reduced, and the nucleo-cytoplasmic ratio was increased, *i.e.*, the changes were toward values characterizing younger larval stages. 3. Similar treatment of juvenile postmetamorphic Xenopus gave comparable but less marked changes in cell and nuclear sizes. The changes were greatest in smaller animals.

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Collagen Digestion by Dog Pancreatic Juice.* (23146)

JON V. STRAUMFJORD, JR. AND JOHN P. HUMMEL
Department of Biochemistry, State University of Iowa, Iowa City

Although collagen has long been recognized to be resistant to enzymic hydrolysis, Ssasdikow(1) reported that trypsin-free fractions of pancreatic extract partially degrade collagen. Weak collagenolytic activity was also found in dog pancreatic juice obtained by Pavlov fistula. Recently Ziffren and Hosie(2) reported degradation of collagen by commercial trypsin preparations and pancreatic juice, and postulated the presence of a heat labile enzyme, collagenase, in pancreatic juice. Banga and Balo(3) noted liberation of carbohydrate from collagen during incubation with an enzyme from a pancreatic extract they called collagenmucoproteinase. Certain bacteria, notably *Clostridia*, produce collagenase as an exoenzyme.

Because of the uncertainty of the identity of the enzyme responsible for collagen degradation in dog pancreatic juice, the present study was undertaken in an attempt to define the nature of "collagenase."

Methods. Collagen was prepared from beef achilles tendon by the method of Buechler(4). Electron microscopy† of the preparation revealed that the great majority of particles showed the characteristic structure of native collagen. Crystalline trypsin, chymotrypsin, and soy-bean inhibitor were obtained from Worthington Biochemical Corp., Freehold, N. J. Carboxypeptidase was obtained from Pentex, Inc., Kankakee, Ill. Bacterial collagenase from *Clostridium histolyticum* was generously supplied by Dr. R. E. Kallio of the Department of Bacteriology. Pancreatic juice was obtained from mongrel dogs by the procedures of Ziffren and Hosie(2). Collagen (50 mg) was suspended in 3 ml of pancreatic juice, enzyme solutions, or buffer solutions, each at pH 8.0. After incubation at 37° for 24 hours with occasional shaking, 7 ml of water was added, the suspension was shaken well and centrifuged. The residuum was saved for further study and a portion of supernatant was autoclaved for 3 hours in 6 N HCl at 15 lb/sq. in. An aliquot of the hydrolysate was neutralized to pH 4-5 with 6 N NaOH and the free hydroxyproline was determined by the method of Neuman and Logan(5). Undigested collagen obtained by

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TABLE I. Digestion of Collagen by Pancreatic Juice and Proteolytic Enzymes.*

Medium	mg collagen digested: Analysis of supernatant		Analysis of residual collagen	
M/15 phosphate, pH 7.0	1	(3)	3 ± 2	(14)
Trypsin in M/15 phosphate, pH 7 (1.3 mg/cc)	4.3 ± .1	(2)	7.5 ± 1	(2)
Carboxypeptidase (.066 mg/cc)	2.8	(1)		
Pancreatic juice, inactive				
Dog 1			4.1 ± 2	(3)
3	3.6	(1)	6.7	(1)
4	3.1 ± .1	(3)	3.0 ± .7	(2)
Mean	3.3 ± .3		4.6 ± 1.6	
Pancreatic juice, active				
Dog 1	5.7	(1)	9	(1)
2	5.1 ± 1.2	(4)	11.6 ± 2.1	(6)
3	4.1	(1)	11.6	(1)
4	4.5 ± .3	(2)	6.9	(1)
Mean	4.9 ± .6		9.8 ± 2	
Pancreatic juice and inhibitor				
Dog 2 active	3.6 ± .8	(3)	8.5 ± 1	(3)
3 inactive	2.9	(1)	8.5	(1)
4 "	3.1 ± .2	(2)	1.5	(1)
4 active	3.2 ± .3	(2)	5.1	(1)
Mean	3.2 ± .2		5.9 ± 2.9	

* Conditions as described in text. Crystalline trypsin inhibitor (2 mg) was added as indicated. The numbers of separate experiments, done in duplicate, are indicated in parentheses.

centrifuging was washed at the centrifuge 3 times with water, discarding the washings. After hydrolysis of undigested collagen with 6 N HCl for 3 hours in the autoclave in the same manner as before, hydroxyproline was determined in an aliquot of hydrolysate diluted 1:100. This method of hydrolysis gave 90% recovery of the hydroxyproline (13.4%) in beef achilles tendon(5). The enzyme results are expressed as mg of collagen dissolved.

