured hemoglobin was in the region of neutrality, and with native hemoglobin at pH 4. Very little proteolysis by trypsin was evident with native hemoglobin at neutrality.

‡ Our cathepsin B + C corresponds to their solution 4, and our cathepsin C to their solution 5.

§ Culture medium contained 20 g/L BBL Trypticase, 0.48 g/L KH_2PO_4, and 2.28 g/L Na_2HPO_4.

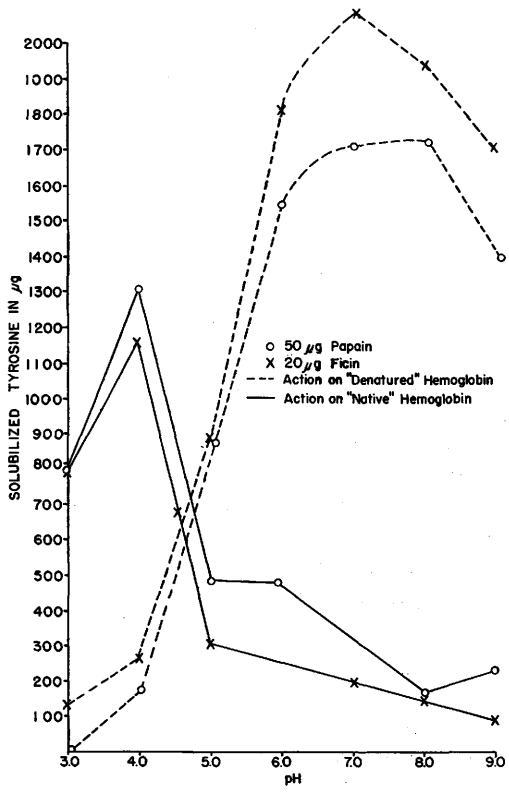

FIG. 2. pH optima of papain and ficin on urea-denatured and native hemoglobin. The ordinate plots amount of acid solubilized tyrosine released from solutions of urea-denatured and native hemoglobin buffered at the desired pH.

Collagen at pH 4.5 and below, in the presence of a low salt concentration, undergoes a visible swelling into a "gel-like" form, reversible by increasing the salt concentration or raising the pH. The physical change in collagen in the same pH range over which the plant cathepsins are active as "collagenases" suggests that an alteration in the state of the substrate is a prerequisite for the digestibility of the substrate by these enzymes. This view is supported by the observation that the proteolysis of the "gel" form of collagen by papain and ficin can be inhibited by raising the salt concentration (Fig. 3) or pH, either of which reverses the "gel" form to the native or "fibrous" form. Increasing sodium chloride concentrations over a similar range did not affect the activity of papain and ficin on hemoglobin.

On the basis of these observations, the "collagenase" action of some animal tissue cathepsins, known to be active at low pH, was studied. Beef spleen cathepsin B + C, and cathepsin C acted in a similar fashion to papain and ficin, readily digesting collagen at acid pH's with an optimum at pH 3.0. Observations on the proteolysis of native and urea-denatured hemoglobin as a function of pH by these animal cathepsins were similar to that found for the plant proteinases.

Simple watery extracts of leucocytes obtained from human purulent fluid, or from beef blood, were repeatedly observed to digest collagen at pH 1.5. Attempts to purify and concentrate this enzyme have not yet been successful.‖

The possibility that the bacterial collagenase preparation also digests collagen by a two step mechanism, *i.e.* an enzymatic alteration of collagen to a digestible form followed by proteolysis, was also investigated. Direct

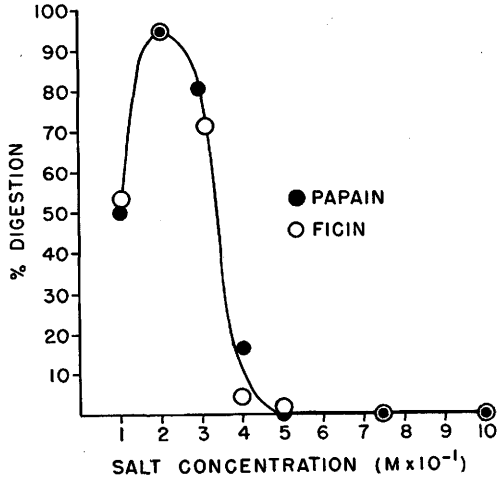

FIG. 3. Digestion of collagen at pH 3.1 by papain and ficin as a function of salt concentration. 0.1 M lactate buffer supplemented with varying concentrations of NaCl. 20 mg Achilles tendon in test. One hr incubation with 1.5 mg papain preparation; ½ hr incubation with 1 mg ficin preparation.

‖ Preparations obtained by disintegrating washed leucocytes with a tuning fork or by grinding with powdered alumina were active on denatured hemoglobin over a wide pH range but did not digest collagen. The method of Tappan, Jones, and Fruton(6) on sediment of purulent exudates yielded a "leucoprotease" active only at neutrality against denatured hemoglobin but with little "cathepsin" like or "collagenase" activity.

proof of this hypothesis, namely separation of the enzymes involved, could not be achieved. A variety of fractionating procedures were not successful in producing a proteinase-free preparation capable of altering the collagen molecule, although proteolytic preparations that are unable to attack native collagen can be derived from collagenase active filtrates(8). The problem is further complicated by the presence of at least 3 different enzymes in the collagenase preparation; a cysteine inhibited collagenase, a cysteine inhibited proteinase; and a cysteine activated proteinase.

Discussion. The observations on the digestion of collagen by papain and ficin at low pH's have led us to the view that the action of the plant cathepsins on collagen is related to the state of the substrate rather than a specific characteristic of the enzymes. According to this view the dissolution of collagen in acid involves two steps: a non-enzymatic controlled equilibrium of collagen with a swollen digestible form, followed by enzymatic proteolysis. It can be shown that the extent of the non-enzymatically controlled equilibrium is determined by the pH and the salt concentration of the solution. The view of a more digestible, yet reversible, form of collagen considerably extends the number of enzymes which can degrade this substrate, for it implies that many proteolytic enzymes capable of acting below pH 4.5 may have the ability to digest collagen. The action of the plant and animal cathepsins as well as that of pepsin(4) as "collagenases" would be accounted for by this mechanism.

Observations on purulent fluids obtained from areas of active suppuration in patients, reveal the presence of a low pH with evidence of considerable proteolysis and collagen breakdown. It is suggested that in areas of suppuration, the accumulated proteinases from the autolyzed inflammatory cells, necrotic tissue, and bacterial cells contribute significantly to the accelerated collagen breakdown by the mechanism described for the cathepsins.

Summary. Papain and ficin digest collagen in the pH 2.0 - 4.5 range at low salt concentration. Evidence is presented that collagen undergoes a reversible alteration at low pH which renders it susceptible to digestion by a number of proteolytic enzymes active at acid pH's. A mechanism for the enzymatic breakdown of collagen in areas of suppuration is suggested. No evidence has been obtained to support a preliminary alteration in collagen as part of the mechanism by which *Clostridium histolyticum* collagenase preparations degrade collagen.

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