Results. The extent of collagen degradation (Table I) as measured by hydroxyproline in the supernatant, was in certain cases much less than the degradation as measured by the difference between collagen added and that recovered after incubation. Mere manipulation and washing made 1.9 mg of the 50 mg of collagen soluble as revealed by recovery of collagen in the residuum; moreover, addition and prompt removal of pancreatic juice followed by the usual washing procedure brought about solubilization of 3.6 mg. Hydrolyzed pancreatic juice contained no significant quantities of free hydroxyproline.

The results obtained by assay of residual collagen were much more variable than those obtained from the supernatant. Both methods of assay, however, demonstrated a clear difference in activity of various pancreatic preparations. For descriptive purposes, these have been called "active" and "inactive." Pancreatic juice from dogs 1 and 3 was initially inactive but became active after repeated freezing and thawing, whereas juice from dog 2 was active immediately following collection. Active pancreatic juice made 6.8 mg or 12% of the collagen soluble by assay of the undigested residue but only 3.9 mg or 6.8% soluble by assay of the supernatant. Inactive pancreatic juice gave about 2.3 mg or 5% digestion with both assay procedures. No difference in carbohydrate content of the supernatant could be detected by using the phenol sulfuric acid method of R. Montgomery.‡ Addition of 0.1 mg of trypsin (sufficient to convert trypsinogen to trypsin) to 20 ml of pancreatic juice from dog 4, caused the preparation to become active. Addition of 2 mg of soybean trypsin inhibitor to active pancreatic juice decreased collagen degradation to the level of inactive pancreatic juice, but had no effect upon inactive preparations. Bacterial collagenase digested collagen rapidly but was not inhibited by soybean trypsin inhibitor.

Trypsin (1.3 mg/3 ml) but not chymotrypsin made about 4.3 mg of collagen soluble under these conditions. Interestingly, carboxypeptidase (0.2 mg/3 ml) also degraded about 2.8 mg of collagen, despite the fact that proline and hydroxyproline peptides are unattacked by this enzyme. Commercial preparations of carboxypeptidase are reported to contain a proteolytic contaminant(6).

Discussion. The study of enzymic degra-

‡ Personal communication.

dation of collagen is complicated by several factors. Apart from the obvious problem of the variable degree of dispersion of the substrate, the chemical constitution of collagen from beef achilles tendon is uncertain. In preparation of collagen the only property employed for its purification is insolubility, a property shared by other structural constituents. Beef achilles tendon collagen is reported to contain about 4% of elastin(7), which is subject to destruction by pancreatic elastase. Approximately 5% of our collagen preparation was insoluble after heating in water for 3 hours at 100°, conditions which convert collagen to gelatin. However, elastin contains only about 1.8% of hydroxyproline; therefore collagen breakdown measured as hydroxyproline is not significantly affected by elastin breakdown. In addition, denatured collagen, which is distinguished from native collagen by differences in appearance by electron microscopy, but which cannot be measured quantitatively, may be more susceptible to enzymic destruction. Neuman and Tytell(8) have emphasized the importance of employing native collagen for collagenase assay. By electron microscopy, our preparation, which was predominately native collagen, contained small amounts of denatured collagen. In the present experiments a decision cannot be made as to whether native or denatured collagen was being degraded.

A major portion of collagen digestion appears to be due to trypsin. This conclusion is based on activation of fresh pancreatic juice by small amounts of trypsin and by inhibition of collagen digestion by activated pancreatic juice in the presence of specific soybean inhibitor.

Indeed the differences observed between assays of hydroxyproline in supernatant solution and in washed residue suggest that tryptic digestion may be manifested not only by dissolution of collagen but also by weakening of the structure so that it becomes more soluble upon subsequent washing. Hide collagen pretreated with trypsin subsequently becomes more soluble in warm water(9).

Ziffren and Hosie(2) reported that dog pancreatic juice digested large quantities of collagen. Their methods differed from ours in 3 respects. (1) The residual collagen, separated by filtration, was transferred to test tubes for subsequent hydrolysis. Losses by manipulation could cause considerable error. (2) The residual collagen was hydrolyzed for 30 minutes with 0.5 N HCl at 15 lb/sq. in. pressure. Under these conditions we found only 5-7% of total hydroxyproline was liberated; even after 3 hours heating only 60% of hydroxyproline was liberated. (3) The colorimetric standard previously employed was not purified hydroxyproline but was a weighed portion of collagen which was also hydrolyzed with 0.5 N HCl for 30 minutes at 15 lb pressure. Moreover a buffer-collagen control was not run with each experiment. One or more of these possible sources of error may have magnified the extent of collagen digestion by pancreatic juice reported previously.

The extent of collagen digestion by pancreatic enzymes other than trypsin is so slight that it is difficult to ascertain the nature of the causative enzyme. Possibly carboxypeptidase, pankrin(10), prolinase, or elastase singly or in combination are responsible. Until methods for the preparation of pure native collagen are developed, or specific inhibitors for those other enzymes are found, attempts to define this small digestion do not appear fruitful. In our opinion, it is unnecessary to ascribe the small amount of collagen degradation caused by inactive or inhibited pancreatic juice to a specific pancreatic collagenase.

Summary. Degradation of collagen after one day incubation with pancreatic juice or various enzymes was studied. About 6% was degraded by trypsin or by pancreatic juice containing active trypsin. Collagen degradation was much less when incubated with fresh inactive pancreatic juice or pancreatic juice containing trypsin inhibitor. The extensive digestion of collagen previously reported could not be confirmed.

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Chemotherapeutic Activity of Combinations of Sulfisoxazole and Oleandomycin. (23147)

E. GRUNBERG, W. F. DELORENZO, D. ELDRIDGE AND E. TITSWORTH

Chemotherapy Laboratories, Hoffmann-La Roche, Inc., Nutley, N. J.

The combined effect of antibacterial agents generally demonstrated by an increased but also occasionally decreased bacteriostatic effect *in vitro* has comparatively rarely been reproduced in animal experiments. This report is concerned with a comparison of combined activity of a sulfonamide and an antibiotic *in vitro* and *in vivo*. The agents used were: (a) sulfisoxazole (3,4-dimethyl-5-sulfanilamido-isoxazole)* which has been described(1) as a substance of low toxicity and marked though not always optimal(2) chemotherapeutic activity *in vitro* and in experimental infections with Gram positive and Gram negative organisms, and (b) oleandomycin, an antibiotic which has been shown by Sobin *et al.*(3) as well as by Fust *et al.*(4) to exhibit *in vitro* and in experimental animals an activity range similar to that of penicillin and erythromycin. Clinically the effect of the antibiotic is particularly marked against Gram positive microorganisms(5).

Materials and methods. The origin of the strains(1) as well as the technics of the broth dilution tests(1) and gradient plates(6) have been described elsewhere. Mueller-Hinton medium(7) was used in all experiments which employed the gradient plate. The methods for the *in vivo* experiments are the same as those given by Schnitzer *et al.*(1) except that the Giorgio strain of *Staphylococcus aureus* (obtained through the courtesy of Dr. R. M. McCune, Jr.) which is of low sensitivity to sulfonamides was used. The infective dose of this organism was 0.5 ml intravenously of a saline suspension containing 250.000.000 cells. Both *in vitro* in the broth dilution method and *in vivo*, sulfisoxazole and oleandomycin were tested alone as well as in the following combinations: (a) a fixed relation of 5 parts sulfisoxazole combined with 1 part oleandomycin, (b) sulfisoxazole in the presence of a constant inactive concentration of oleandomycin, and (c) oleandomycin in the presence of a constant, inactive concentration of sulfisoxazole. *In vitro* the combination of sulfisoxazole and oleandomycin was also tested in the proportion of 1:1.

* Gantrisin®.

Results. Activity *in vitro*. A. Broth dilution method: In the case of *Streptococcus hemolyticus* 4 and *Pneumococcus pneumoniae* 6301, sulfisoxazole showed comparatively low activity (25-50 mg/ml) while oleandomycin was highly effective (0.25-0.5 μg/ml). With *Escherichia coli* J and *Eberthella typhi* F the range of activity was 3.9 to 15.6 μg/ml for sulfisoxazole and 31.2-62.5 μg/ml for oleandomycin. The inhibiting dose against *Staphylococcus aureus* 209 was 3.9 μg/ml for sulfisoxazole and 0.031 μg/ml for oleandomycin. In every case, the combination gave the effect of the more potent component.

B. Gradient plate technic. The results of experiments with *Staphylococcus aureus* 209, *Escherichia coli* J and *Eberthella typhi* F,